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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.

ATTACHMENTS

d58dbheux.pdf

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PROTOCOL CITATION

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KEYWORDS

Neurons, Mitochondria, Membrane, PINK1, Parkin, Ubiquitin

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MATERIALS TEXT

MATERIALS:

For Mitochondrial depolarisation and Membrane isolation:

1. Mitochondrial depolarisation: [M]**10 Micromolar (µM)**

Antimycin A from Streptomyces sp. **Sigma –**

Aldrich Catalog #A8674 ; [M]**1 Micromolar (µM)**

Oligomycin A **Sigma –**

Aldrich Catalog #75351 in

Dimethyl sulfoxide (DMSO) **Sigma**

Aldrich Catalog #D2650 .

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

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

4. MitoBuffer:

A	B
Sucrose	270 mM
HEPES	20 mM
EDTA	3 mM
Sodium orthovanadate	1 mM
2-glycerophosphate	10 mM
Sodium fluoride	50 mM
Sodium pyrophosphate	5 mM
Chloroacetamide	200 mM
Pomplete protease inhibitors	1X
Phosphatase inhibitors cocktail 2 and 3	1 X

5. PBS + inhibitors:

A	B
DPBS, no calcium, no magnesium (Gibco™ #14190094)	
Sodium orthovanadate	1 mM
2-glycerophosphate	10 mM
Sodium fluoride	50 mM
Sodium pyrophosphate	5 mM
PMSF	10 mM
Chloroacetamide	200 mM
Complete protease inhibitors	1X
Phosphatase inhibitors cocktail 2 and 3	1 X

DPBS no calcium no magnesium **Gibco - Thermo**

Fischer Catalog #14190094

6. Table of reagents:

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A	B	C
REAGENT	**COMPANY**	**CAT. NUMBER**
D (+)-SACCHAROSE (SUCROSE)	VWR	27480.36
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

D-( )-Sucrose AnalaR NORMAPUR® analytical reagent **VWR Chemicals Catalog #27480.360**

Imidazole **Sigma Aldrich Catalog #56750**

HEPES **Formedium Catalog #HEPES10**

Sodium Orthovanadate **Sigma Aldrich Catalog #S6508-10G**

Sodium pyrophosphate decahydrate **Sigma Aldrich Catalog #221368**

2-Chloroacetamide **Sigma – Aldrich Catalog #C0267**

Phosphatase Inhibitor Cocktail 2 **Sigma Aldrich Catalog #P5726**

cOmplete™ EDTA-free Protease Inhibitor Cocktail **Roche Catalog #11873580001**

Sodium fluoride **Sigma – Aldrich Catalog #S7920**

β-Glycerophosphate disodium salt hydrate **Sigma – Aldrich Catalog #G9422**

Phenylmethanesulfonyl fluoride solution **Sigma – Aldrich Catalog #93482**

Phosphatase Inhibitor Cocktail 3 **Sigma – Aldrich Catalog #P0044**

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

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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).

Microcentrifuges ventilated/refrigerated Micro Star 17 / 17R **VWR international**

5. **Ltd Catalog #521-1647**

Dounce Dura Grind® Tissue

6. Grinder **EMS Catalog #64791-07**
7. Probe sonicator, Branson Digital Sonifier.

CONSUMABLES:

1. Cell culture multidishes, 6 well (Thermo Scientific™ #140675).

Nunc™ Cell-Culture Treated Multidishes, 6 well **Thermo Fisher Catalog #140675**

2.

50 mL Stripette™ Serological Pipets Polystyrene Individually Paper/Plastic Wrapped Sterile 25/Ba **Corning Catalog #4490**

3.

25 mL Stripette™ Serological Pipets Polystyrene Individually Paper/Plastic Wrapped Sterile 25/Ba **Corning Catalog #4489**

4. .

10 mL Stripette™ Serological Pipets Polystyrene Individually Paper/Plastic Wrapped Sterile 50/Ba **Corning Catalog #4488**

5.

5 mL Stripette™ Serological Pipets Polystyrene Individually Paper/Plastic Wrapped Sterile 50/Bag **Corning Catalog #4487**

15 mL conical centrifuge tube **greiner bio-**

6. **one Catalog #188271**

50 mL conical centrifuge tube **greiner bio-**

7. **one Catalog #227261**
8.

PIPETTE TIPS 100- 1000 µL BLUE SUITABLE FOR EPPENDORF STERILE 60 PIECES PER RACK **greiner bio-one Catalog #686271**

PIPETTE TIP 10 - 100 µL SUITABLE FOR EPPENDORF 96 PIECES / ST RACK **greiner bio-**

, **one Catalog #685261**

1.5ml Safe-lock

9. tubes **Eppendorf Catalog #0030120086**

Cell Lifters **Thermo**

10. **Fisher Catalog #08100240**

5 mL Open-Top Thinwall Ultra-Clear Tube 13 x 51mm - 50Pk **Beckman**

11. **Coulter Catalog #344057**

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Membrane Isolation For Neurons-Mitochondrial depolarisation ◊TIMING 1-9h, day of experiment

1

To depolarize or uncouple mitochondrial membrane potential in neurons, cultures could be until to **09:00:00** with a combination of **10 Micromolar (µM)** Antimycin A and **1 Micromolar (µM)** Oligomycin dissolved in DMSO at **37 °C** .

Membrane Isolation For Neurons-Mitochondrial isolation ◊TIMING 1-1.5h 1h 8m 5s

2 

Gently aspirate media from wells and add **1 mL** of PBS+inhibitors in each well at **Room temperature** .

Note: Do not use cold PBS, otherwise neuronal cells can detach from the dishes.

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

4  3m

Centrifuge neuronal cells at **500 x g** for **00:03:00** at **4 °C** .

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

6 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.

7  5m

Pellet nuclei and the remaining intact cells from lysate at **2000 rpm** for **00:05:00** at **4 °C** .

The subsequent supernatant is the post-nuclear supernatant (PNS).

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8 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.

9  1h

Spin at **200000 x g** (**40000 rpm**) for **01:00:00** at **4 °C** .

10 Remove the supernatant, this represents the cytosolic fraction.

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

12  5s

For Western Blotting or proteomic experiments: transfer the pellet using a tip of a pipette to an Eppendorf tube and add **200 µl** - **300 µl** of MitoBuffer. Sonicate the membrane pellet with a probe sonicator 20% amplitude for **00:00:05** or until the pellet is completely resuspended.

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

14 Membrane lysates can be stored at **-80 °C** .

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