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Mitochondrial Isolation and Real-Time Monitoring of MOMP

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Abstract

Isolated model systems have proven to be the standard in the apoptosis field to deconstruct MOMP into individual steps and to study the behavior of a subset of MOMP regulators. Here we describe the method to isolate, JC-1-label, and purify mouse liver mitochondria and subsequently describe how to utilize the JC-1-labeled mitochondria for real-time MOMP measurements.

Keywords

Isolated mitochondria; JC-1 labeling; Real-time MOMP measurements; BCL-2 family; BAX

1 Introduction

The mitochondrial pathway of apoptosis is dependent on a complex interplay between a combination of proteins, lipids, and mitochondria [1–4]. Cellular system models are not ideal to investigate the complex interplay between these factors. As such, isolated organellar systems are ideal because purified recombinant proteins (e.g., caspase 8-cleaved BID), isolated lipids (e.g., sphingosine-1-phosphate), and small molecules [5] can be examined in known quantities in biochemically defined environments [4, 6, 7].

In this chapter, we describe a protocol to isolate mitochondria and investigate the effects of BCL-2 proteins on MOMP in real time (Fig. 1). We divide the protocol into three sections: (1) “Liver mitochondria isolation” in which a liver is dissected from a mouse to purify mitochondria; (2) “Mitochondria labeling with JC-1” describes how to label; and (3) “Real-time MOMP measurements” demonstrates how to monitor MOMP in real time using a plate reader in a 96-well format.

2 Materials

2.1 Liver Mitochondria Isolation

1. C57BL/6 mice (*see* Note 1).
2. Phosphate Buffered Saline (PBS): 2.7 mM KCl, 1.5 mM KH₂PO₄, 136.9 mM NaCl, 8.9 mM Na₂HPO₄·7H₂O.
3. Potter-Elvehjem Dounce homogenizer 2 mL.
4. Swinging bucket centrifuge.
5. Spectrophotometer.
6. Trehalose Isolation Buffer (TIB): 200 mM trehalose, 68 mM sucrose, 10 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% BSA, 1 tablet of protease inhibitor cocktail (*see* Notes 2 and 3).
7. HEKM buffer: 10 mM HEPES, pH 7, 0.2 mM EDTA, 200 mM KCl, 5 mM MgCl₂.

2.2 Mitochondria Labeling

1. JC-1: 5, 5', 6, 6'-Tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolocarbocyanine iodide.
2. Sepharose CL-2B resin.
3. Gravity poly-prep chromatography column.
4. FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone.
5. Triton X-100.

2.3 MOMP Measurements

1. Recombinant protein/peptide/drugs, e.g., BAX [6] (*see* Note 4).
2. β -octylglucoside (OG).
3. Oligomycin in ethanol (25 mM stock).
4. KCL in ddH₂O (3 M stock).
5. HEKM buffer: 10 mM HEPES, pH 7, 0.2 mM EDTA, 200 mM KCl, 5 mM MgCl₂.

3 Methods

Animal handling, euthanasia, and dissection must be done following the Institutional Animal Care and Use Committee (IACUC) guidelines.

3.1 Liver Mitochondria Isolation

1. Visualize the liver, bile duct, and gallbladder. The bile duct and gallbladder must be removed or kept separate from the liver in order to avoid contamination of the mitochondria with bile.
2. Excise the four liver lobes and transfer immediately to a 50 mL conical tube containing 15 mL ice-cold PBS buffer (*see* Notes 5 and 6).
3. Transfer the liver to a clean petri dish placed on ice and drain off the excess PBS.
4. Mince the liver using a clean razor blade until a homogeneous paste is formed.
5. Transfer half of the liver paste into a chilled Potter-Elvehjem Dounce homogenizer containing 10 mL TIB.
6. Insert the pestle into the homogenizer and gently push it downward. Resistance is common during the first strokes, and no excessive force should be applied to the liver sample. Retract the pestle to resuspend the liver sample and once again, gently push downward. Slowly repeat this motion until all the material is able to go past the pestle without strong resistance.
7. Homogenize the paste five times, transfer into a prechilled 15 mL conical tube, and store on ice until further processing.
8. Repeat **steps 6 and 7** with the second half of the liver paste.
9. The following centrifugation steps are required to obtain the mitochondrial fraction (Fig. 2). All the steps should be performed in a swinging bucket centrifuge at 4 °C. Resuspension of pellets is done gently by pipetting using a P1000 pipette.
 - a. $600 \times g$ for 10 min. Transfer the supernatant (S/N) to a clean, prechilled 15 mL conical tube.
 - b. $3500 \times g$ for 10 min. Discard the S/N. Resuspend the pellet in 10 mL TIB.
 - c. $1500 \times g$ for 5 min. Transfer the S/N to a clean, prechilled 15 mL conical tube.
 - d. $5500 \times g$ for 10 min. Discard the S/N. Resuspend the pellet in 10 mL TIB buffer.
 - e. Repeat **steps 9b–9d**, but resuspend the final pellet in 500 μ L TIB.
10. To quantify the amount of isolated mitochondria in the resuspended pellet (**step 9e**), add 5 μ L of the resuspended pellet to 995 μ L of TIB (1:200 dilution) and

measure the OD₅₂₀ with a spectrophotometer (*see* Note 7). Dilute the mitochondria with TIB accordingly to standardize the sample.

11. At this point the isolated mitochondria can be aliquoted (50 μ L), frozen in dry ice–ethanol, and stored at -80°C (*see* Note 8).

3.2 Mitochondria Labeling with JC-1 and Purification

Labeling mitochondria with JC-1, a radiometric mitochondrial potential indicator that changes color as membrane potentials increase during MOMP, allows for standardization of mitochondrial fractions and to record MOMP in real time.

1. Dilute 50 μ L of mitochondria in 250 μ L TIB.
2. Add JC-1 to the diluted mitochondria to a final concentration of 15 μ M and incubate for 10 min at 30°C (*see* Note 9).
3. Centrifuge the mitochondria at $5550 \times g$ for 10 min at room temperature and resuspend the pellet in 25 μ L TIB.
4. Preequilibrate a 2 mL CL-2B gravity column with two column volumes of TIB. Load the resuspended JC-1-labeled mitochondria onto the column, and allow sample to flow through (*see* Note 10).
5. Using a clean Pasteur pipette, slowly apply four column volumes of TIB to the column and collect $20 \times 100 \mu\text{L}$ ($\sim 2\text{--}3$ drops) fractions in microcentrifuge tubes.
6. To determine which fractions contain labeled mitochondria, prefill a 96-well plate with 95 μ L of 0.1% Triton X-100 in water and add 5 μ L from each fraction to a separate well (*see* Note 11).
7. Measure the fluorescence using a spectrophotometer (Ex: 561 nm/Em: 620 nm) to identify the mitochondria-containing fractions.
8. Combine the fractions containing JC-1-labeled mitochondria and standardize the samples using relative JC-1 fluorescence intensity (*see* Note 12).

3.3 Real-Time MOMP Measurements

Real-time MOMP quantification is determined by measuring the mitochondrial membrane potential (ψ_M) using the fluorescent JC-1 dye. The JC-1 dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~ 529 nm) to red (~ 590 nm). MOMP and the subsequent loss of ψ_M are indicated by a decrease in the red–green fluorescence intensity ratio. There is an abundance of options for investigating a subset of inducers and regulators of MOMP in real time. It is important to include internal and experimental controls. An example is given in Table 1, and many commercially available proteins, peptides, and small molecules can be tested (*see* Table 2 for examples) (*see* Note 13).

1. Prepare a $2 \times$ protein/inducer solution in TIB (supplemented with 25 μ M oligomycin and an additional 200 mM KCl; 12.5 μ M oligomycin and 110 mM KCl final concentrations; used to prevent ψ_M reactivation, and to dissociate

cytochrome *c* from the membrane, respectively) and dispense 50 µL in each well of a 96-well plate. The plate should contain the following internal and experimental controls (Fig. 3; Table 1).

- a. Negative internal control: TIB only.
 - b. Positive internal control: 2× FCCP (20 µM in TIB).
 - c. Negative experimental control: purified recombinant BAX (20–50 nM final concentration; a titration will be needed to identify a concentration that does not permeabilize as different preparations display different activities).
 - d. Positive experimental control: β-octylglucoside-activated BAX (BAX^{OG}, 20–50 nM final concentration; a titration will be needed to identify a concentration that permeabilizes as different preparations display different activities).
2. Prepare a 2× suspension of JC-1 loaded mitochondria (~1.25 Relative Fluorescence Units (RFU) per µL final concentration) and add 50 µL to each well (*see* Note 14).
 3. Measure the fluorescence every 5 min in a plate reader for 60 min (Ex: 561 nm/Em: 620 nm) at 37 °C.
 4. Calculate the percentage of mitochondria that have undergone MOMP using the following formula:

$$\% \text{ MOMP} = \left(\text{RFU}_{\text{buffer}} - \text{RFU}_{\text{sample}} \right) / \left(\text{RFU}_{\text{buffer}} - \text{RFU}_{\text{FCCP}} \right)$$

where RFU_{buffer} and RFU_{FCCP} represent the fluorescence of the negative and positive internal controls, respectively, and RFU_{sample} is the fluorescence of the sample.

4 Notes

1. Mice were purchased from The Jackson Laboratory (another source is Charles River Laboratories). Female mice at 6 weeks of age were used for liver mitochondria isolation.
2. Protease inhibitor cocktail should be added fresh according to manufacturer's instructions.
3. TIB should be prepared using BSA-Fraction V to eliminate fatty acid and lipid contaminants, which promote nonspecific BAK/BAX activation.
4. Recombinant BAX protein was expressed and purified as described in Suzuki et al. [6].
5. In case of blood contamination, wash the liver in ice-cold PBS until no more blood is washed out.

6. From this point, all the steps must be performed using reagents and material at 4 °C to minimize the activation of proteases and phospholipases.
7. An OD₅₂₀ value of 0.25 equals ~20 µg/µL of protein.
8. Freshly isolated mitochondria should be used within 2 h to ensure integrity of the outer membrane. If frozen mitochondria are used, thaw the sample in a 30 °C water bath and continue using TIB in the subsequent procedures.
9. The stock solution of JC-1 at 200 µM is prepared in dimethyl-sulfoxide (DMSO) and diluted accordingly in TIB.
10. Make sure that the resin does not run dry before loading the JC-1-labeled mitochondria.
11. 0.1% Triton X-100 dissolves mitochondria.
12. Determine the fluorescence of each combined sample as described in **steps 6 and 7** in Subheading 3.2. Combine the fractions containing labeled mitochondria and use a standard number of RFU per sample in the 96-well experiment, which approximately corresponds to 50 µg of mitochondria protein.
13. For the positive internal control, we use FCCP that uncouples the electron transport chain from oxidative phosphorylation in mitochondria by depolarizing the mitochondrial membrane. To generate a positive experimental control, we use detergent-activated recombinant BAX [2]. The detergent, β-octylglucoside (OG), artificially triggers BAX activation, and therefore BAX^{OG} (20–50 nM) can be used as a reliable positive control. For 100 µL of 2 µM BAX^{OG}: ~4.3 µg BAX + 0.7% OG in HEKM buffer, incubate for 60 min at 4 °C, aliquot and store at –80 °C.
14. The amount of JC-1-labeled mitochondria of ~50–100 RFUs per reaction is ideal.

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References

1. Luna-Vargas MP, Chipuk JE (2016) The deadly landscape of pro-apoptotic BCL-2 proteins in the outer mitochondrial membrane. *FEBS J* 283:2676–2689. 10.1111/febs.13624 [PubMed: 26662859]
2. Renault TT, Floros KV, Chipuk JE (2013) BAK/BAX activation and cytochrome c release assays using isolated mitochondria. *Methods* 61:146–155. 10.1016/j.ymeth.2013.03.030 [PubMed: 23567751]
3. Renault TT et al. (2015) Mitochondrial shape governs BAX-induced membrane permeabilization and apoptosis. *Mol Cell* 57:69–82. 10.1016/j.molcel.2014.10.028 [PubMed: 25482509]

4. Kuwana T et al. (2005) BH3 domains of BH3-only proteins differentially regulate BAX-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 17:525–535. 10.1016/j.molcel.2005.02.003 [PubMed: 15721256]
5. Reyna DE et al. (2017) Direct activation of BAX by BTSA1 overcomes apoptosis resistance in acute myeloid leukemia. *Cancer Cell* 32:490–505 e410 10.1016/j.ccell.2017.09.001 [PubMed: 29017059]
6. Suzuki M, Youle RJ, Tjandra N (2000) Structure of BAX: coregulation of dimer formation and intracellular localization. *Cell* 103:645–654 [PubMed: 11106734]
7. Chipuk JE et al. (2008) Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. *Proc Natl Acad Sci U S A* 105:20327–20332. 10.1073/pnas.0808036105 [PubMed: 19074266]

Fig. 1.

Overview of the mouse liver mitochondria isolation and real-time MOMP study. **Step 1** in the method is the isolation of mitochondria from mouse liver. Followed by **step 2** in which mitochondria are labeled and purified. Finally, **step 3** involves the real-time MOMP measurements

Fig. 2.

A schematic diagram depicting how to isolate liver mitochondria during the different centrifugal steps using a swinging bucket centrifuge at 4 °C. (1) repeat the centrifuge steps from $3500 \times g$ for 10 min through $5500 \times g$ for 5 min. (2) final step is resuspending the pellet in 500 µL TIB

Internal controls	Experimental controls
Negative: Buffer Positive: 10 μ M FCCP	Negative: 20-50 nM BAX Positive: 20-50 nM BAX ^{OG}

Fig. 3. Setup for a real-time MOMP measurement in a 96-well plate. For the internal control, buffer and 10 μ M FCCP were used as negative and positive controls, respectively. For the experimental control, 20–50 nM BAX and 20–50 nM BAX^{OG} were used as negative and positive controls, respectively. The fluorescence is measured every 5 min (Ex: 561 nm and Em: 620 nm) for an hour and is plotted over time

Table 1

An experimental design to measure MOMP in real time with the appropriate internal and experimental controls

Wells	Conditions	TIB buffer (μl)	FCCP [20μM] (μl)	BAX [2μM] (μl)	BAX ^{OG} [2μM] (μl)	2× protein/ inducer (μl)	2× JC-1 loaded mitochondria [~2.5 RFU/μ]	Total (μl)
1	Negative internal control: TIB buffer	50					50	100
2	Positive internal control: FCCP [10 μM final]		50				50	100
3	Negative experimental control: BAX [50 nM final]	47.5		2.5			50	100
4	Positive experimental control: BAX ^{OG} [50 nM final]	47.5			2.5		50	100
5	Protein/inducer 1×					50	50	100

Table 2

An overview of commercially available recombinant BCL-2 proteins, BH3 peptides, and small molecules targeting BCL-2 proteins

Human Recombinant Proteins/Peptides	Amino Acids	Tags	Manufacturer	Cat.#
BAX	1–192	-	Not Available	Reference #6
BCL-2	2–211	C-term 10-His tag	R&D Systems	827-BC-050
BCL-xL	2–212	C-term 6-His tag	R&D Systems	894-BX-050
Full-length BID	1–195	-	R&D Systems	846-BD-050
Caspase-8 cleaved BID	1–60, 61–195	-	R&D Systems	882-B8–050
BAD BH3 peptide	140–165	-	AnaSpec	AS-64082
BAK BH3	71–87	-	AnaSpec	AS-61616
BAX BH3 peptide, wild type	55–74	-	AnaSpec	AS-62266
BID-BH3-peptide	81–101	-	AnaSpec	AS-61711
BIM BH3, Peptide IV	141–166	-	AnaSpec	AS-62279
Noxa BH3, Peptide 1	17–43	-	AnaSpec	AS-62282
PUMA BH3	129–154	-	AnaSpec	AS-62404
Small Molecules	Target	Manufacturer		Cat.#
A-1210477	MCL-1	APExBIO Technology		B6011
ABT-199 (Venetoclax)	BCL-2	APExBIO Technology		A8194
ABT-263 (Navitoclax)	BCL-xL/BCL-2/BCL-w	APExBIO Technology		A3007
ABT-737	BCL-xL/BCL-2/BCL-w	APExBIO Technology		A8193
BAM7	BAX	APExBIO Technology		A3218
WEHI-539	BCL-xL	APExBIO Technology		A3935