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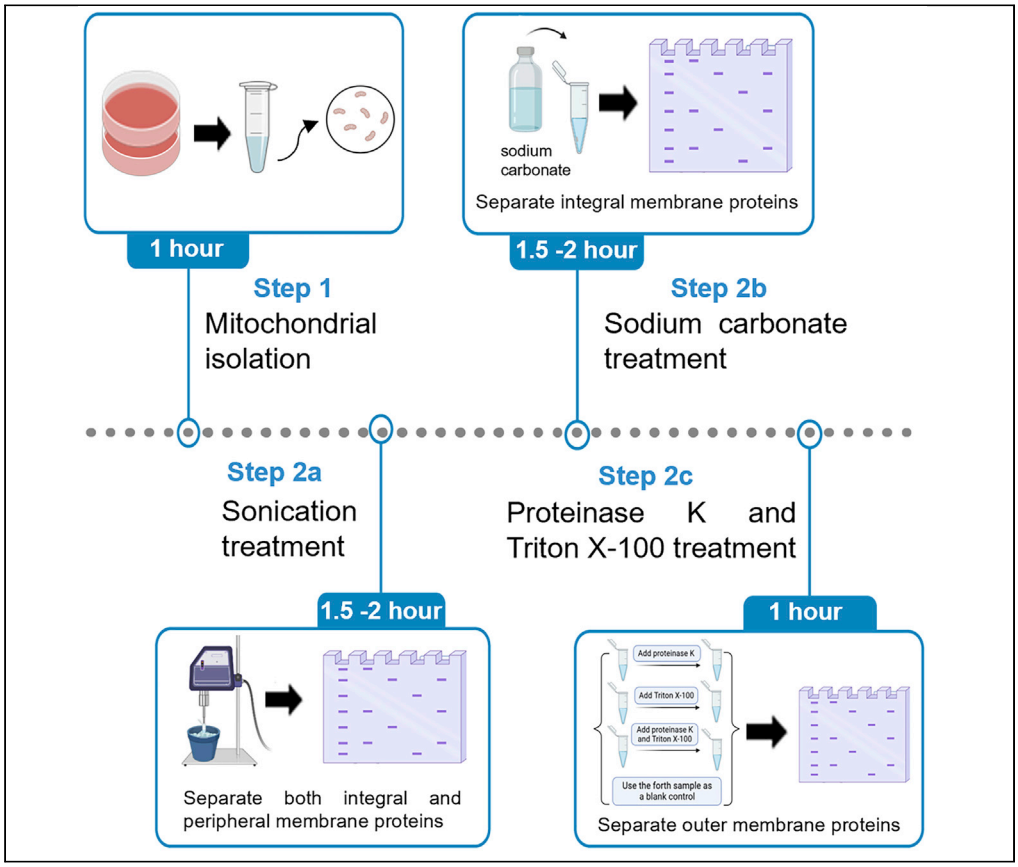
Protocol for mitochondrial isolation and sub-cellular localization assay for mitochondrial proteins



Here, we provide a protocol to isolate mitochondria from cultured cells and extract differently located mitochondrial proteins. We detail steps to separate both integral and peripheral membrane proteins from soluble proteins using sonication. We describe the separation of integral membrane proteins from the peripheral membrane and soluble proteins using sodium carbonate extraction. Furthermore, we detail the use of proteinase K and Triton X-100 to distinguish outer membrane proteins from mitochondrial proteins.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for mitochondrial isolation and mitochondrial proteins localization

Analysis of mitochondrial proteins sub-cellular location by western blot

Isolating mitochondria from cells for various use, such as enzyme activity detection

Sub-cellular localization provides insights into the role of mitochondrial proteins

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Protocol for mitochondrial isolation and sub-cellular localization assay for mitochondrial proteins

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SUMMARY

Here, we provide a protocol to isolate mitochondria from cultured cells and extract differently located mitochondrial proteins. We detail steps to separate both integral and peripheral membrane proteins from soluble proteins using sonication. We describe the separation of integral membrane proteins from the peripheral membrane and soluble proteins using sodium carbonate extraction. Furthermore, we detail the use of proteinase K and Triton X-100 to distinguish outer membrane proteins from mitochondrial proteins.

BEFORE YOU BEGIN

Mitochondria can be isolated from various types of cultured cells, and in this protocol, we choose HEK293T cells to demonstrate all the processes (Figure 1).

The main materials needed are listed in the "key resources table"; more detailed information on fabricating reagents for the following steps is shown in "materials and equipment."

Isolation of mitochondria from cultured cells

Timing: 60 min

1. Prepare the buffers and tubes needed for the procedure (see materials and equipment).

⚠ CRITICAL: Precool all the materials needed on ice 10 min before starting the procedure. Mix the reagents taken from the refrigerator upside down before using them.

2. Collect cultured cells from several 100 cm^2 cell culture dishes with approximately 80% density of cells. After removing the culture medium, wash it twice with phosphate buffer saline (PBS).

Note: The number of cultured cells needed varies. The bigger the cell, the more dishes are needed. For example, HEK293T cells in a total of 1×10^8 from cell culture dishes (ten 100 cm^2 cell culture dishes at approximately 80% density of cells) are needed in mitochondrial isolation.

⚠ CRITICAL: From this step onward, all activities should be performed on ice to minimize the activation of destructive proteinases.

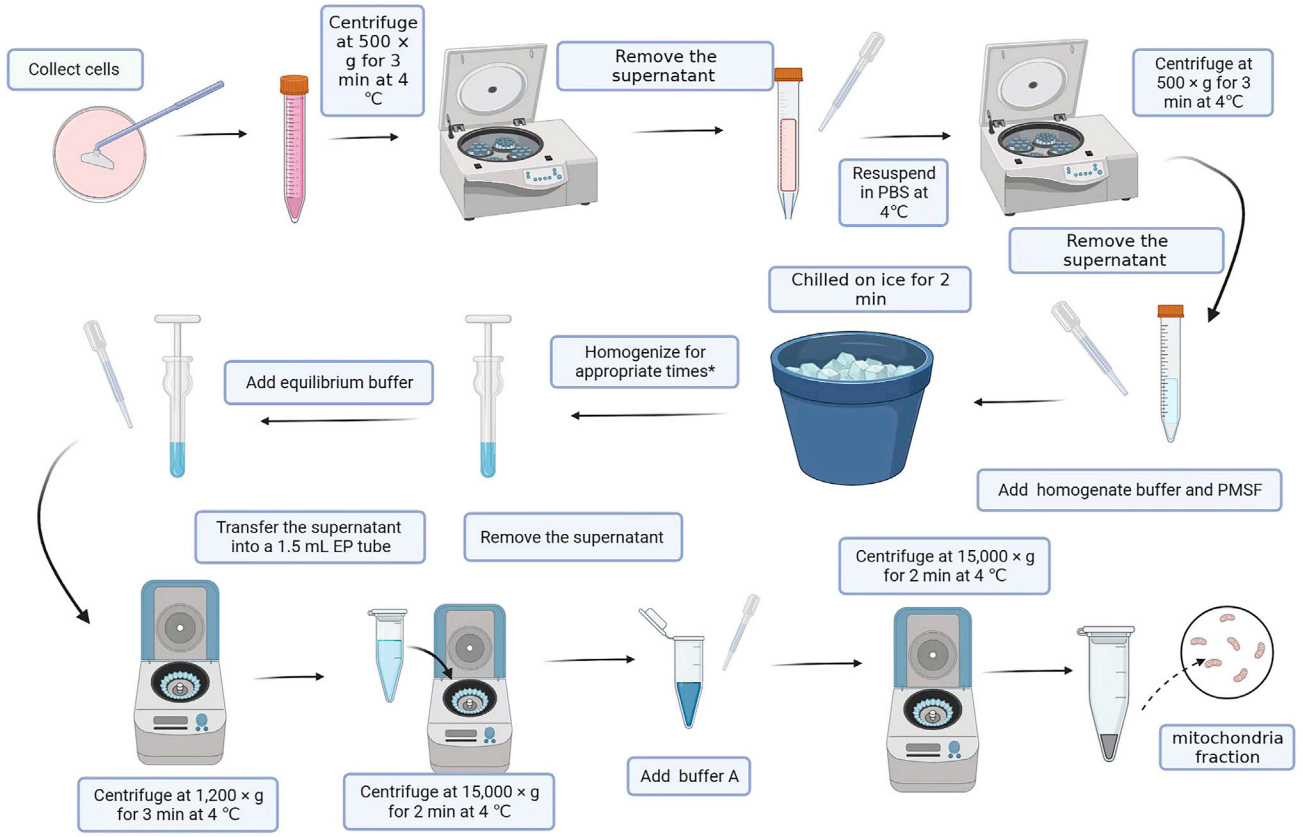

Figure 1. Schematic workflow of mitochondrial isolation from cultured cells

3. Remove PBS and use a cell lifter to scrape off the adherent cells. Subsequently, transfer the suspension to a 15 mL tube using a pipette, and centrifuge the tube at 500 × *g* for 3 min at 4°C. Remove the supernatant, then add PBS, and repeat the centrifugation process.

   Note: Make sure that all the cells are collected and adherent cells are scraped off in the same direction in each dish.

4. Remove the supernatant, add 2 mL of homogenate buffer and 20 μL of phenylmethylsulfonyl fluoride (PMSF) (100 mM), resuspend the pellet, and chill it on ice for 2 min.
5. Homogenize the cells for appropriate times by using a Dounce tissue grinder.

   Note: Different cells have different homogenization times. They need to be determined by Trypan Blue staining, in which 80% of cells stained in the field of vision observed under a microscope is the best. For HEK293T cells, about 20 times of homogenization is usually adopted (Figure 2).

6. Add 200 μL of equilibrium buffer to the Dounce tissue grinder at the end of homogenization.

   Note: After centrifugation, the fraction of cells disrupted in homogenization is in the pellet, while the mitochondria needed are in the suspension. Therefore, remove the pellet and retain the suspension.

7. Centrifuge at 1,200 × *g* for 3 min at 4°C and transfer the supernatant to a 1.5 mL tube. Repeat the centrifugation process until no sediment can be observed in order to remove the whole cells.

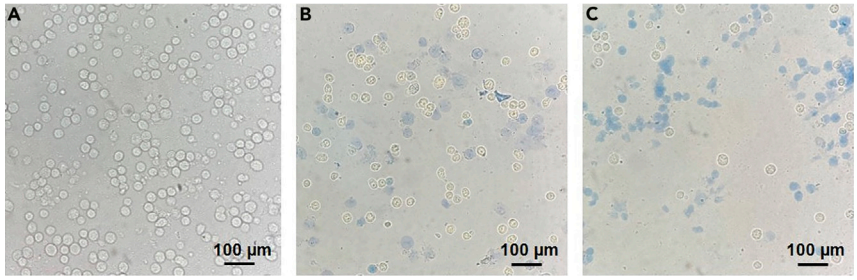

Figure 2. Evaluation of cell homogenizing effect using trypan blue staining

(A–C) Cells before homogenization (A), cells stained by trypan blue before homogenization (B), and cells stained by trypan blue after homogenization (C).

8. Transfer the supernatant to a fresh 1.5 mL tube and centrifuge at 15,000 × *g* for 2 min at 4°C.
9. Remove the supernatant, then resuspend the pellet in 500 μL of buffer A, and centrifuge at 15,000 × *g* for 2 min at 4°C.
10. At this stage, the pellet is the crude mitochondria (mitochondrial enriched fractions) and can be used for electrophoresis and subsequent experiments.

Note: If not used right away, crude mitochondria should be stored frozen at −80°C for at most 1 week.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
0.01 M PBS (powder, pH 7.2–7.4)	Solarbio Life Science	Cat#P1010
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	Cat#P7626
Dehydrated alcohol (CH3CH2OH)	ANTE	N/A
Hydrochloric acid (HCl)	http://www.zh-chem.com	N/A
Trizma base	Sigma-Aldrich	Cat#V900483
Sodium chloride (NaCl)	Sigma-Aldrich	Cat#V900058
Magnesium chloride (MgCl2)	Sigma-Aldrich	Cat#208337
Trypan blue stain (0.4%)	Gibco	Cat#15250061
Sucrose	Sigma-Aldrich	Cat#V900116
Ethylenedinitrilotetraacetic acid (EDTA)	Sigma-Aldrich	Cat#V900106
Potassium chloride (KCl)	Sigma-Aldrich	Cat#P5405
Sodium phosphate dibasic (Na2HPO4)	Sigma-Aldrich	Cat#V900061
Sodium carbonate (Na2CO3)	Sigma-Aldrich	Cat#S7795
Proteinase K	Beyotime Biotechnology	Cat#ST533
Triton X-100	Sigma-Aldrich	Cat#93443
Experimental models: Cell lines		
Cultured cells, e.g., HEK293T cells	Stem Cell Bank, Chinese Academy of Sciences	Cat#BFN60700191
Software and algorithms		
BioRender	BioRender.com	N/A
Other		
Cell culture dish	Sigma-Aldrich	Cat#CLS430167
Cell lifer	Sigma-Aldrich	Cat#CLS3008
15 mL centrifuge tube	Sigma-Aldrich	Cat#430791
Eppendorf 5424R Microcentrifuge	Eppendorf	Cat#5406000119
Vent filter for reservoir	Pall Life Sciences	Cat#LWFS32501

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
15 mL Dounce tissue grinder	Duran Wheaton Kimble Life Science	Cat#357544
Nikon Eclipse 80i fluorescence microscope	Nikon	N/A
1.5 mL centrifuge tube	Maisinuo	Cat#HZX018-2
Scientz-IID ultrasonic homogenizer	Ningbo Scientz Biotechnology	N/A
Type 42.2 Ti Rotor	Beckman Coulter	Cat#343007
Open-top thickwall polypropylene tube	Beckman Coulter	Cat#343621
Beckman Coulter Optima XPN-100 ultracentrifuge	Beckman Coulter	Cat#A94469
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat#23225
SpectraMax iD3 Multi-Mode Microplate Reader	Molecular Devices	N/A
PowerPac Universal power supply	Bio-Rad	Cat#1645070
Protec OptiMax 2010-NDT tabletop film processor	Protec	N/A
X-OMAT BT Film (5 × 7 in.)	Carestream	N/A
Epson Perfection V850 Pro photo scanner	Epson	Cat#B11B224201

MATERIALS AND EQUIPMENT

Isolation of mitochondria from cultured cells

100 mM PMSF

Reagent	Final concentration	Amount
PMSF	100 mM	870.95 mg
100% dehydrated alcohol	N/A	50 mL
Total	**N/A**	**50 mL**

Store at −20°C with a one-year shelf life.

⚠ CRITICAL: PMSF is harmful to the mucosa of the respiratory tract, eyes, and skin and can be lethal if inhaled, ingested, or absorbed through the skin. In case of eye or skin contact with PMSF, rinse immediately with plenty of water. Clothing contaminated with PMSF should be discarded.

Homogenate buffer

Reagent	Final concentration	Amount
Tris-HCl (pH 7.8)	350 mM	2,120 mg
NaCl	250 mM	730.50 mg
$MgCl_2$	50 mM	238.03 mg
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**

This buffer needs to prepare fresh the day before or on the day of experiment and precooled at 4°C.

Equilibrium buffer

Reagent	Final concentration	Amount
Tris-HCl (pH 7.8)	3.5 mM	21.20 mg
NaCl	2.5 mM	7.31 mg
$MgCl_2$	0.5 mM	2.38 mg
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**

This buffer needs to prepare fresh the day before or on the day of experiment and precooled at 4°C.

Buffer A		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.4)	10 mM	60.57 mg
EDTA	1 mM	14.61 mg
Sucrose	320 mM	5476.80 mg
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**

This buffer needs to prepare fresh the day before or on the day of experiment and precooled at 4°C.

Localization of membrane-associated proteins by sonication

1 × TD		
Reagent	Final concentration	Amount
Trizma base	49.99 mM	302.80 mg
NaCl	274.13 mM	801 mg
KCl	20.12 mM	75 mg
Na_2HPO_4	13.95 mM	99 mg
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**

Store at 4°C with a six-month shelf life.

Localization of membrane-associated proteins by sodium carbonate

100 mM Na_2CO_3		
Reagent	Final concentration	Amount
Na_2CO_3	100 mM	529.95 mg
ddH_2O	N/A	50 mL
Total	**N/A**	**50 mL**

Store at 4°C with a six-month shelf life.
100 mM PMSF and 1 × TD buffer formulations are described above.

Outer membrane proteins localization using proteinase K and Triton X-100

100 mM PMSF and 1 × TD buffer formulations are described above.

STEP-BY-STEP METHOD DETAILS

Although six discrete mitochondrial compartments can be recognized in some articles,[1] to simplify the structure, we roughly divide a mitochondrion into four compartments: outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and matrix. Mitochondrial proteins can be categorized into soluble and membrane proteins (MPs). Soluble proteins are localized in intermembrane space and mitochondrial matrix, whereas membrane proteins are associated with inner or outer mitochondrial membranes. Moreover, membrane proteins can be either peripheral or integral. Peripheral MPs crowd the surface of the membrane through electrostatic, hydrogen-bonding, or hydrophobic interactions with lipid head groups or other integral MPs and can be detached from the membrane by relatively mild treatments such as changing the ionic strength or pH in the buffer. Integral MPs are firmly embedded in the lipid bilayer by hydrophobic interactions between the hydrocarbon chains of lipids and the hydrophobic domains of proteins and can only be removed by detergent solubilization.[2–6] In addition, isotopes are also used in localization by labeling proteins in mitochondria.[7] The three methods to extract and localize proteins from mitochondria are discussed subsequently.

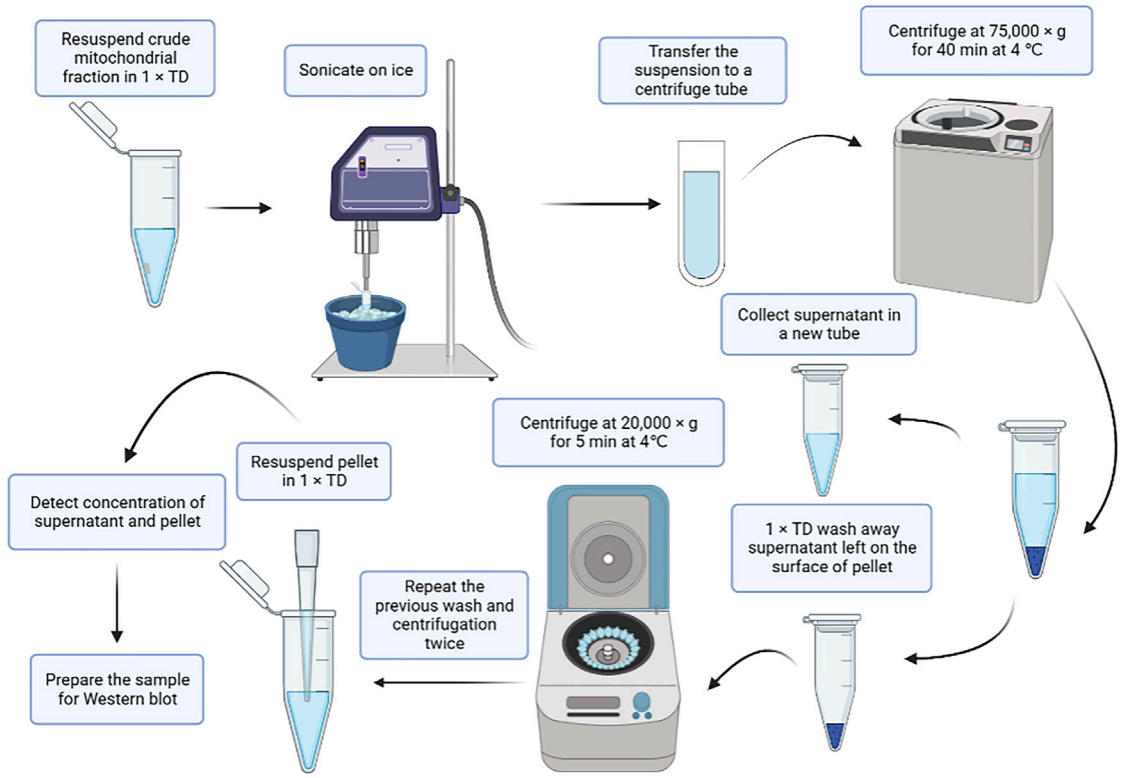

Figure 3. Localization of membrane-associated proteins by sonication schematic workflow

Localization of membrane-associated proteins by sonication

Timing: between 90 and 120 min

Sonication can break mitochondrial through high frequency ultrasonic vibration energy generated by ultrasonic generator. Thus, sonication can distinguish between the membrane proteins (integral and peripheral) in the pellet (P) and soluble proteins in the supernatant (S) (Figure 3).

1. Obtain cultured cells from several 100 cm^2 cell culture dishes with approximately 80% density of cells, according to the aforementioned corresponding steps to collect the crude mitochondrial fraction.

   Note: For HEK293T cells, a total of 3×10^7 (three 100 cm^2 cell culture dishes at approximately 80% density of cells) are needed in mitochondrial isolation to perform sonication.

2. Resuspend the crude mitochondria fraction obtained in step 1 in 150 μL of 1 × TD and collect in a 1.5 mL precooled tube. Clean the ultrasonic homogenizer and set the power to 150 W and amplitude to 30% before homogenizing the samples on ice for 1 min with a pause of 1 s every 1.5 s.

   Note: Typically, before drying the probe of the ultrasonic homogenizer, wash the probe with 15 mL of 75% ethanol and subsequently with water at high pressure. Do not forget to keep a portion as the control group.

3. Allow the 1.5 mL tube containing mitochondrial suspension collected in step 2 to stand on the ice and turn on the ultrasonic homogenizer to sonicate the suspension (Figure 4).

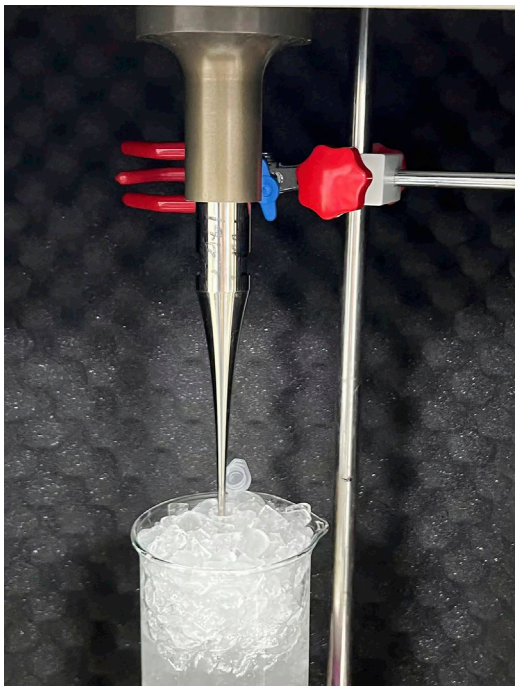

Figure 4. Sonication of the sample on ice

⚠ CRITICAL: The probe is close to the bottom of the liquid and don't touch the tube wall to avoid foam in the process of ultrasound. This entire step is performed on ice to prevent overheating caused by sonication. Be cautious during the procedure to avoid splashes or extremely high temperatures.

4. After sonication, transfer the suspension to the special centrifuge tube of the ultra-speed Type 42.2 Ti Rotor and centrifuge for 40 min at 75,000 × *g* in Beckman Coulter Optima XPN-100 ultracentrifuge at 4°C (Figure 5). Then collect the supernatant (S) in a fresh 1.5 mL tube.
5. Use 100 μL 1 × TD to gently rinse off the remaining supernatant from the surface of the pellet; subsequently, centrifuge at 20,000 × *g* for 5 min at 4°C and pipette off the supernatant.

Note: Gently add 1 × TD against the wall to avoid dispersing the pellet.

⚠ CRITICAL: In case of accidental dispersion and resuspension of the pellet into the supernatant, centrifuge further to maximize the purity of the pellet.

6. Repeat step 5 twice for a total of three times.
7. Fully resuspend the pellet (P) in 20 μL of 1 × TD.
8. Identify the concentration of the supernatant (S) and pellet (P) using the Pierce BCA Protein Assay Kit.
9. Prepare the sample for the Western blot.

Localization of membrane-associated proteins by sodium carbonate

Timing: between 90 and 120 min

The sodium carbonate can break mitochondrial membranes through low surface tension based on the principle of osmotic pressure. Thus, this experiment can separate the integral proteins in the pellet (P) from peripheral and soluble proteins in the supernatant (S) (Figure 6).

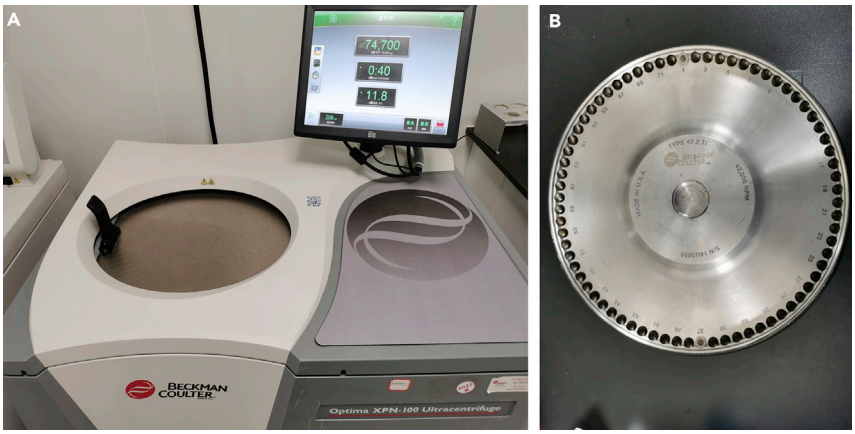

Figure 5. Beckman Coulter centrifugal machine and Type 42.2 Ti Rotor
(A) Beckman Coulter centrifugal machine.
(B) Type 42.2 Ti Rotor.

10. Obtain cultured cells from several 100 cm^2 cell culture dishes with approximately 80% density of cells, according to the aforementioned corresponding steps to collect the crude mitochondrial fraction.

Note: For HEK293T cells, a total of 3×10^7 (three 100 cm^2 cell culture dishes at approximately 80% density of cells) are needed in mitochondrial isolation for the sodium carbonate extraction experiment performed at one pH gradient.

11. Resuspend crude mitochondrial fraction obtained in step 10 in 30 μL of Na_2CO_3 (100 mM).

Note: Do not forget to keep a portion as the control group. In addition, the volume mentioned here is just sufficient for one pH gradient; in case the sodium carbonate extraction experiment is performed at different pH values, the volume of reagents needed should be prepared after calculation.

12. Incubate on ice for 30 min. After 1 μL of PMSF (100 mM) is added, mix the sample well.
13. Transfer the suspension to the special centrifuge tube of the ultra-speed Type 42.2 Ti Rotor and centrifuge for 40 min at 75,000 × *g* in Beckman Coulter Optima XPN-100 ultracentrifuge at 4°C (Figure 5). Collect the supernatant (S) in a fresh 1.5 mL tube.
14. Gently wash away the supernatant left on the surface of the pellet using 100 μL 1 × TD and then centrifuge at 20,000 × *g* for 5 min at 4°C.

Note: Gently add 1 × TD against the wall to avoid dispersing the pellet.

⚠ CRITICAL: In case of accidental dispersion and resuspension of the pellet into the supernatant, centrifuge further to maximize the purity of the pellet.

15. Repeat step 14 twice for a total of three times.
16. Thoroughly resuspend the pellet (P) in 20 μL of 1 × TD.
17. Identify the concentration of the supernatant (S) and pellet (P) using the Pierce BCA Protein Assay Kit.
18. Prepare the sample for the Western blot.

Outer membrane proteins localization using proteinase K and Triton X-100

Timing: 60 min

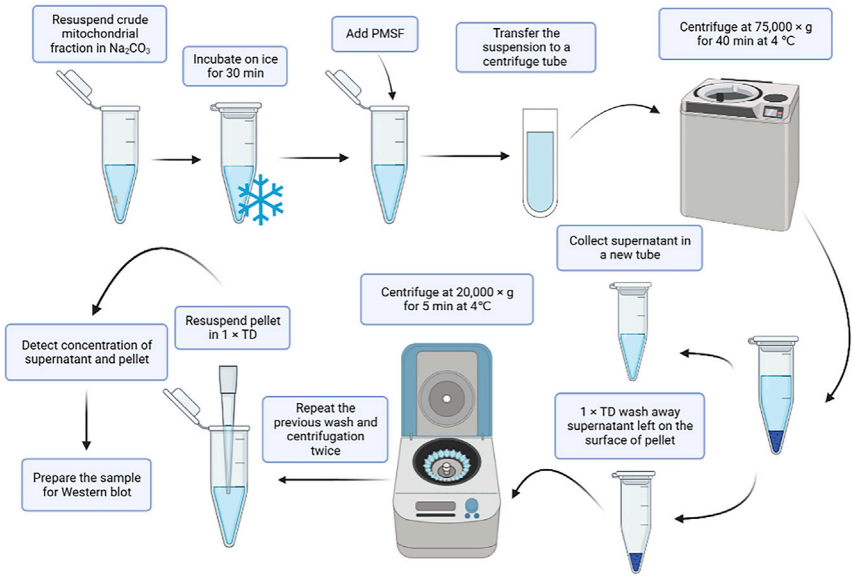

Figure 6. Localization of membrane-associated proteins by sodium carbonate schematic workflow

Proteinase K can only digest outer membrane proteins, but after adding Triton X-100 to dissolve mitochondrial membranes, proteinase K can pass through all compartments to digest all mitochondrial proteins. Therefore, proteinase K and Triton X-100 are used to distinguish between outer membrane proteins and other mitochondrial proteins (Figure 7).

19. Obtain cultured cells from several 100 cm^2 cell culture dishes with approximately 80% density of cells, according to the aforementioned corresponding steps to collect the crude mitochondrial fraction.

Note: For HEK293T cells, a total of 5 × 10^7 (five 100 cm^2 cell culture dishes at approximately 80% density of cells) are needed in mitochondrial isolation to perform this experiment.

20. Fully resuspend crude mitochondrial fraction obtained in step 19 in 120 μL of 1 × TD and equally divide them into four samples.
21. Add 3.33 μL of proteinase K solution (1 mg/mL) (10 μg of proteinase K/mg of mitochondrial protein) in the first sample, 3.33 μL of 20% Triton X-100 (2 mg of Triton X-100/mg of mitochondrial protein) in the second sample, and 3.33 μL of proteinase K solution (1 mg/mL) and 3.33 μL of 20% Triton X-100 in the third sample, and use the fourth sample as a blank control.
22. Store the four samples at room temperature for 1 h and mix them well every 5 min by using a pipette.

Note: The room temperature mentioned here maintains between 20°C and 27°C.

23. Add 1 μL of 100 mM PMSF into the four samples and mix well.
24. Prepare the sample for the Western blot.

EXPECTED OUTCOMES

Sonication can distinguish between the membrane proteins (integral and peripheral) in the pellet (P) and soluble proteins in the supernatant (S). In addition, the sodium carbonate extraction experiment can separate the integral proteins in the pellet (P) from peripheral and soluble proteins in the supernatant (S). Combining the two methods, mitochondrial proteins theoretically can be divided

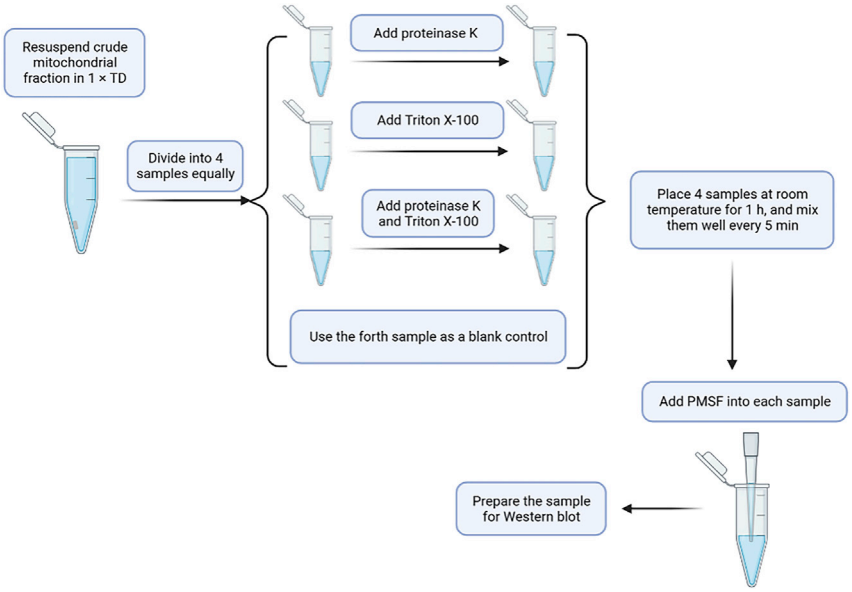

Figure 7. Schematic workflow of outer membrane protein localization using proteinase K and Triton X-100

into integral and peripheral membrane proteins and soluble proteins. In contrast, proteinase K and Triton X-100 are used to distinguish between outer membrane proteins and other mitochondrial proteins (Figure 8). In our recent work, we have performed sub-cellular localization of SERAC1 using proteinase K and Triton X-100, and it is easy to conclude that SERAC1 is both soluble and outer membrane protein and is supposed to be located at more than just the outer mitochondrial membrane.[8]

LIMITATIONS

There are various experiments performed on sodium carbonate extraction at pH 11.5. However, the strength of proteins connected to the membrane and the environment can vary significantly. Performing the sodium carbonate extraction experiment at different pH gradients favors both finding the optimum pH for your experiment and avoiding interpreting some peripheral proteins with looser connections to the membrane as soluble proteins or with tighter connections as integral proteins. Although Triton X-100 exerts the function of solubilization, mitochondrial inner and outer membranes differ in various aspects, leading to selective solubilization in some components and quite

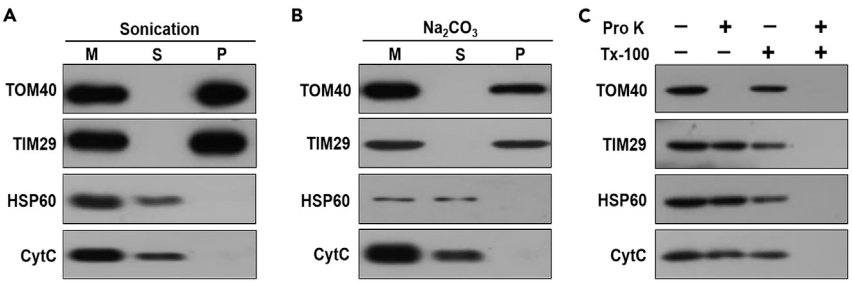

Figure 8. Expected outcomes of protein localization using sonication, sodium carbonate or proteinase K and Triton X-100

(A–C) Mitochondria from HEK293T cells were sonicated (A) or treated with Na2CO3 (B), proteinase K or/and Triton X-100 (C), and then left untreated (M) or separated into supernatant (S) and pellet (P) and analyzed by Western blot. TOM40, TIM29, HSP60 and CytC are used as markers representing outer membrane, inner membrane, mitochondrial matrix and mitochondrial intermembrane proteins, respectively.

different behaviors toward Triton X-100.[9] Mitochondrial fractions were incubated with 0.38 mg, 2 mg, 2 mg and 5 mg Triton X-100/mg of protein in pig liver, yeast, cell lines and beef heart, respectively.[10–13] A suitable concentration of Triton X-100 applied to different samples needs to be tested.

TROUBLESHOOTING

Problem 1

Cells are in critical condition when isolating mitochondria (step 2).

Potential solution

Control the density of cells to at least reach 80%, and adherent cells are the desired culture condition, but cells are not supposed to be dense.

Problem 2

The number of cells scraped off from dishes is less than expected (step 3).

Potential solution

Flush the dish several times with PBS to maximize the cells collected. The osmotic pressure of PBS is similar to that of cells; thus, pipetting PBS surrounding cells while transferring the cells to a tube is reasonable and acceptable.

Problem 3

Overly homogenize when isolating mitochondria (step 5).

Potential solution

Extract the supernatant every several rounds (depending on the strength used) during homogenization, stain it with Trypan Blue, and observe under a microscope.

Problem 4

Obtain less sample than expected after ultracentrifugation (steps 4 and 13).

Potential solution

Please try your best to shorten the time in this process, because the special centrifuge tube is open-top, and practicing longer means evaporating more.

Problem 5

Acquire impure substances (supernatant or pellet) after ultracentrifugation in the sonication or sodium carbonate extraction experiment (steps 4 and 13).

Potential solution

Carefully aspirate the supernatant and strictly avoid touching the pellet by leaving some supernatant on its surface. In addition, when collecting the pellet, thoroughly rinse it with 1 × TD. Repeat this process several times until the substance is pure.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ya Wang (yawang@wmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new data or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

H.F. supervised this work. S.Z., D.Z., X.H., and D.L. performed experiments and wrote the manuscript. H.F. and Y.W. edited the manuscript. All authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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