# Organelle isolation: functional mitochondria from mouse liver, muscle and cultured filroblasts

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**Mitochondria participate in key metabolic reactions of the cell and regulate crucial signaling pathways including apoptosis. Although several approaches are available to study mitochondrial function *in situ* are available, investigating functional mitochondria that have been isolated from different tissues and from cultured cells offers still more unmatched advantages. This protocol illustrates a step-by-step procedure to obtain functional mitochondria with high yield from cells grown in culture, liver and muscle. The isolation procedures described here require 1–2 hours, depending on the source of the organelles. The polarographic analysis can be completed in 1 hour.**



## INTRODUCTION

Mitochondria are central organelles controlling the life and death of the cell. They participate in key metabolic reactions, synthesize most of the ATP and regulate a number of signaling cascades, including apoptosis[1].

Since the early years of "hard-core" bioenergetics when mechanisms behind energy conservation were avidly investigated, mitochondrial research has benefited from the availability of preparations of organelles isolated from tissues. We owe this to the pioneering work of George Palade and coworkers, who in the late 1940s developed a protocol to isolate mitochondria, based on differential centrifugation[2]. They built on the earlier work of Bensley and Hoerr[3], who isolated a mixed membranous fraction by centrifugation from freeze-thawed guinea-pig liver that was probably enriched in mitochondria. The intuition of Palade was to apply differential centrifugation to allow for separation of the constituents of the cell based on their different sedimentation properties following mechanical homogenization of the tissue. This approach was a real Copernican revolution for mitochondrial research, allowing the isolation of pure organelles with high yields. As a practical consequence, in the subsequent 20 years, we saw such amazing discoveries: the mechanism of energy conservation[4]; the identification of mitochondrial DNA[5,6] and of import of mitochondrial precursor proteins[7]; the definition of mitochondrial ultrastructure, with the development of the so-called "Palade's model"[8]; and last but not least, the discovery of inner mitochondrial membrane channels[9].

After almost 15 years during which mitochondria left the center stage of biomedical research, they made their grand *reentrée* in the 1990s, following the discovery that they amplify apoptosis by releasing cytochrome *c* and other intermembrane space proteins required to activate fully effector caspases[10,11]. Although it appears clear that mitochondria play a crucial role in apoptosis, the precise mechanism by which cytochrome *c* is released remains a matter of intense debate and research[12]. Moreover, evidence is mounting on the role of this organelle in several pathophysiological processes, including neurodegeneration[13], neuronal morphogenesis and plasticity[14] and infertility[15]. These findings, added to the results of old and new areas of research, aimed at unraveling the basic biological mechanisms of mitochondrial function. From the transport of metabolites and ions, to the elucidation of the mechanisms and proteins involved in protein import, and to the dynamic behavior of mitochondria, all of these fields benefit greatly from the availability of isolated, pure organelles.

This protocol describes how to obtain functional, purified, intact mitochondria from three different sources: liver[16], skeletal muscle[17] and cultured cells[18]. These variants intend to be exemplificative and not exhaustive, as they do not cover the different sources from which mitochondria can be isolated. For example, isolation of mitochondria from yeast cells is tailored on the mechanical and osmotic characteristics of these lower eucaryotes[19]. Since our intention is to give a general framework for different organs and for cultured cells that can be in any case modified by the individual researcher, following exactly these protocols is best suited only for isolation of organelles from the described tissues and cells. However, our experience indicates that the protocol used with fibroblasts can be adopted without modification to isolate mitochondria from other cell lines such as HeLa and the prostate cancer cell line LnCaP. On the other hand, the protocols to isolate mitochondria from organs other than muscle and liver differ from the ones described here. We therefore strongly advise the reader to refer to published protocols specific for brain[20], brown adipose tissue[21], and heart[22].

It should be stressed that protocols available to isolate mitochondria are somewhat differ from ours, especially in the speeds of the differential centrifugation steps and in the sugar used as osmolyte in the isolation buffer. While in our experience small changes in the sedimentation speeds (600 vs. 800*g*, 7,000 vs. 8,000*g*) do not affect quality and yield of the mitochondrial preparation, it has been reported that the use of monosaccharides such as mannitol results in better coupled isolated mitochondria[23,24]. In our experience the use of mannitol did not improve the quality of our mitochondrial preparations. Should the reader find that quality or yield of mitochondria isolated using our protocol is unsatisfactory, it is advisable to try to substitute sucrose with a monosaccharide like mannitol. The ultimate goal of a mitochondrial isolation is to obtain organelles as pure and as functional as possible. We strongly advise, especially if mitochondria are used in functional assays (e.g., release of cytochrome *c*, mitochondrial fusion, protein import and

production of reactive oxygen species), to always measure the coupling of the preparation using an oxygen electrode. These protocols therefore end with a description of how to measure mitochondrial respiration to ascertain the quality of the preparation. Well-coupled mitochondria are the first step to achieving reliable, reproducible results in assays aimed at investigating the mechanisms of mitochondrial involvement in complex biological phenomena.

In conclusion, these protocols represent a valuable starting point to obtain pure mitochondria from tissues and cells. Isolated mitochondria can then be used to study the function of the organelle, response to apoptotic stimuli, characteristics of cytochrome *c* release, protein import and many other aspects of mitochondrial biology and pathophysiology that require a source of pure and functional organelles.

## MATERIALS

### REAGENTS

- Cell line of interest or liver or muscle isolated from mice
- Mice of the desired genetic background (Charles River or Jackson Laboratories)
- Dulbecco's phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS, Invitrogen, cat. no. 14200-067)
- Sucrose (Sigma, cat. no. 84100)
- Potassium phosphate monobasic (Pi, Sigma, cat. no. P5379)
- Sigma7-9 (Tris, Sigma, cat. no. T1378)
- 4-Morpholinepropanesulfonic acid (MOPS; Sigma, cat. no. M1254)
- Disodium ethylenediaminetetraacetate dihydrate (EDTA; Sigma, cat. no. ED2SS)
- Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA; Sigma, cat. no. E4378)
- Potassium chloride (Baker, cat. no. 0208)
- Magnesium chloride hexahydrate (Sigma, cat. no. M9272)
- Bovine serum albumin (BSA; Sigma, cat. no. A6003)
- Dulbecco's modified Eagle's medium (Invitrogen, cat. no. 11971025)
- 200 mM L-glutamine (Invitrogen, cat. no. 25030024),
- Fetal bovine serum (Invitrogen, cat. no. 10270106)
- 5,000 U $ml^{-1}$ penicillin/5,000 μg $ml^{-1}$ streptomycin (Invitrogen, cat. no. 15070063)
- 10 mM minimal essential medium nonessential amino-acid solution (Invitrogen, cat. no. 11140)
- 0.25% (w/v) trypsin–EDTA solution (Invitrogen, cat. no. 25200072)
- Adenosine 5′-diphosphate sodium salt (ADP; Sigma, cat. no. A2754)
- Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma, cat. no. C2920)
- Glutamic acid (Sigma, cat. no. 27647)
- Malic acid (Sigma, cat. no. M1000)
- Succinic acid (Sigma, cat. no. S3674)
- Rotenone (Sigma, cat. no. R8875)
- L-Ascorbic acid (Sigma, cat. no. 255564)
- *N,N,N,N*-Tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD; Sigma, cat. no. T3134)
- Antimycin A (Sigma, cat. no. A8674)

### EQUIPMENT

- 500 $cm^2$ dishes for cell culture (Nunclon, cat. no. 16 6508)
- 18-cm cell scrapers (Falcon, cat. no. 353085)
- Motor-driven tightly fitting glass/Teflon Potter Elvehjem homogenizer (**Fig. 1**)
- Clark-type oxygen electrode (Hansatech Oxygraph; **Fig. 2**)
- 50 ml polypropylene Falcon tubes
- 14 ml polypropylene Falcon tubes
- 1.5 ml microfuge test tube
- 30 ml round-bottomed glass centrifuge tube (Kimble, cat. no. 45500-30)
- Rubber adapter sleeve for centrifuge tube (Kimble, cat. no. 45500-15)
- Refrigerated centrifuge for 50 ml Falcon tubes and glass centrifuge tube
- Hamilton syringe: 10 μl (Hamilton, cat. no. 701 N) and 50 μl (Hamilton cat. no. 705 N)

### REAGENT SETUP

**Cell culture medium** Use the medium recommended for your favorite cell line. For the cell lines mentioned in this protocol, use Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 0.1 mM minimal essential medium nonessential amino acids, 2 mM L-glutamine, penicillin–streptomycin 50 U $ml^{-1}$ and 50 μg $ml^{-1}$, respectively.

**Cells** Two or three days before performing the experiments, plate cells in 500 $cm^2$ tissue-culture dishes. Use 70 ml of cell culture medium for each plate.

▲ **CRITICAL** Ensure that the cells are spread thoroughly wide on the plates: for high yield of isolated mitochondria, it is crucial to reach almost 100% confluence on the day of the experiment.

**1 M sucrose** Dissolve 342.3 g of sucrose in 1 liter of distilled water; mix well and prepare 20 ml aliquots; store them at −20 °C.

**0.1 M Tris/MOPS** Dissolve 12.1 g of Tris in 500 ml of distilled water, adjust pH to 7.4 using MOPS powder, bring the solution to 1 liter and store at 4 °C.

**1 M Tris/HCl** Dissolve 121.14 g of Tris in 500 ml of distilled water, adjust pH to 7.4 using HCl; bring the solution to 1 liter and store at room temperature.

**0.1 M EGTA/Tris** Dissolve 38.1 g of EGTA in 500 ml of distilled water, adjust pH to 7.4 using Tris powder, bring the solution to 1 liter and store at 4 °C.

**0.5 M $MgCl_2$** Dissolve 101.7 g of $MgCl_2$ in 1 liter of distilled water and store at 4 °C.

**1 M KCl** Dissolve 74.6 g of KCl in 1 liter of distilled water and store at 4 °C.

**1 M EDTA** Dissolve 372.2 g of EDTA in 500 ml of distilled water, adjust pH to 7.4 using Tris powder, bring the solution to 1 liter and store at 4 °C.

**10% BSA** Dissolve 10 g of BSA in 100 ml of distilled water and store at −20 °C.

**1 M Pi** Dissolve 136.1 g of $KH_2PO_4$ in 500 ml of distilled water, adjust pH to 7.4 using Tris powder, bring the solution to 1 liter and store at 4 °C.

**10 mM ADP** Dissolve 4.7 mg of ADP in 1 ml of distilled water. Adjust pH to 7.4, prepare 100 μl aliquots and store in the dark at −20 °C for up to 6 months.

**20 mM FCCP** Dissolve 5.1 mg of FCCP in 1 ml of absolute ethanol. The color of the solution is faint yellow. Store at −20 °C. Dilute the stock solution to 100 μM by adding 10μl of 20 mM FCCP in 2 ml of absolute ethanol, just prior to use.

**Figure 1** | Glass/Teflon Potter Elvehjem homogenizers. The homogenizer on the left (5 ml) is most suitable for isolation of mitochondria from cells, whereas the one on the right (30 ml) is more appropriate for isolation from tissues.

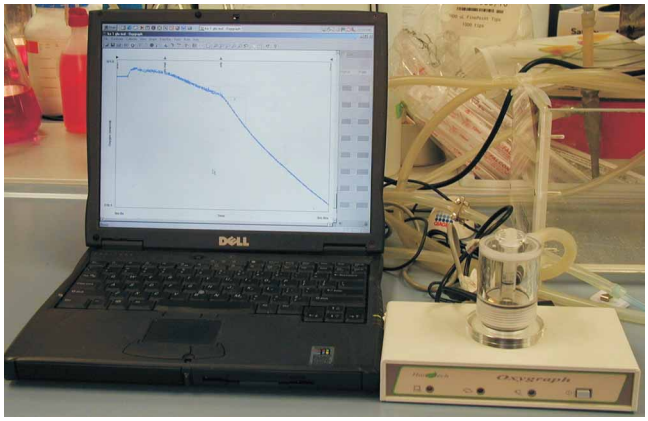

**Figure 2 |** A Clarke-type oxygen electrode connected to a laptop and a water bath. The trace on the screen corresponds to the recording of the experiment running when the photograph was taken.

**0.25 M glutamate/0.125 M malate** Dissolve 9.2 g of glutamic acid and 4.2 g of malic acid in 100 ml of distilled water. Adjust pH to 7.4 with Tris base to achieve complete dissolution of the salts. Add water to bring the volume to 250 ml, prepare 10 ml aliquots and store at −20 °C for up to 6 months.
**0.5 M succinate stock solution (100×)** Dissolve 3.0 g of succinic acid in 30 ml of distilled water. Adjust pH with Tris base to achieve complete solubilization of the salts. Add water to make up the volume to 50 ml, prepare 10 ml aliquots and store at −20 °C for up to 6 months.
**2 mM rotenone stock solution** Dissolve 4.7 mg of rotenone in 6 ml of absolute ethanol. Mix well for complete dissolution. ▲ CRITICAL Rotenone in organic solvents decomposes and is oxidized upon exposure to light and air. The solution, previously transparent, becomes brownish. It is imperative to protect the stock solution from direct light using an aluminum foil. ! CAUTION Rotenone is highly toxic: avoid skin contact and inhalation.

## PROCEDURE

**1|** Mitochondria can be isolated from a variety of cells or tissues. Option A describes isolation of mitochondria from mouse embryonic fibroblasts (MEFs) (see **Fig. 3** for a timeline); option B describes isolation of mitochondria from mouse liver (see **Fig. 4** for a timeline); and option C describes isolation of mitochondria from mouse skeletal muscle (see **Fig. 5** for a timeline).

### (A) Isolation of mitochondria from MEFs ● TIMING approximately 2 h

(i) Remove the medium from the cells and wash the cells once with PBS.
(ii) Remove PBS and detach the cells using a cell scraper.
(iii) Transfer the cell suspension to a 50 ml polypropylene Falcon tube.
(iv) Wash the plate once with PBS and scrape the dish to detach the remaining cells.
(v) Transfer the cells to the same polypropylene Falcon tube defined in Step 3. In our experience, seeding 120 × $10^6$ MEFs per dish 2 days before the experiment results in a good yield of mitochondria (approximately 3 mg of mitochondrial protein).
(vi) Centrifuge cells at 600*g* at 4 °C for 10 min.
(vii) Discard the supernatant and resuspend cells in 3 ml of ice-cold $IB_c$.

**600 mM ascorbate stock solution** Dissolve 5.2 g of ascorbic acid in 50 ml of distilled water, adjust pH to 7.4 and store at −20 °C for up to 6 months.
**30 mM TMPD stock solution** Dissolve 0.36 g of TMPD in 50 ml of distilled water; adjust pH to 7.4; store at −20 °C for up to 6 months. The color of the solution is deep blue owing to the oxidation of the compound by oxygen.
**25 mg $ml^{-1}$ antimycin A stock solution** Dissolve 50 mg of antimycin A in 2 ml of absolute ethanol. Dilute the stock solution to 25 µg $ml^{-1}$, by adding 2 µl of 25 mg $ml^{-1}$ Antimycin A in 2 ml of absolute ethonal, just prior to use.
! CAUTION Antimycin A is highly toxic: avoid skin contact and inhalation.
**Buffer for cell and mouse liver mitochondria isolation ($IB_c$)** Prepare 100 ml of IB by adding 10 ml of 0.1 M Tris–MOPS and 1 ml of EGTA/Tris to 20 ml of 1 M sucrose. Bring the volume to 100 ml with distilled water. Adjust pH to 7.4.
**Buffer 1 for muscle mitochondria isolation ($IB_m1$)** Prepare 100 ml of $IB_m1$ by mixing 6.7 ml of 1 M sucrose, 5 ml of 1 M Tris/HCl, 5 ml of 1 M KCl, 1 ml of 1 M EDTA and 2 ml of 10% BSA. Adjust pH to 7.4. Bring the volume to 100 ml with distilled water.
**Buffer 2 for muscle mitochondria isolation ($IB_m2$)** Prepare 100 ml of $IB_m12$ by mixing 25 ml of 1 M sucrose, 3 ml of 0.1 M EGTA/Tris and 1 ml of 1 M Tris/HCl. Adjust pH to 7.4. Bring the volume to 100 ml with distilled water.
**Experimental buffer for cell and mouse-liver mitochondria ($EB_c$)** To prepare 100 ml of $EB_c$, mix 12.5 ml of 1 M KCl, 1 ml of 1 M Tris/MOPS, 10 ml of 100 µl 0.1 M EGTA/Tris and 100 µl of Pi. Adjust pH to 7.4. Bring the volume to 100 ml with distilled water.
**Experimental buffer for muscle mitochondria ($EB_m$)** To prepare 100 ml of $EB_m$, add 1 ml of 1 M Tris/HCl, 1 ml of 0.5 M $MgCl_2$, 200 µl of 1 M Pi and 20 µl of 0.1 M EGTA/Tris to 25 ml of 1 M sucrose. Adjust pH to 7.4. Bring the volume to 100 ml with distilled water. ▲ CRITICAL Wash all glassware three times with bidistilled water to avoid $Ca^{2+}$ contamination. $Ca^{2+}$ overload is the most common cause for the dysfunction of isolated mitochondria. ▲ CRITICAL Prepare all the buffers the same day of the experiment, to avoid bacterial/yeast growth in stored buffers. ▲ CRITICAL Since pH depends on temperature, measure the pH of all solutions at 25 °C.

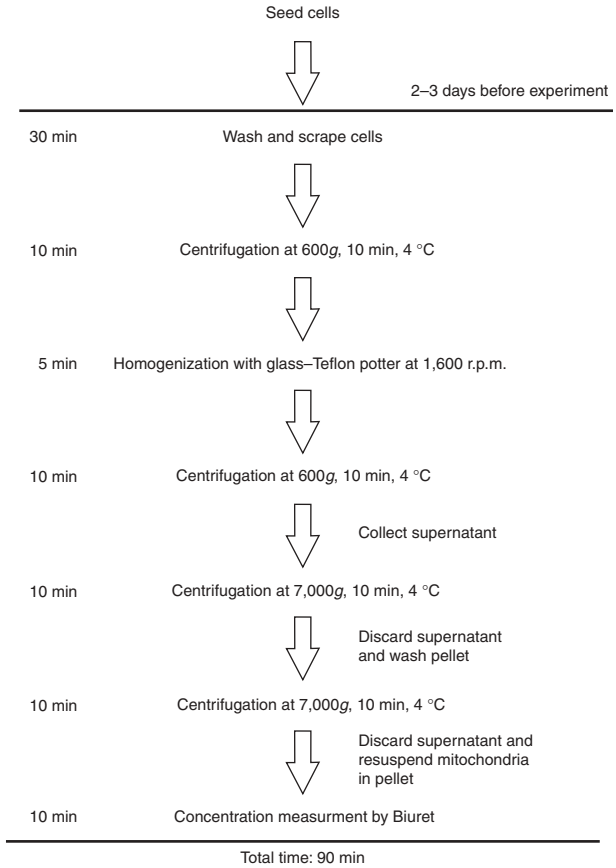


**Figure 3 |** Timing of isolation of mitochondria from MEFs.

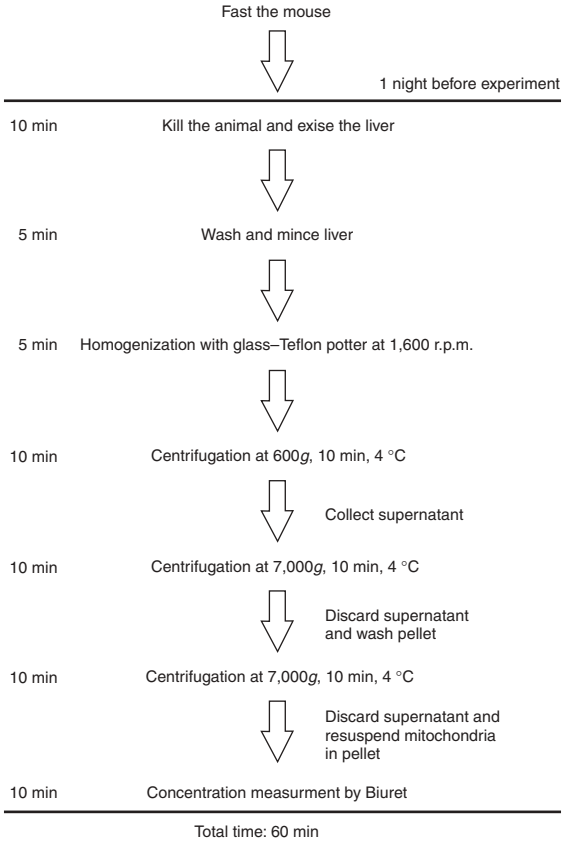


**Figure 4** | Timing of isolation of mitochondria from mouse liver.

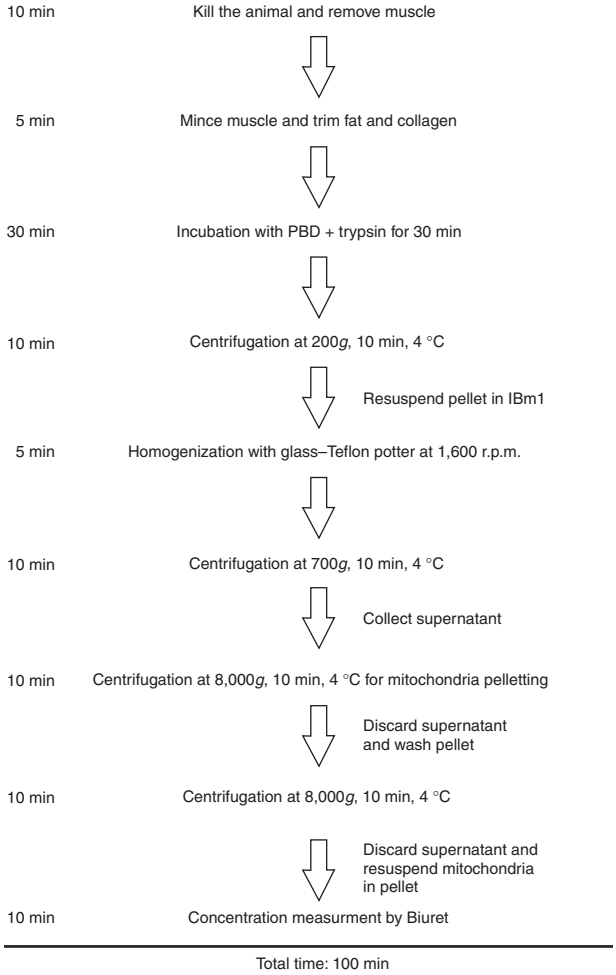


**Figure 5** | Timing of isolation of mitochondria from mouse skeletal muscle.

(viii) Homogenize the cells using a Teflon pestle operated at 1,600 r.p.m.; stroke the cell suspension placed in a glass potter 30–40 times the cell suspension placed in a glass potter.
▲ **CRITICAL STEP** The Teflon–glass coupling represents the best compromise between homogenization of the cells and the preservation of mitochondrial integrity. Harsher techniques, including glass pestle in a glass potter, can easily damage mitochondria.
▲ **CRITICAL STEP** Precool the glassware in an ice-bath 5 min before starting the procedure. Homogenization as well as the following steps must be performed at 4 °C to minimize the activation of damaging phospholipases and proteases.
! **CAUTION** Wear protecting gloves while you are using the homogenizer to avoid possible injuries in the unlikely event that the potter breaks down.
**? TROUBLESHOOTING**

(ix) Transfer the homogenate to a 50 ml polypropylene Falcon tube and centrifuge at 600*g* for 10 min at 4 °C.

(x) Collect the supernatant, transfer it to a glass centrifuge tube and centrifuge it at 7,000*g* for 10 min at 4 °C.
**? TROUBLESHOOTING**

(xi) Discard the supernatant and wash the pellet with 200 µl of ice-cold $IB_c$. Resuspend the pellet in 200 µl of ice-cold $IB_c$ and transfer the suspension to a 1.5 ml microfuge tube.

(xii) Centrifuge the homogenate at 7,000*g* for 10 min at 4 °C.

(xiii) Discard the supernatant and resuspend the pellet containing mitochondria. You can use a glass rod to loosen the pellet paste. Avoid adding IB and try to resuspend the mitochondria in the small amount of buffer that remains after discarding the supernatant. Use a 200 µl pipettor and avoid the formation of bubbles during the resuspension.

(xiv) Transfer the mitochondrial suspension to a microfuge and store it on ice.
▲ **CRITICAL STEP** Avoid diluting mitochondria with buffer. Mitochondria retain their functionality for a longer time, probably as a consequence of lower exposure to oxygen, when they are stored in a concentrated form.

(xv) Measure mitochondria concentration using the Biuret methods.
■ **PAUSE POINT** Mitochondria are now ready to be used in experiments: use the preparation within 1–3 h for better functional responses.
▲ **CRITICAL STEP** The typical yield of this preparation is ~50 mg $ml^{-1}$ in a total volume of approximately 0.1 ml.

▲ CRITICAL STEP The Biuret method for measurement of mitochondrial concentration is accurate in the range of protein concentrations obtained from this protocol; other methods like the Bradford method can be used, but the mitochondrial lysate must be diluted in order to avoid saturation of the probe.

**(B) Isolation of mitochondria from mouse liver ● TIMING approximately 1 h**

(i) Starve the mouse overnight before the isolation experiment.

(ii) Kill an adult mouse (about 30 g) by cervical dislocation and rapidly explant the liver from the peritoneal cavity. Find the gallbladder and remove it using a scalpel. Immerse the liver in 50 ml of ice-cold $IB_c$ in a small beaker.
▲ CRITICAL STEP Local and national regulations on animal care and handling vary. Check that you hold the appropriate authorization to perform animal experiments.

(iii) Rinse the liver free of blood by using ice-cold $IB_c$. Usually, four or five washes are sufficient to completely clarify the $IB_c$.

(iv) Mince the liver into small pieces using scissors. This should be performed while keeping the beaker in an ice bath.

(v) Discard the $IB_c$ used during the mincing and replace it with 5 ml of ice-cold fresh $IB_c$. Transfer the suspension to the glass potter.
▲ CRITICAL STEP Homogenization, as well as the following steps, must be performed at 4 °C to minimize activation of damaging phospholipases and proteases.

(vi) Homogenize the liver using a Teflon pestle operated at 1,600 r.p.m., stroke the minced liver 3–4 times.
▲ CRITICAL STEP The optimal ratio between tissue and isolation buffer ranges between 1:5 to 1:10 (w:v).
▲ CRITICAL STEP Precool the glassware in an ice-bath 5 min before starting the procedure. Homogenization and the following steps must be performed at 4 °C to minimize activation of damaging phospholipases and proteases.
! CAUTION Wear protecting gloves while you are using the homogenizer to avoid possible injuries in the unlikely event that the potter breaks down.
? TROUBLESHOOTING

(vii) Transfer the homogenate to a 50 ml polypropylene Falcon tube and centrifuge at 600*g* for 10 min at 4 °C.

(viii) Transfer the supernatant to glass centrifuge tubes and centrifuge at 7,000*g* for 10 min at 4 °C.
? TROUBLESHOOTING

(ix) Discard the supernatant and wash the pellet with 5 ml of ice-cold $IB_c$.

(x) Centrifuge at 7,000*g* for 10 min at 4 °C.

(xi) Discard the supernatant and resuspend the pellet, containing mitochondria. You can use a glass rod to loosen the pellet paste. Avoid adding IB and try to resuspend the mitochondria in the small amount of buffer that remains after discarding the supernatant. Use a 1 ml pipettor and avoid the formation of bubbles during the resuspension process.

(xii) Transfer mitochondrial suspension into a 14 ml Falcon tube and store on ice.
▲ CRITICAL STEP Avoid diluting mitochondria with buffer as mitochondria retain their functionality for a longer time when kept concentrated, minimizing exposure to oxygen.
■ PAUSE POINT Mitochondria are now ready to be used in experiments; use the preparation within 1–3 h for better functional responses.

(xiii) Measure mitochondrial concentration using the Biuret methods.
▲ CRITICAL STEP The usual concentration of mitochondria in this kind of preparation is about 80 mg $ml^{-1}$ and the total volume is about 1 ml.
▲ CRITICAL STEP The Biuret method for measurement of mitochondrial concentration is accurate in the range of protein concentrations obtained from this protocol; other methods like the Bradford method can be used, but the mitochondrial lysate must be diluted in order to avoid saturation of the probe.

**(C) Isolation of mitochondria from mouse skeletal muscle ● TIMING approximately 1.5 h**

(i) Kill the mouse by cervical dislocation. Using a scalpel, rapidly remove the skeletal muscles of interest and immerse them in a small beaker containing 5 ml of ice-cold PBS supplemented with 10 mM EDTA. A timeline of this protocol is outlined in **Figure 6**.
▲ CRITICAL STEP Local and national regulations on animal care and handling vary. Check that you hold the appropriate authorizations to perform animal experiments.
▲ CRITICAL STEP The use of EDTA instead of EGTA chelates also $Mg^{2+}$, which is extremely abundant in muscle tissue (given the high content in ATP). $Mg^{2+}$ can influence mitochondrial function as well as the kinetics of cytochrome *c* release[25].

(ii) Mince the muscles into small pieces using scissors and trim visible fat, ligaments and connective tissue.

(iii) Wash the minced muscles twice or thrice with ice-cold PBS supplemented with 10 mM EDTA.

(iv) Resuspend the minced muscles in 5 ml of ice-cold PBS supplemented with 10 mM EDTA and 0.05% trypsin for 30 min.

(v) Centrifuge at 200*g* for 5 min and discard the supernatant.

(vi) Resuspend the pellet in $IB_m1$.

(vii) Homogenize the muscles using a Teflon pestle operated at 1,600 r.p.m.; stroke the minced muscle ten times.
▲ CRITICAL STEP The optimal ratio between tissue and isolation buffer ranges between 1:5 and 1:10 (w:v).
▲ CRITICAL STEP Precool the glassware in an ice-bath 5 min before starting the procedure. Homogenization, and the following steps, must be performed at 4 °C to minimize the activation of damaging phospholipases and proteases.
! CAUTION Wear protecting gloves while you are using the homogenizer to avoid possible injuries in the unlikely event that the potter breaks down.
Precool the glassware in an ice-bath for 5 min before starting the following steps.
? TROUBLESHOOTING
(viii) Transfer the homogenate to a 50 ml polypropylene Falcon tube and centrifuge at 700*g* for 10 min at 4 °C.
(ix) Transfer the supernatant to glass centrifuge tubes and centrifuge at 8,000*g* for 10 min at 4 °C.
? TROUBLESHOOTING
(x) Discard the supernatant and resuspend the pellet in 5 ml of ice-cold $IB_m2$.
(xi) Centrifuge at 8,000*g* for 10 min at 4 °C.
(xii) Discard the supernatant and resuspend the pellet containing mitochondria. You can use a glass rod to loosen the pellet paste. Avoid adding IB and try to resuspend the mitochondria in the small amount of buffer that remains after discarding the supernatant. Use a 200 µl pipettor and avoid the formation of bubbles during the resuspension process.
(xiii) Transfer mitochondrial suspension into a 14 ml Falcon tube and keep it on ice.
(xiv) Measure mitochondrial concentration using the Biuret methods.
▲ CRITICAL STEP This preparation normally yields 0.8 ml of 50 mg $ml^{-1}$ mitochondria.
▲ CRITICAL STEP The Biuret method for measurement of mitochondrial concentration is accurate in the range of protein concentrations obtained from this protocol; other methods like the Bradford method can be used, but the mitochondrial lysate must be diluted in order to avoid saturation of the probe.

**Measuring mitochondrial respiration ● TIMING approximately 1 h**

**2|** Calibrate the Clarke-type oxygen electrode. Procedures vary from instrument to instrument. You should follow the manufacturer's instructions for the instrument you are using.

**3|** Equilibrate temperature and oxygen tension of EBc or EBm by placing open beakers containing the buffers in the water bath connected to the oxygraph. After 20–30 min, the temperature of the buffers is likely to be in equilibrium with that of the water bath.

**4|** Add an appropriate volume of EB to the oxygraph chamber. Use 0.5 ml for the mitochondria isolated from cells and 1 or 2 ml for the liver and muscle mitochondria. Close the oxygraph chamber.

**5|** Start the recording of the oxygen consumption.
▲ CRITICAL STEP Verify that the recording is stable and that no drifts are apparent. Drifts can mask the oxygen consumption by the mitochondrial preparation and thereby complicate the interpretation of the results.
? TROUBLESHOOTING

**6|** Wait for 2 min to obtain a stable baseline.

**7|** Using an appropriate Hamilton microsyringe, add mitochondria to obtain a final concentration of 1 mg $ml^{-1}$. A fast, transitory decrease in the oxygen content of the chamber will be observed, caused by anaerobiosis of the isolated mitochondria; this will be followed by a slower decrease caused by the respiration of the mitochondria. This is supported by endogenous substrates and is commonly referred to as "state 1" respiration[26].

**8|** Record oxygen consumption till it stops.
! CAUTION In liver mitochondria state 1 respiration commonly does not stop.

**TABLE 1** | Substrates and inhibitors of the respiratory chain.

| | Substrate (final concentration) | Inhibitor (final concentration) |
|---|---|---|
| Complexes I, III, IV | Glutamate (5 mM)/malate (2.5 mM) | /// |
| Complexes II, III, IV | Succinate (5 mM) | Rotenone (2 µM) |
| Complex IV | Ascorbate (6 mM)/TMPD (300 µM) | Antimycin A (0.25 µg $ml^{-1}$) |

The span of the respiratory chain examined by each combination of substrate/inhibitor is indicated along with the final concentration to use in the oxygraphy experiments.

**9|** Using Hamilton microsyringes, add the appropriate concentrations of respiratory substrates and inhibitors for the complexes of the respiratory chain you wish to study (refer to **Table 1**). The mitochondrial suspension will now start consuming oxygen as a consequence of the basal activity of the respiratory chain in counteracting the inner mitochondrial membrane proton leak. This represents the so-called "state 2" respiration[26].

▲ CRITICAL STEP The rate of oxygen consumption should now be faster than the rate observed with buffer alone. This indicates that you have obtained functional, respiring mitochondria.

? TROUBLESHOOTING

**10|** Record for 5 min.

**11|** Add ADP to obtain a final concentration of 100–150 μM. Faster consumption of oxygen will be observed. This has been caused by proton back-diffusion through the stalk portion of the ATPase, which has been compensated by faster electron flow through the respiratory chain to the terminal electron acceptor, $O_2$. This is classically referred to as "state 3" respiration[26].

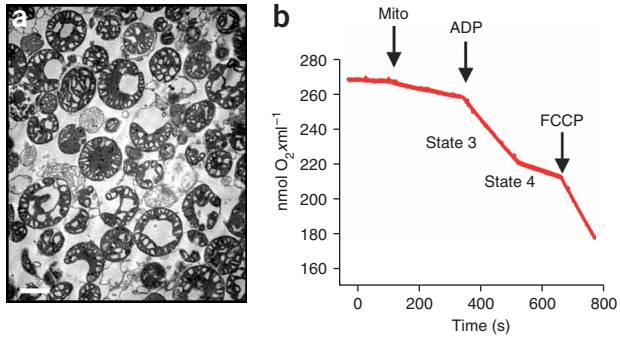


**Figure 6 |** Ultrastructure and oxygen consumption of mouse liver mitochondria isolated according to the protocol presented. (**a**) Mitochondrial ultrastructure. Mouse liver mitochondria (0.5 mg $ml^{-1}$) incubated in EB supplemented with 5 mM glutamate and 2.5 mM malate for 5 min were fixed by adding glutaraldehyde (final concentration 2.5% (v/v)). Transmission electron micrographs were acquired from randomly selected fields, as described[18]. (**b**) Oxygen consumption of 1 ml EB supplemented with 5 mM glutamate and 2.5 mM malate. Where indicated (arrows), mouse liver mitochondria (MLM, final concentration 1 mg $ml^{-1}$), ADP (100 μM) and FCCP (60 nM) were added. Respiration after ADP stimulation is indicated as "state 3", whereas respiration after consumption of added ADP is indicated as "state 4."

▲ CRITICAL STEP The rate of oxygen consumption should now be faster than the rate observed with substrates alone, indicating that we have obtained well coupled mitochondria. The increase in respiration, observed with ADP, varies from tissue to tissue and from substrate to substrate. As a general rule, and for the sole purpose of quality control of the preparation, the minimum requirements to proceed with the experiment are as follows: using glutamate malate as a substrate, maintaining a ratio of 2 in mitochondria isolated from cell lines and a ratio of 4 in mitochondria isolated from tissues.

? TROUBLESHOOTING

**12|** Wait until the respiration slows down and returns to a rate comparable to that before the addition of ADP. This is caused by the consumption of the added ADP. The respiration, which follows ADP exhaustion, is classically referred to as "state 4" respiration[26].

**13|** Wait for 3 min.

**14|** Add the uncoupler FCCP to obtain a final concentration of 60–100 nM.

**15|** The respiration will speed up and reach values slightly higher than those observed during the recording of state-3 respiration.

? TROUBLESHOOTING

**16|** Record for a further 5 min and then stop recording.

● TIMING

Step 1A: approximately 2 h, depending on the amount of cells to be used; however, cells will need to be seeded 2 or 3 d in advance to let them grow

Step 1B: approximately 1 h; however the mouse will need to be fasted from the night before

Step 1C: 1.5 h, depending on the amount of muscle to be minced

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

**TABLE 2 |** Troubleshooting table.

| Step | Problem | Possible causes | Solution |
|---|---|---|---|
| 1Aviii, 1Bvi, 1Cvii | Low yield of isolated mitochondria | Low cell density during homogenization | Low cell density may result in better homogenization. However, mitochondria are usually of lower quality, probably as a consequence of mechanical damage during preparation |

**TABLE 2** | Troubleshooting table (continued).

| Step | Problem | Possible causes | Solution |
|---|---|---|---|
| 1Ax, 1Bviii, 1Cix | Low quality of isolated mitochondria | Pellet after centrifugation is lost | When the supernatant is poured off, the loose upper part of the mitochondrial pellet may be detached as well. Intact mitochondria tend to sediment more quickly than damaged mitochondria. The loose part of the pellet probably contains a high proportion of damaged (uncoupled) mitochondria and can be lost without affecting the overall quality of the mitochondrial preparation |
| 1Ax, 1Bvii | Low quality of isolated mitochondria | Lipid contamination | The white foamy material near the top of the tube consists of lipids. Mixing of lipids with the mitochondria suspension will cause some degree of uncoupling. Therefore avoid contact with mitochondria: remove the foamy material by wiping the inside of the tube with a Kimwipe |
| 5 | Oxygen consumption baseline is not stable | Bacterial or yeast contamination of your buffer | Verify if your buffers are contaminated, by repeating the recording with bidistilled water in the oxygraph chamber |
| | | Inadequate calibration of the instrument | Re-calibrate the instrument |
| | | Tears in the polyethylene membrane of the electrode | Check response of the oxygraph by transiently stopping stirring: due to the immediate drop in the local oxygen concentration the recording should immediately fall and return to the original baseline only when the stirrer is restarted. If this *manoeuvre* does not give the expected results, inspect and if necessary substitute the membrane of the electrode |
| 9 | Mitochondrial preparation is not consuming oxygen | Overestimation of final protein concentration (therefore added too little protein in the oxygraph chamber) | Try to double mitochondrial concentration in the chamber |
| | | Mechanical and osmotical damage to mitochondria during isolation | Substitute 0.2 M sucrose with 0.3 M mannitol in the isolation buffer |
| | | Contamination by other intracellular membranes, such as endoplasmic reticulum or nuclei | In steps 1Axi, 1Bix or 1Cx, wash the mitochondrial pellet with twice the amount of isolation buffer |
| 11 | ADP-stimulated respiration rate is too low | The most trivial explanation is that you omitted Pi from your buffer | Add Pi and check the respiration |
| | | High percentage of mitochondria with ruptured outer membranes that leaked cytochrome *c* | Add exogenous cytochrome *c* and check the respiration; if respiration starts, the outer membrane is leaky. See troubleshooting for step 9 (mechanical and osmotic damage) |
| | | Unusually high basal respiration, as a consequence of uncoupling by $Ca^{2+}$ overload | Follow carefully all the indicated critical steps to avoid the indicated contaminations; try washing glassware with isolation buffer, supplemented with EGTA |
| | | Unusually high basal respiration, as a consequence of uncoupling by fatty acids | Include 0.1% fatty acid free albumin in the EB; if this procedure works increase FCCP concentration since albumin binds reversibly to FCCP |
| 15 | Uncoupled respiration rate is lower than ADP-stimulated respiration | Too much FCCP used | Since at high doses FCCP is also an inhibitor of the respiratory chain, you can overcome this problem by titrating down the concentration of FCCP used |

## ANTICIPATED RESULTS

The goal of a mitochondrial preparation is to obtain a good amount of relatively pure, well coupled mitochondria. The quality of the obtained organelles can be checked by using oxygraphy to measure their oxygen consumption. For example, mitochondria isolated from mouse liver and energized with glutamate/malate respond to stimulation of ATPase by added ADP with a sixfold

increase in the rate of oxygen consumption (**Fig. 6b**). This usually reflects mitochondria that are highly pure and intact. A closer look by conventional electron microscopy at the morphology and at the purity of the organelles isolated from other intracellular membranes revealed that most of the organelles displayed an intact inner and outer membrane and that the level of contamination by other membranes was kept to a minimum (**Fig. 6a**).

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