Isolation of Membrane-enriched fractions from mouse cortical neurons.

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ABSTRACT

Upon mitochondrial damage, activation of the PINK1 kinase and Parkin ubiquitin ligase induces ubiquitylation of multiple proteins at the mitochondria to stimulate their elimination by mitophagy. Protein ubiquitylation is a highly dynamic, reversible and complex post-translation modification (PTM) and it is frequently linked with phosphorylation. The major challenges, for biochemical and quantitative proteomic analysis of cellular proteins that are ubiquitylated and phosphorylated in response to mitochondrial damage in a PINK1-Parkin-dependent manner, involve the spatial configuration and stoichiometry of these post-translational modifications occurring on the mitochondria. Here, we describe an optimised protocol to isolate membrane-enriched fractions that provides high mitochondrial yield from primary cells, such as neuronal cultures. This protocol, in combination with other enrichment strategies, will facilitate proteomic and biochemical workflows for investigation of molecular events defined by PINK1/Parkin pathway.

KEYWORDS

Neurons, mitochondria, membrane, PINK1, Parkin, Ubiquitin

MATERIALS

For Mitochondrial depolarisation and Membrane isolation

1. **Mitochondrial depolarisation:** 10 µM Antimycin A (Sigma-Aldrich #A8674); 1 µM Oligomycin (Sigma-Aldrich #75351) in DMSO (Sigma-Aldrich #D2650).

2. **HB (hypotonic Buffer) Buffer**: 8.55% (w/v) sucrose: 42.25g sucrose for 500mL + 1:100 Imidazole (300mM Stock). Filter the solution and store at 4°C.

3. **HB Buffer + inhibitors**: HB + 200mM Chloroacetamide, 1X complete protease inhibitors, 1 X phosphatase inhibitors cocktail 2 and 3 (add the day of the experiment).

4. **MitoBuffer**: 270mM sucrose, 20mM HEPES, 3mM EDTA, 1mM Sodium orthovanadate, 10mM 2-glycerophosphate, 50mM Sodium fluoride, 5mM sodium pyrophosphate, 200mM Chloroacetamide, 1X complete protease inhibitors, 1 X phosphatase inhibitors cocktail 2 and 3.

5. **PBS + inhibitors**: DPBS, no calcium, no magnesium (Gibco™ #14190094), 1mM Sodium orthovanadate, 10mM 2-glycerophosphate, 50mM Sodium fluoride, 5mM sodium pyrophosphate, 10mM PMSF, 200mM Chloroacetamide, 1X complete protease inhibitors, 1 X phosphatase inhibitors cocktail 2 and 3.

6. **Table of reagents**

REAGENT	COMPANY	CAT. NUMBER
D (+)-SACCHAROSE (SUCROSE)	VWR	27480.360
IMIDAZOLE	Merck (Sigma-Aldrich)	56750
HEPES	Formedium	HEPES10
SODIUM ORTHOVANADATE	Merck (Sigma-Aldrich)	S6508
SODIUM FLUORIDE	Merck (Sigma-Aldrich)	S7920
2-GLYCEROPHOSPHATE DISODIUM SALT HYDRATE	Merck (Sigma-Aldrich)	G9422
PMSF	Merck (Sigma-Aldrich)	93482
SODIUM PYROPHOSPHATE DECAHYDRATE	Merck (Sigma-Aldrich)	221368
2-CHLOROACETAMIDE	Merck (Sigma-Aldrich)	C0267
PHOSPHATASE INHIBITOR COCKTAIL 2	Merck (Sigma-Aldrich)	P5726
PHOSPHATASE INHIBITOR COCKTAIL 3	Merck (Sigma-Aldrich)	P0044
COMPLETE PROTEASE INHIBITORS	Merck (Roche)	11873580001

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Cell lines

1. Mouse cortical Neurons established from C57BL6J, PINK1 WT and KO, Parkin WT and KO mice as published in protocols.io (dx.doi.org/10.17504/protocols.io.bswanfae)

STOCK SOLUTION PREPARATION

- **Antimycin A**: Prepare 50mM of Antimycin A in DMSO; aliquot and store at − 20 °C.
- **Oligomycin**: Prepare 10 mM of Oligomycin in DMSO; aliquot and store at − 20 °C.

EQUIPMENT

1. Eppendorf refrigerated centrifuge 5810R.
2. Beckman SW 55 Ti Swinging-Bucket Rotor 55000 rpm.
3. Optima L-90K Ultracentrifuge.
4. Cell culture incubator 5% CO2, 95% humidity HERAcell®CO2 incubator (150 L).
5. Microcentrifuges, Micro Star 17R (VWR #521-1647).
6. Dounce Dura Grind® Tissue Grinder (EMS #64791-07).
7. Probe sonicator, Branson Digital Sonifier.

CONSUMABLES

1. Cell culture multidishes, 6 well (Thermo Scientific™ #140675).
2. 50mL Stripette® Serological Pipets (Corning #4490).
3. 25mL Stripette® Serological Pipets (Corning #4489).
4. 10mL Stripette® Serological Pipets (Corning #4488).
5. 5mL Stripette® Serological Pipets (Corning #4487).
6. 15ml CELLSTAR® tubes (Greiner bio-one #188271).
7. 50ml CELLSTAR® tubes (Greiner bio-one # 227261).
8. Standard 1ml and 200µl Pipette tips (Greiner bio-one # 686271, #685261).
9. 1.5ml Eppendorf tubes (Eppendorf™ # 0030120086).
10. Disposable Cell Lifter (FisherBrand #08100240).
11. Beckman centrifuge tubes (Beckman# 344057).

MEMBRANE ISOLATION FOR NEURONS

Mitochondrial depolarisation ◊TIMING 1-9h, day of experiment

To depolarize or uncouple mitochondrial membrane potential in neurons, cultures could be until to 9 hours with a combination of 10 µM Antimycin A and 1 µM Oligomycin dissolved in DMSO at 37°C.

Mitochondrial isolation ◊TIMING 1-1.5h

1. Gently aspirate media from wells and add 1mL of PBS+inhibitors in each well at Room Temperature **Note:** Do not use cold PBS, otherwise neuronal cells can detach from the dishes.

2. Scrape neuronal cells and collect in a 50mL labelled Falcon tube.

3. Centrifuge neuronal cells at 500g for 3 min at 4°C.

4. Resuspend pellets in 2 mL of HB buffer + inhibitors freshly added.

5. Homogenise cells using a stainless steel Dounce homogeniser tissue grinder with 40 stroke. **Note:** It is recommended to slowly press and twist the pestle on the sample. When the pestle is raised and turned, a strong vacuum force is generated creating shearing forces that will help to generate a fine homogenate. During this action, do not completely remove the pestle, this to avoid the generation of bubbles and to retain cellular organelles intact. Check cell lysis with Trypan blue (4ul lysate + 4ul trypan blue), 90% of cells should be disrupted.

6. Pellet nuclei and the remaining intact cells from lysate at 2000rpm for 5min at 4°C. The subsequent supernatant is the post-nuclear supernatant (PNS).

7. For total membrane isolation, transfer the PNS to an ultracentrifuge tube. **Note:** Fill tube at least 4/5 with HB buffer to prevent tube collapse and equalize weight to <0.01g.

8. Spin at 200000g (40k rpm) for 1h at 4°C.

9. Remove the supernatant, this represents the cytosolic fraction.

10. Pellet represents the membrane fractions. Freeze the pellet or resuspend the pellet according to the experiment.

11. For Western Blotting or proteomic experiments: transfer the pellet using a tip of a pipette to an Eppendorf tube and add 200uL-300uL of MitoBuffer. Sonicate the membrane pellet with a probe sonicator 20% amplitude for 5 seconds or until the pellet is completely resuspended.

12. Proceed with protein quantification by using the Coomassie Protein Assay and sample preparation according to the experiment.

13. Membrane lysates can be stored at -80°C.