# Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells

Mariusz R Wieckowski[1,4], Carlotta Giorgi[2–4], Magdalena Lebiedzinska[1], Jerzy Duszynski[1] & Paolo Pinton[2]

[1]Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland. [2]Department of Experimental and Diagnostic Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI) and Emilia Romagna Laboratory BioPharmaNet, University of Ferrara, Ferrara, Italy. [3]Vita-Salute San Raffaele University, Center of Excellence in Cell Development, and IIT Network, Research Unit of Molecular Neuroscience, Milan, Italy. [4]These authors contributed equally to this work. Correspondence should be addressed to M.R.W. (m.wieckowski@nencki.gov.pl) and P.P. (pnp@unife.it).

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**Many cellular processes require the proper cooperation between mitochondria and the endoplasmic reticulum (ER). Several recent works show that their functional interactions rely on dynamic structural contacts between both organelles. Such contacts, called mitochondria-associated membranes (MAMs), are crucial for the synthesis and intracellular transport of phospholipids, as well as for intracellular $Ca^{2+}$ signaling and for the determination of mitochondrial structure. Although several techniques are available to isolate mitochondria, only few are specifically tuned to the isolation of MAM, containing unique regions of ER membranes attached to the outer mitochondrial membrane and mitochondria without contamination from other organelles (i.e., pure mitochondria). Here we provide optimized protocols to isolate these fractions from tissues and cells. These procedures require 4–5 h and can be easily modified and adapted to different tissues and cell types.**



## INTRODUCTION

The association of endoplasmic reticulum (ER) and mitochondria was first described in 1958 (ref. 1). Later, in the early 70s, various groups observed the close apposition of the outer mitochondrial membrane and the ER in rat liver and cultured rat hepatocytes[2–4]. Twenty years later, the mitochondria-associated membrane (MAM) fraction was first separated and characterized by Vance[5]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the MAM fraction has shown both differences and similarities to the mitochondrial and ER protein profile and composition. Structural and functional interactions of mitochondria with the ER have been demonstrated for rat[5] and mouse liver[6], rat brain[7], Chinese hamster ovary cells[8] and also for yeast[9–11]. It is important to note that direct fusion between membranes of the ER and the mitochondria has not been reported; membranes invariably always maintained organelles as separate structures. In living HeLa cells, ~20% of the mitochondrial surface is in direct contact with the ER[12]. A groundbreaking electron microscopic study yielded important information concerning the 'real' structure of subcellular organelles and confirmed the existence of numerous physical links between the mitochondria and the ER[13].

### Background information about the MAM fraction

As reported in the literature, the MAM fraction, enriched in phospholipid synthesis enzymes (i.e., diacylglycerol acyltransferase, phosphatidylserine synthase and cholesterol acyltransferase), has often been described as a center of nonvesicle interorganelle lipid trafficking[14]. The abnormal accumulation of lipids and proteins in storage bodies in neuronal ceroid lipofuscinosis (NCL) appeared to be the consequence of impaired interorganelle lipid transport, especially between the ER and the mitochondria. Indeed, analysis of subcellular fractions of livers obtained from the animal model related to this neurodegenerative disease (mnd/mnd mouse) clearly showed that the affected lipid trafficking was associated with a reduced amount of MAM[14]. It is interesting to note that proteins present at contact sites between organelles (i.e., the MAM fraction) also enable the selective release and uptake of $Ca^{2+}$ from the ER and the mitochondria, respectively. Close apposition of inositol 1,4,5-trisphosphate ($IP_3$)-gated channels receptor ($IP_3R$) from the ER to the mitochondrial surface enables the uptake of $Ca^{2+}$ by mitochondria via a low-affinity $Ca^{2+}$ uniporter in a very efficient way, because it can be exposed to a microdomain of $Ca^{2+}$ concentration higher than in the bulk cytosol after cell stimulation[12].

Thus, numerous proteins have been recently proposed to participate in the interaction and communication between the mitochondria and the ER, highlighting the emerging role of this region in bioenergetics, cell survival and cell death[15,16]. $IP_3Rs$ have been shown to be localized in the proximity of mitochondria[17], and its co-localization[18] and interaction with the outer mitochondrial membrane (OMM) resident voltage-dependent anion channel (VDAC) have been recently proved[19]. The assembly of $IP_3R$–VDAC complexes is mediated by ER and mitochondria resident stress related chaperones. Sigma-1 receptors (Sig1R) are enriched in the MAM fraction and recruit $Ca^{2+}$-binding chaperones grp78, ankyrin-B and type 3 $IP_3R$ (refs. 20–22). Also, the OMM-associated fraction of the matrix chaperone grp75 bridges the gap between $IP_3R$ and VDAC[19]. In addition, other $Ca^{2+}$-binding ER resident chaperones have been found in the MAM fraction, e.g., calnexin (CNX), calreticulin and ERp44[23–25]. The multifunctional cytosolic sorting protein PACS-2 is another protein that has been found in the MAM fraction[26]. This fraction can also contain adenine nucleotide translocase (ANT) and cyclophylin D, the components of mitochondrial contact sites with similar composition to the mitochondrial permeability transition pore (MPTP). Such close apposition of the MPTP to the ER can sensitize mitochondria to $Ca^{2+}$ signals[27]. Recently, the mitochondrial GTPase mitofusin 2 has been shown to be enriched in MAM as well as localized on the ER, where it interacts with mitofusins on mitochondria to form interorganellar bridges[28].

All these findings highlight the importance of having a method to isolate this fraction and unravel the mechanism and function of MAM.

### Previous results obtained using this protocol

This procedure describes how to obtain highly purified MAM and mitochondria from rat liver and cultured cells. It is important

to note that the protocol can be easily modified to isolate MAM from other organs, tissues or cell types.

Recently, we have successfully used this procedure to study its molecular composition[19] and propose a role of MAM fraction in cellular response to oxidative stress connected with the phosphorylation of p66Shc protein. The data obtained indicate that a significant portion of p66Shc is present in the MAM fraction and that its level depends on the age of animal[29]. Moreover, it has been shown that, in mouse embryonic fibroblast (MEF) cells responding to the oxidative stress, a part of the cytosolic pool of p66Shc protein translocates to crude mitochondrial fraction (containing MAM)[30]. Further purification of crude mitochondria, resulting in the isolation of highly purified mitochondria and MAM, showed that most of p66Shc was present in MAM, whereas highly purified mitochondria contained only a low amount of p66Shc[29]. This strongly suggests that the MAM fraction, because of its characteristic composition, can also be involved in signal transduction pathways transmitting different signals to the mitochondria.

Various protocols are available to obtain mitochondria[31], but these are mostly limited to the isolation of a crude mitochondria fraction that does not permit an accurate analysis of MAM composition. It should be noted that with the present protocol it is possible to obtain a pure mitochondria fraction, wherein the ER, the nuclear and other nonmitochondrial markers disappear. Finally, it should be stressed that the protocol originally described for MAM isolation[5] refers only to isolation of this fraction from the liver and not from cell cultures. Based on the experience of our laboratories, we propose a well-tried isolation protocol, which enables the isolation of high-purity MAM and mitochondria fractions from animal tissues and cell cultures. This protocol is potentially useful for a wide range of studies in different physiological and pathological cellular processes.

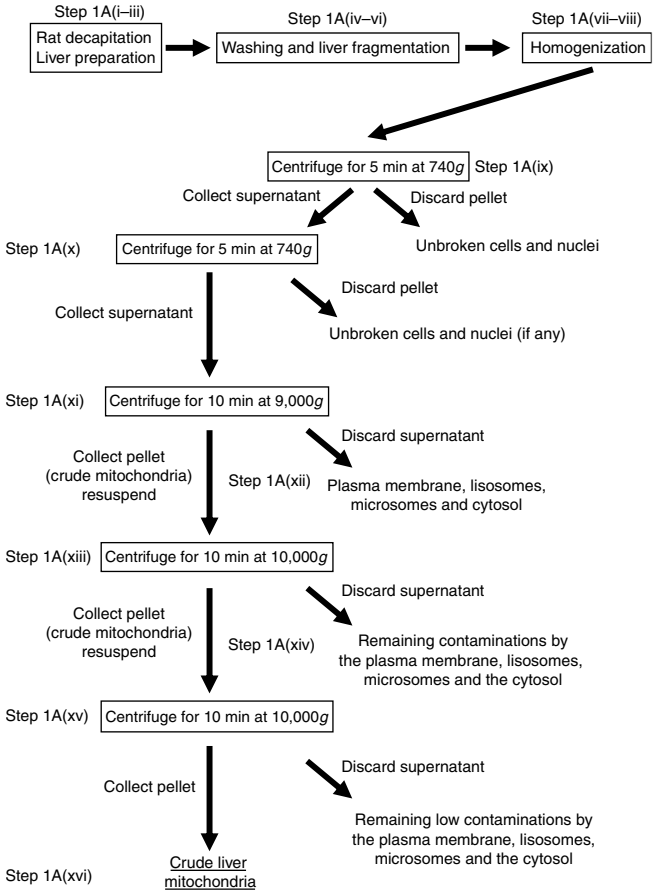


**Figure 1** | Timeline and schematic steps of crude mitochondria isolation from rat liver.

### Overview of procedure

The procedure can be divided into 2 main sections. In the first, a crude mitochondrial fraction is isolated from tissue (**Fig. 1**) or cells (**Fig. 2**). In the second, crude mitochondria are fractionated to the pure mitochondria and MAM fraction. These fractions can be purified from crude mitochondria isolated from different tissues and cell lines (see **Fig. 3** for a timeline).

## MATERIALS

Biological material—rat or mouse liver or cell line of interest **! CAUTION** Experiments using rodents must conform to National and Institutional regulations.

**REAGENTS**

- Bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A6003) **▲ CRITICAL** BSA is used to remove (bind) free fatty acids. For this reason it is important to use BSA essentially for free fatty acids.
- D-Mannitol (Sigma-Aldrich, cat. no. M4125)
- Disodium ethylenediaminetetraacetate dihydrate (EDTA; Sigma-Aldrich, cat. no. ED2SS)
- Dulbecco's modified Eagle's medium (Invitrogen, cat. no. 11971025)
- Dulbecco's phosphate-buffered saline (D-PBS), liquid, without $Ca^{2+}$ and $Mg^{2+}$ (Invitrogen, cat. no. 70011036)
- Dulbecco's phosphate-buffered saline (D-PBS), liquid, with $Ca^{2+}$ and $Mg^{2+}$ (Invitrogen, cat. no.14040091)
- Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA; Sigma-Aldrich, cat. no. E4378)
- Fetal bovine serum (Invitrogen, cat. no. 10270106)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- L-glutamine 200 mM (100×) liquid (Invitrogen, cat. no. 25030024)
- Penicillin (5,000 U $ml^{-1}$)–streptomycin (5,000 mg $ml^{-1}$) (Invitrogen, cat. no. 15070063)
- Percoll (Sigma-Aldrich, cat. no. P1644)
- Phosphatase inhibitor cocktail 3(100×) (Sigma, cat. no. P0044)
- Protease inhibitor cocktail (100×) (Sigma, cat. no. P8340)
- Sucrose (Merck, cat. no. 100892.9050) **▲ CRITICAL** To isolate intact mitochondria, it is necessary to use sucrose containing low-calcium contamination.
- Trizma-Base (Sigma-Aldrich, cat. no. T1503)
- Trypsin, 0.25% (1×) with EDTA $4Na^{+}$, liquid (Invitrogen, cat. no. 25200072)

**EQUIPMENT**

- Cell-culture dishes, 10 cmΦ (Sarstedt, cat. no. 83.1802)
- Cell scrapers (Sarstedt, cat. no. 83.1830)
- Stirrer motor with electronic speed controller (Cole-Palmer, cat. no. EW-04369-10)
- Motor-driven tightly fitting glass/Teflon Potter Elvehjem homogenizer
- Loose fitting glass/Teflon Potter Elvehjem homogenizer
- Oak Ridge Nalgene 30-ml tubes (for Sigma rotor angular 6 × 30 ml, Model 12139)
- Ultra-Clear 14-ml polypropylene tubes (Beckman, cat. no. 344060) for SW40 rotor
- Polycarbonate tubes with cap assembly (Beckman, cat. no. 355618) for 70-Ti rotor
- 1.5-ml Eppendorf microfuge test tubes (Eppendorf AG, Hamburg, Germany, cat. no. 0030 120.086)
- 70-Ti rotor (Fixed angle, 8 × 39 ml, 70,000 r.p.m., 504,000*g*) (Beckman, cat. no. 337922)
- SW 40 rotor (Swinging bucket, 6 × 14 ml, 40,000 r.p.m., 285,000*g*) (Beckman, cat. no. 331302)

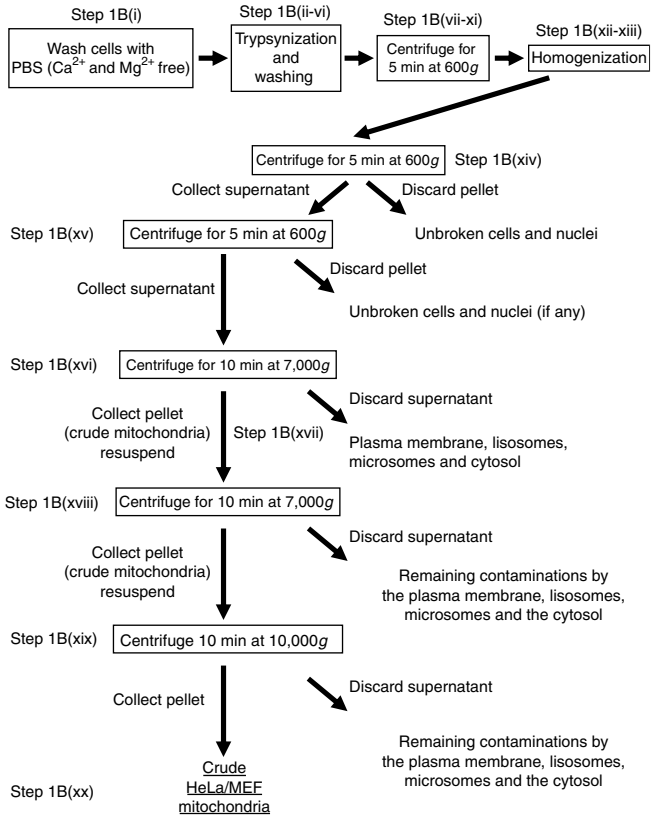


**Figure 2** | Timeline and schematic steps of crude mitochondria isolation from HeLa/mouse embryonic fibroblast (MEF) cells.

- Sigma rotor angular 6 × 30 ml (Sigma, cat. no. 12139)
- Beckman Coulter Optima L-100 XP Ultracentrifuge (Beckman Coulter, Inc.)
- Refrigerated Sigma low-speed centrifuge (Sigma (Braun), Model 2K15, tabletop)
- Low-speed MPW 342 centrifugewith rotor no. 12108
- Nikon TMS (PRECOPTIC Co.–Nikon, Poland; cat. no. 310681) microscope

**REAGENT SETUP**

**1-M Tris–HCl (pH 7.4)** Dissolve 121.14 g of Trizma-Base in 500 ml of bi-distilled water, adjust pH to 7.4 using HCl, bring the solution to 1 liter with bi-distilled water and store at 4 °C. The solution remains stable for a long period of time.

**0.5-M HEPES (pH 7.4)** Dissolve 59.57 g of HEPES in 400 ml of bi-distilled water, adjust pH to 7.4 using KOH, bring the solution to 500 ml with bi-distilled water and store at 4 °C. The solution remains stable for a long period of time.

**100-mM EGTA (pH 7.4)** Dissolve 3.8 g of EGTA in 70 ml of bi-distilled water, adjust pH to 7.4 with KOH, bring the solution to 100 ml with bi-distilled water and store at 4 °C. The solution remains stable for a long time period.

**Starting buffer (SB): 225-mM mannitol, 75-mM sucrose and 30-mM Tris–HCl pH 7.4 (for $IB_{cells}$-1, $IB_{cells}$-2, $IB_{liver}$-1, $IB_{liver}$-2 and $IB_{liver}$-3)** Dissolve 20.5 g of mannitol, 13 g of sucrose in 400 ml of bi-distilled water and add 15 ml of Tris–HCl (1 M pH 7.4). Leave the buffer for about 30 min at 4 °C to cool down. Check the pH of the buffer and adjust if necessary with KOH (if too low) or HCl (if too high) and bring the solution to a final volume of 500 ml with bi-distilled water and store at 4 °C. ▲ CRITICAL In all, 300–400 ml is enough for one isolation of MAM and pure mitochondria from the liver and cells. ▲ CRITICAL Wash all glasswares with bi-distilled water to avoid $Ca^{2+}$ contamination. $Ca^{2+}$ contamination can cause swelling of the mitochondria and rupture of the outer mitochondrial membrane. ▲ CRITICAL This buffer needs to be prepared fresh the day before or on the day of experiment and must be free of protease and phosphatase inhibitor cocktails to avoid sample alteration. ▲ CRITICAL Extreme care should be taken to avoid contamination with the ice and tap water in all preparations.

**$IB_{cells}$-1: 225-mM mannitol, 75-mM sucrose, 0.1-mM EGTA and 30-mM Tris–HCl pH 7.4** Mix 150 ml of SB and 60 µl of 100-mM EGTA (pH 7.4). ▲ CRITICAL EGTA is recommended to remove traces of $Ca^{2+}$. It can be replaced by EDTA, but at a lower concentration ~0.05–0.1 mM but not higher than 0.1 mM. ▲ CRITICAL This buffer needs to be prepared fresh the day before or on the day of experiment and must be free of protease and phosphatase inhibitor cocktails to avoid sample alteration.

**$IB_{cells}$-2: 225-mM mannitol, 75-mM sucrose and 30-mM Tris–HCl pH 7.4** $IB_{cells}$-2 has the same composition as SB and so is made up the same way.

**$IB_{liver}$-1: 225-mM mannitol, 75-mM sucrose, 0.5% BSA, 0.5-mM EGTA and 30-mM Tris–HCl pH 7.4** Dissolve 0.75 g of albumin in 150 ml of SB, add 750 µl of 100-mM 0.5 mM EGTA (pH 7.4) and store at 4 °C. ▲ CRITICAL EGTA is recommended to remove traces of $Ca^{2+}$. It can be replaced by EDTA, but at a lower concentration, not higher than 0.1 mM. ▲ CRITICAL This buffer needs to be prepared fresh the day before or on the day of experiment and must be free of protease and phosphatase inhibitor cocktails to avoid sample alteration.

**$IB_{liver}$-2: 225-mM mannitol, 75-mM sucrose, 0.5% BSA and 30-mM Tris–HCl pH 7.4** Dissolve 0.25 g of albumin in 150 ml of SB and store at 4 °C. ▲ CRITICAL This buffer needs to be prepared fresh the day before or on the day of experiment and must be free of protease and phosphatase inhibitor cocktails to avoid sample alteration.

**$IB_{liver}$-3: 225-mM mannitol, 75-mM sucrose and 30-mM Tris–HCl pH 7.4** $IB_{liver}$-3 has the same composition as SB and so is prepared the same way.

**MRB (mitochondria resuspending buffer): 250-mM mannitol, 5-mM HEPES (pH 7.4) and 0.5-mM EGTA** To prepare 100 ml of MRB solution dissolve 4.56 g of mannitol in 80 ml of bi-distilled water, add 1 ml of 0.5-M HEPES (pH 7.4). Leave the buffer for about 30 min at 4 °C to cool down. Check the pH of the buffer and adjust if necessary with KOH (if too low) or HCl (if too high) and bring the solution to a final volume of 100 ml with bi-distilled water and store at 4 °C. ▲ CRITICAL This buffer needs to be prepared fresh the day before or on the day of experiment and must be free of protease and phosphatase inhibitor cocktails to avoid sample alteration.

**Percoll medium: 225-mM mannitol, 25-mM HEPES (pH 7.4), 1-mM EGTA and 30% Percoll (vol/vol)** Dissolve 2.052 g of mannitol in 25 ml of bi-distilled water, add 2.5 ml of 0.5-M HEPES (pH 7.4) and 0.5 ml of 100-mM EGTA (pH 7.4). Leave the buffer for about 30 min at 4 °C to cool down. Check the pH of the buffer and adjust if necessary with KOH (if too low) or HCl (if too high) and bring this basal solution to a final volume of 35 ml with bi-distilled water and store at 4 °C.

On the day of experiment prepare 16 ml of Percoll medium (8 ml for one ultracentrifuge tube) by mixing 4.8 ml of Percoll and 11.2 ml of basal solution.

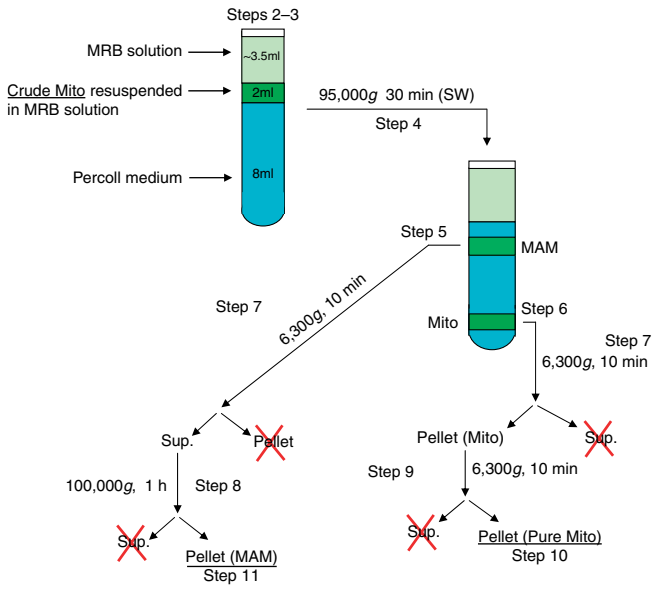


**Figure 3** | Scheme for mitochondria-associated membrane (MAM) and pure mitochondria isolation from crude mitochondria.

## PROCEDURE

### Preparation of crude mitochondrial fraction

**1|** Isolate crude mitochondria from different tissues (e.g., rat liver) as described in option A (see **Fig. 1** for a timeline) or cells (e.g., HeLa or MEFs) as described in option B (see **Fig. 2** for a timeline). In our experience, 60–80 HeLa confluents plates (10 cmΦ) and 150 confluents plates (10 cmΦ) for MEFs are enough to isolate satisfying amounts of crude mitochondrial fraction needed to purify MAM fraction and pure mitochondrial fraction.

**(A) Isolation of crude mitochondria from rat liver ● TIMING ~1.5 h**

(i) Starve the animal overnight before the isolation of mitochondria.
▲ **CRITICAL STEP** High levels of glycogen present in the liver of non-starved animals affect the mitochondria isolation.

(ii) Kill the animal by decapitation and immediately wash the liver in ice-cold $IB_{liver}$-1.

(iii) Remove gallbladder and transfer the liver into a 50-ml beaker.

(iv) Wash the liver 3–4 times using ice-cold $IB_{liver}$-3 to remove the blood.

(v) Cut the liver into small pieces using scissors.

(vi) Discard the bloody $IB_{liver}$-3 and wash once again with fresh 10 ml of ice-cold $IB_{liver}$-1. Discard the bloody $IB_{liver}$–1 solution.

(vii) Transfer the liver pieces to the 50-ml glass/Teflon Potter Elvehjem homogenizer. Add $IB_{liver}$-1 in the ratio 4 ml of buffer per gram of liver
▲ **CRITICAL STEP** Precool the glassware and homogenizer with pestle in an ice-bath 5 min before starting the procedure. Homogenization as well as the following steps must be carried out at 4 °C (i.e., in cold room) to minimize the activation of proteases and phospholipases.

(viii) Homogenize the liver pieces using a Teflon pestle by eight strokes at 1,500 r.p.m. The integrity of homogenized cell can be checked under a light microscope.
**! CAUTION** Wear protecting gloves while using the homogenizer to avoid possible injuries in the unlikely event that the potter breaks down.
▲ **CRITICAL STEP** Pestle homogenization at higher force and speed can affect mitochondria integrity. The movement of the pestle should be slow and done with 'fluidity'.
**? TROUBLESHOOTING**

(ix) Transfer the homogenate to a 30-ml polypropylene centrifugation tube and centrifuge at 740*g* for 5 min at 4 °C (refrigerated Sigma low-speed centrifuge).

(x) Collect the supernatant and discard the pellet (containing unbroken cells and nuclei). Centrifuge again at 740*g* for 5 min at 4 °C (refrigerated Sigma low-speed centrifuge).

(xi) Collect the supernatant, discard the pellet (if present) and centrifuge at 9,000*g* for 10 min at 4 °C (refrigerated Sigma low-speed centrifuge).

(xii) Discard the supernatant (this is a cytosolic fraction containing lysosomes and microsomes) and gently resuspend the pellet containing mitochondria in 20 ml of ice-cold $IB_{liver}$-2.
■ **PAUSE POINT** Store the supernatant at 4 °C (on ice) up to 1.5 h if there is a plan to proceed with further separation of cytosolic, lisosomal and ER fractions. To perform such subfractionation centrifuge supernatant at 20,000*g* for 30 min at 4 °C. Pellet consists of lisosomal fraction containing also plasma-membrane contamination. Further centrifugation of the obtained supernatant (100,000*g* for 1 h) results in isolation ER (pellet) and cytosolic fraction (supernatant).
▲ **CRITICAL STEP** Resuspension of mitochondria at this step performed with pipettor or tight potter can result in the rupture of OMM and detachment of MAM fraction from mitochondria. It is necessary to transfer the mitochondrial pellet from the centrifuge tube to the homogenizer using a 1-ml 'automatic' pipette with the cut-out end of the tip. For resuspension process use loose fitting glass/Teflon Potter Elvehjem homogenizer (two to three hand-made strokes are enough) or alternatively a soft paintbrush.

(xiii) Centrifuge mitochondrial suspension at 10,000*g* for 10 min at 4 °C (refrigerated Sigma low-speed centrifuge).

(xiv) Discard the supernatant and gently resuspend the crude mitochondrial pellet in 20 ml of $IB_{liver}$-3.
▲ **CRITICAL STEP** It is necessary to transfer the mitochondrial pellet from the centrifuge tube to the homogenizer using a 1-ml 'automatic' pipette with the cut-out end of the tip. For resuspension process use loose fitting glass/Teflon Potter Elvehjem homogenizer (two to three hand-made strokes are enough) or alternatively a soft paintbrush.

(xv) Centrifuge mitochondrial suspension again at 10,000*g* for 10 min at 4 °C (refrigerated Sigma low-speed centrifuge).
▲ **CRITICAL STEP** It is important to perform all three centrifugation steps (one at 9,000*g* and two at 10,000*g*) to remove any microsomal contamination, which can be coisolated in the next step with the MAM fraction.
**? TROUBLESHOOTING**

(xvi) Discard the supernatant and resuspend the crude mitochondrial pellet in 2 ml of ice-cold MRB.
▲ **CRITICAL STEP** It is necessary to transfer the mitochondrial pellet from the centrifuge tube to the homogenizer using a 1-ml 'automatic' pipette with the cut-out end of the tip. For resuspension process use loose fitting glass/Teflon Potter Elvehjem homogenizer (two to three hand-made strokes are enough) or alternatively a soft paintbrush.

■ **PAUSE POINT** Store a small amount (0.5 ml) of crude mitochondrial fraction for further investigations (western blot) in a 1.5-ml Eppendorf microfuge. Freeze at −20 °C if not used immediately.

**(B) Isolation of crude mitochondria from HeLa or MEF cells ● TIMING ~2.5 h**

(i) Remove the medium and wash cells twice with PBS (without $Ca^{2+}$ and $Mg^{2+}$).

(ii) Remove PBS and add 1 ml of trypsin solution per 10-cmΦ plate.

(iii) Incubate plates in the incubator (about 3 min) to detach cells.

(iv) Add 6 ml of DMEM to stop trypsinization and detach cells.

(v) Transfer the cell suspension to 50-ml polypropylene tube.

(vi) Wash plates once with PBS (with $Ca^{2+}$ and $Mg^{2+}$), detach the remaining cells with the use of scraper and transfer cells to the same polypropylene tube.

(vii) Centrifuge cells at 600*g* for 5 min at 4 °C (low-speed MPW 342 centrifuge with rotor no. 12108).

(viii) Discard the supernatant and resuspend cells in 30 ml of PBS (with $Ca^{2+}$ and $Mg^{2+}$).

(ix) Centrifuge cells at 600*g* for 5 min at 4 °C (low-speed MPW 342 centrifuge with rotor no. 12108).

(x) Resuspend cells in small volume (5 ml) of PBS (with $Ca^{2+}$ and $Mg^{2+}$) and combine all cell suspension in one 50-ml polypropylene tube.

(xi) Centrifuge cells at 600*g* for 5 min at 4 °C (low-speed MPW 342 centrifuge with rotor no. 12108).

(xii) Discard the supernatant and resuspend cell pellet in 20 ml of ice-cold $IB_{cells}$–1.

▲ **CRITICAL STEP** Precool the glassware and homogenizer with pestle in an ice-bath 5 min before starting the homogenization step (xiii). Homogenization as well as the following steps must be carried out at 4 °C (i.e., in cold room) to minimize the activation of proteases and phospholipases.

(xiii) Homogenize cells (at 4,000 r.p.m.) using a Teflon pestle. Every 25 strokes, control cell integrity under the microscope. Finish homogenization when 80–90% of cell damage has been attained. The integrity of homogenized cell can be checked under a light microscope.

▲ **CRITICAL STEP** Pestle homogenization at higher force and speed can affect mitochondrial integrity. The movement of the pestle should be slow and done with 'fluidity'.

**!** **CAUTION** Wear protecting gloves while using the homogenizer to avoid possible injuries in the unlikely event that the potter breaks down.

**?** **TROUBLESHOOTING**

(xiv) Transfer the homogenate to a 30-ml polypropylene centrifugation tube and centrifuge at 600*g* for 5 min at 4 °C (refrigerated Sigma low-speed centrifuge).

(xv) Collect supernatant and discard the pellet (containing unbroken cells and nuclei). Centrifuge again at 600*g* for 5 min at 4 °C (refrigerated Sigma low-speed centrifuge).

(xvi) Collect supernatant, discard the pellet (if present) and centrifuge at 7,000*g* for 10 min at 4 °C (refrigerated Sigma low-speed centrifuge).

(xvii) Discard the supernatant (this is a cytosolic fraction containing lysosomes and microsomes) and gently resuspend the pellet containing mitochondria in 20 ml of ice-cold $IB_{cells}$-2.

■ **PAUSE POINT** Store the supernatant at 4 °C (on ice) up to 1.5 h if there is a plan to proceed with further separation of cytosolic, lisosomal and ER fractions. To perform such subfractionation centrifuge supernatant at 20,000*g* for 30 min at 4 °C. The pellet consists of lisosomal and plasma membrane fractions. Further centrifugation of the obtained supernatant (100,000*g* for 1 h) results in the isolation of ER (pellet) and cytosolic fraction (supernatant).

▲ **CRITICAL STEP** Resuspension of the mitochondria carried out at this step with pipettor or tight potter can result in the breaking of OMM and detaching of MAM fraction from mitochondria. It is necessary to transfer the mitochondrial pellet from the centrifuge tube to the homogenizer using a 1-ml 'automatic' pipette with the cut- out end of the tip. For resuspension process use loose fitting glass/Teflon Potter Elvehjem homogenizer (two to three hand-made strokes are enough) or alternatively a soft paintbrush.

(xviii) Centrifuge mitochondrial suspension at 7,000*g* for 10 min at 4 °C (refrigerated Sigma low-speed centrifuge).

(xix) Discard the supernatant, resuspend the mitochondrial pellet as before, in 20 ml of ice-cold $IB_{cells}$-2 and centrifuge mitochondrial suspension at 10,000*g* for 10 min at 4 °C.

**?** **TROUBLESHOOTING**

(xx) Discard the supernatant and resuspend gently the crude mitochondrial pellet in 2 ml of ice-cold MRB buffer.

▲ **CRITICAL STEP** It is necessary to transfer the mitochondrial pellet from the centrifuge tube to the homogenizer using a 1-ml 'automatic' pipette with the cut-out end of the tip. For resuspension process use loose fitting glass/Teflon Potter Elvehjem homogenizer (two to three hand-made strokes are enough) or alternatively a soft paintbrush.

■ **PAUSE POINT** Store a small amount (0.2 ml) of crude mitochondrial fraction for further investigations (western blot) in 1.5-ml Eppendorf microfuge. Freeze at −20 °C if not used immediately.

**Fractionation of crude mitochondria** ● TIMING ~2–2.5 h

**2|** Add 8 ml of Percoll medium to the 14-ml thin-wall, Polyallomer ultracentrifuge tubes.

**3|** Layer suspension of mitochondria collected in options A (xvi) or B (xx) on top of 8 ml of Percoll medium in the ultracentrifuge tube (see **Fig. 3**). Afterward, layer the MRB solution (about 3.5 ml) gently on top of the mitochondrial suspension to fill up the centrifuge tube (the suspension should remain 4–5 mm below the top of the tubes).
▲ CRITICAL STEP The filling up of tubes is important to protect them against damage during centrifugation.
? TROUBLESHOOTING

**4|** Centrifuge at 95,000*g* for 30 min at 4 °C in a Beckman Coulter Optima L-100 XP Ultracentrifuge (SW40 rotor, Beckman, Fullerton, CA, USA). A dense band containing purified mitochondria is localized approximately at the bottom of the ultracentrifuge tube (see **Figs. 3** and **4**). MAM is visible as the diffused white band located above the mitochondria (see **Figs. 3** and **4**).

**5|** Collect the MAM fraction from the Percoll gradient with a Pasteur pipette and dilute ten times with MRB.
? TROUBLESHOOTING

**6|** Collect pure mitochondria band with a Pasteur pipette and dilute ten times with MRB.

**7|** Centrifuge MAM and mitochondrial suspension at 6,300*g* for 10 min at 4 °C (refrigerated Sigma low-speed centrifuge).

**8|** Transfer the MAM supernatant to a polycarbonate tubes with cap assembly discard the pellet (containing mitochondrial contamination) and centrifuge at 100,000*g* for 1 h (70-Ti rotor, Beckman) at 4 °C.

**9|** Discard the mitochondrial supernatant, obtained in Step 7 (containing MAM contamination), resuspend gently the pellet in 20 ml of MRB and centrifuge at 6,300*g* for 10 min at 4 °C (refrigerated Sigma low-speed centrifuge).
▲ CRITICAL STEP It is necessary to transfer the mitochondrial pellet from the centrifuge tube to the homogenizer using a 1-ml 'automatic' pipette with the cut-out end of the tip. For resuspension process use loose fitting glass/Teflon Potter Elvehjem homogenizer (two to three hand-made strokes are enough) or alternatively a soft paintbrush.

**10|** Discard supernatant (from Step 9) and resuspend the pellet of pure mitochondria in a small volume of MRB (300–500 µl).
▲ CRITICAL STEP The typical yield of this preparation is ~40 mg $ml^{-1}$ in a total volume of ~0.5 ml from the liver and ~15 mg $ml^{-1}$ in a total volume of ~0.3 ml from the cell culture.
▲ CRITICAL STEP It is necessary to transfer the mitochondrial pellet from the centrifuge tube to the homogenizer using a 1-ml 'automatic' pipette with the cut-out end of the tip. For resuspension process use loose fitting glass/Teflon Potter Elvehjem homogenizer (two to three hand-made strokes are enough) or alternatively a soft paintbrush.

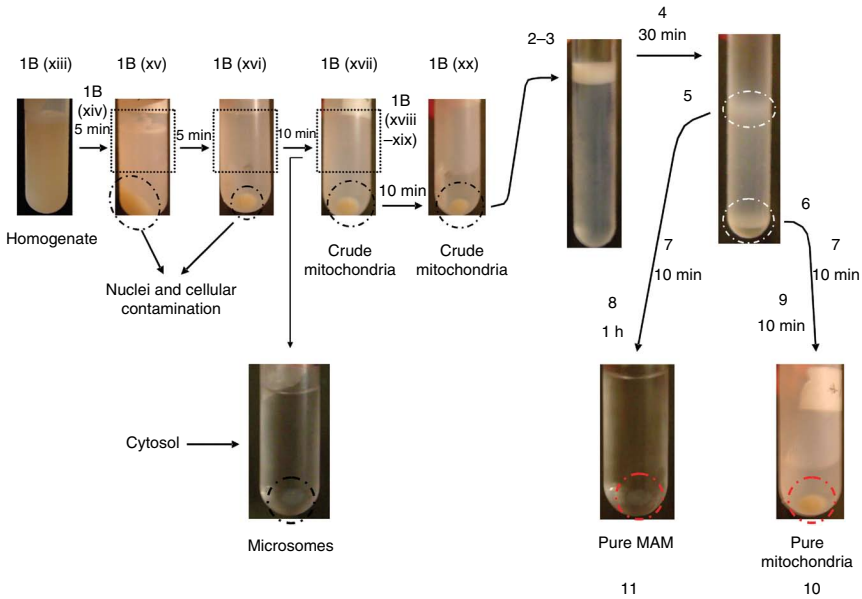


**Figure 4** | Pictures of different kinds of pellet and fractions obtained during the isolation process. Diagram shows typical result of HeLa cells fractionation. The final steps of presented procedure result in isolation of pure mitochondria, microsomes (endoplasmic reticulum (ER)), cytosolic and mitochondria-associated membrane (MAM) fraction. The size of obtained pellets depends on the amount of material used for fractionation. Moreover, the amount of different fractions depends on tissue or cell types used.

■ **PAUSE POINT** Store the pure mitochondrial fraction for further investigations (western blot) in 1.5-ml Eppendorf microfuge. Freeze at −20 °C if not used immediately.
**? TROUBLESHOOTING**

**11|** Discard the supernatant (from Step 8) and resuspend the pellet of MAM in a small volume of MRB (100–200 µl). **Figure 4** presents a picture of the different kinds of pellet obtained during all steps of the fractionation.
▲ **CRITICAL STEP** The typical yield of this preparation is ~1 mg $ml^{-1}$ in a total volume of ~0.2 ml from the liver and 1 mg $ml^{-1}$ in a total volume of ~0.1 ml.
■ **PAUSE POINT** Store the isolated MAM fraction for further investigations (western blot) in 1.5-ml Eppendorf microfuge. Freeze at −20 °C if not used immediately.
**? TROUBLESHOOTING**

● **TIMING**
Step 1(A), Isolation of crude mitochondria from rat liver: ~1.5 h
Step 1(B), Isolation of crude mitochondria from cells: ~2.5 h, depending on the type and amount of cells to be used
Steps 2–11, Fractionation of crude mitochondria: ~2–2.5 h

**? TROUBLESHOOTING**
Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

| Step | Problem | Possible causes | Solution |
|---|---|---|---|
| 1A(viii)<br>1A(xv)<br>1B(xiii)<br>1B(xix)<br>3 and 5 | Low quality of isolated MAM | Gradient break (MAM band appear fragmented) | Layer carefully mitochondrial suspension on Percoll medium in Step 3 |
| | | Crude mitochondria are contaminated by other organelles | Dilute tissue/cell homogenate before first centrifugation (1A(viii); 1B(xiii)). If necessary add one more centrifugation step (10 min at 10,000*g*) after 1A(xv) or 1B(xix) |
| 1A(viii)<br>1B(xiii)<br>5, 11 | Low amount of isolated MAM | Pellet after centrifugation is lost | When the supernatant is poured off, the MAM pellet—a thin bright 'membrane' —may be detached. Remove the MAM membrane directly without discarding the supernatant in Step 5 |
| | | Not enough material was used for isolation | Use more cells or tissue for isolation |
| | | Homogenization was insufficient | Homogenize longer. Check cell disruption under microscope (1A(viii) and 1B(xiii)) |
| 1A(viii)<br>1B(xiii)<br>10 | Low amount of isolated pure mitochondria pellet | Pellet after centrifugation is lost | Presence of little amount of Percoll tends to draw down pure mitochondrial pellet that could have detached. Remove the mitochondrial pellet directly without discarding the supernatant in Step 9 |
| | | Not enough material was used for isolation | Use more cells or tissue for isolation |
| | | Homogenization was insufficient | Homogenize longer. Check cell disruption under microscope (1A(viii) and 1B(xiii)) |

MAM, mitochondria-associated membrane.

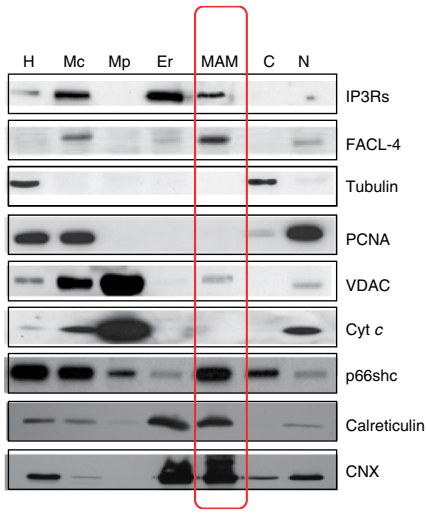


**Figure 5** | Intracellular distributions of p66Shc. Protein components of subcellular fractions prepared from mouse embryonic fibroblast (MEF) cells revealed by immunoblot analysis. H: homogenate; Mc: crude mitochondrial fraction; Mp: pure mitochondrial fraction; ER; MAM: mitochondria-associated membrane; C: cytosol; N: nuclear fraction. A total of 30 µg of proteins were loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (BioRad, Hercules, CA, USA) and transferred onto nitrocellulose membranes. Membranes were blocked using 5% non-fat milk in phosphate-buffered saline (PBS) with 0.01% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Proteins were detected with anti-p66Shc (mouse monoclonal, 1:1,000; Abcam), anti-inositol 1,4,5-trisphosphate-gated channels receptor ($IP_3R_3$) (1:500; BD-Pharmingen, San Diego, CA, USA), anti-PCNA (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) anti-pan voltage-dependent anion channel (VDAC) (1:5,000; Abcam, Cambridge, UK), anti-cytochrome *c* (1:1,000; BD-Pharmingen), anti-long-chain fatty-acid CoA synthases (FACL-4) (1:1,000; Santa Cruz Biotechnology), anti-tubulin (1:10,000; Santa Cruz Biotechnology), anti-calreticulin (1:1,000; Santa Cruz Biotechnology), anti-calnexin (CNX) (1:1,000; Stressgen, Victoria, BC, Canada), followed by secondary horseradish peroxidase (HRP)-conjugated antibodies (1:5,000) from Santa Cruz Biotechnology.

## ANTICIPATED RESULTS

The quality of MAM preparation can be checked by western blot analysis. By using different markers for the fractions obtained, it is possible to control the purity of MAM fraction and the presence of contaminations from other compartments. **Figure 5** reports, as an example, analysis of p66shc intracellular localization in MEF cells. As mentioned above, p66shc is localized predominantly in the MAM fraction and only a small amount of protein is present in the pure mitochondrial fraction. The following markers distribution thus characterize a good fractionation:

- Long-chain fatty-acid CoA synthases (FACL)-4 as MAM marker should be enriched in this fraction.
  Long-chain fatty-acid CoA synthases also known as acyl-CoA synthetases or palmitoyl-CoA ligases is important for the synthesis of cellular lipids and for β-oxidation degradation. FACL-4 protein is a single-pass membrane protein localizing to the mitochondrion, microsome or peroxisome, but extremely enriched in the MAM fraction.
- Inositol 1,4,5-trisphosphate-gated channels receptor as ER marker should be present at about 20% in MAM fraction of than present in the ER. Inositol 1,4,5-trisphosphate-gated channels receptor is an ER channel responsible for the agonist-dependent ER-$Ca^{2+}$ release.
- Calreticulin as ER marker and MAM marker should be present at comparable levels in these fractions.
  Calreticulin is an ER-$Ca^{2+}$-buffering protein binding misfolded proteins and prevents them from being exported from the ER to the golgi apparatus.
- Calnexin as ER marker and MAM marker should be present at comparable levels in these fractions.
  Calnexin is a chaperone protein involved in protein folding and protein quality control.
- Voltage-dependent anion channel as mitochondrial marker should be present in "crud" mitochondria (Mc), but must be enriched in "pure" mitochondria (Mp) fraction. It should be also present in the MAM fraction. The VDAC, also known as mitochondrial porin, is localized on the outer mitochondria membrane and is responsible of the permeability of that membrane.
- Cytochrome *c* as a mitochondrial marker should be extremely enriched in the Mp fraction and lacked in MAM fraction.
  Cytochrome *c* is a component of electron-transfer chain localized in the mitochondrial inter-membrane space.
- Tubulin as a cytosolic marker should be absent in Mp, MAM and ER.
- Proliferating cell nuclear antigen (PCNA) as a nuclear marker should be absent in Mp and MAM.

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