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# PROCEDURE TO INDUCE MITOCHONDRIAL DEPOLARISATION AND LYSING OF MOUSE CORTICAL NEURONS

In 1 collection

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## ABSTRACT

Mutations in PINK1 cause early-onset Parkinson's disease. PINK1 becomes stabilised and active upon mitochondrial depolarisation. This leads to phosphorylation of ubiquitin and Parkin via Serine 65 residues and a feed forward mechanism whereby PINK1 phosphorylates newly formed polyubiquitin chains, generating phospho-ubiquitin, which further promotes Parkin recruitment and activation. Once activated, Parkin ubiquitylates proteins at the outer face of the outer mitochondrial membrane (OMM) and then initiates a downstream pathway that eventually leads to mitophagy, a mitochondria-specific type of autophagy. Notably, much of previous investigation into PINK1/Parkin activity has been performed in non-neuronal human cancer cells where Parkin and/or PINK1 is over-expressed. Here we report a protocol for generation of mouse embryonic cortical neuronal cultures that produce high cell yields and can be used for studying endogenous PINK1 and Parkin signalling by biochemical methods and proteomics

## ATTACHMENTS

[Mouse PINK1 pathway protocol \(166 - 337\).pdf](#)

## DOI

[dx.doi.org/10.17504/protocols.io.bsvcne2w](https://dx.doi.org/10.17504/protocols.io.bsvcne2w)

## PROTOCOL CITATION

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## COLLECTIONS ⓘ

 **Cell-based analysis of PINK1-Parkin pathway activation in primary mouse cortical neurons**

## KEYWORDS

Neurons, PINK1, Parkin, Mitochondrial stress, ubiquitin

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PARENT PROTOCOLS

Part of collection

[Cell-based analysis of PINK1-Parkin pathway activation in primary mouse cortical neurons](#)

MATERIALS TEXT

Please refer PROCEDURE TO ISOLATE AND CULTURE NEURONS FROM EMBRYONIC MOUSE CORTEX for materials and methods.




## Mitochondrial depolarisation

12h

12h

1

◇TIMING ⌚01:00:00 - ⌚09:00:00 , day of experiment in this section.

To depolarize or uncouple mitochondrial membrane potential in neurons, cultures were treated for ⌚03:00:00 to ⌚09:00:00 with a combination of 10 µM Antimycin A and 1 µM Oligomycin dissolved in DMSO at  37 °C .

## Lysing of mouse neuronal cultures

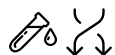
1h



2

◇TIMING ⌚01:00:00 - ⌚01:30:00 in this section.

Gently aspirate the medium from wells which neurons are plated.

3



Wash twice by adding 1 mL of warmed DPBS containing protease inhibitors, phosphatase inhibitors and 200 mM Chloroacetamide.

Note: Chloroacetamide is added to the PBS and the lysis Buffer to inactivate deubiquitinase enzymes and enhance the detection of Ubiquitin signal.

4



Place the 6 well multidishes **On ice** and add **50 µl** of Lysis Buffer. Carefully scrape the cells and collect the lysate in a **1.5 mL** microcentrifuge tube.

5



30m

Sonicate the cell lysate with a probe sonicator 5 seconds 20% amplitude and incubate for **00:30:00** **On ice**.

6



30m

Centrifuge the cell lysate at **17000 x g** in a refrigerated centrifuge for **00:30:00**. Supernatants were collected and protein concentration was determined by using the Coomassie Protein Assay.

7

Cell lysates are stored at **-80 °C**.

#### Immunoblotting of mouse neuronal cultures

7h 13m

8



⚡TIMING **05:00:00** - **48:00:00** in this section.

**40 µg** of cell lysates are diluted 4X into 4X LDS loading buffer supplemented with fresh 10% 2-mercaptoethanol.

9



23m

Samples are boiled for **00:03:00** at **97 °C** or for **00:20:00** at **37 °C**.

Do not boil the sample for an extended time, ubiquitin can refold on membrane and obscure epitope

10



2h

Analyse samples by running on Nu-page Bis-Tris 4-12% gels for a better resolution of ubiquitin chains, at 120 V for ~


🕒 02:00:00 .

- 11 Transfer gel on PVDF membrane for phospho-ubiquitin and ubiquitin signal and nitrocellulose membrane for phospho-Parkin and Parkin signal. Transfer in Towbin buffer at 80 V for 🕒 01:30:00 🧊 On ice or in cold room. <sup>1h 30m</sup>

Note: Prepare only 1 membrane per transfer tank -avoid multiple membranes for transfer in same tank as this reduces ubiquitin transfer.

- 12  1h

Incubate membrane with blocking buffer 5% milk in 0.1% TBS-Tween for 🕒 01:00:00 at 🧊 Room temperature .


- 13   1h

Remove blocking buffer, if primary antibodies are in 5% BSA, rinse twice with 0.1% TBS-Tween to remove any traces of milk, add primary antibodies and incubate 🕒 Overnight at 🧊 4 °C .

Note: Prepare phospho-Ubiquitin (1:2000), Ubiquitin (1:1000), GAPDH (1:5000) and Parkin (1:1000) in 5% BSA (TBS-Tween). Prepare phosphoParkin (1:2000) in 5% milk (TBS-Tween). To avoid non-specific signal, it is recommended to preincubate phospho-Parkin antibody with a membrane for 2 days before using it.

- 14  10m

Remove primary antibody and wash 3 times with 0.1%TBS-Tween for 🕒 00:10:00 .

- 15  1h

Add HRP-conjugate secondary antibodies for 🕒 01:00:00 at RT diluted 1:5000 in 1% BSA (0,1% TBS-Tween).

- 16  10m

Remove secondary antibody and wash 3 times with 0.1%TBS-Tween for 🕒 00:10:00 .

- 17 Develop signal using ECL western Blotting reagents and analysing with Chemidoc.

Note: Depending on signal, film can be best for sensitivity.

