Jul 31, 2021

PROCEDURE TO INDUCE MITOCHONDRIAL DEPOLARISATION AND LYSING OF MOUSE CORTICAL NEURONS

In 1 collection

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dx.doi.org/10.17504/protocols.io.bsvcne2w

ASAP2020 alessi

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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

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PROTOCOL CITATION

Odetta Antico, Miratul M. Muqit 2021. PROCEDURE TO INDUCE MITOCHONDRIAL DEPOLARISATION AND LYSING OF MOUSE CORTICAL NEURONS . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bsvcne2w

COLLECTIONS

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

KEYWORDS

Neurons, PINK1, Parkin, Mitochondrial stress, ubiquitin

CREATED

Feb 28, 2021

LAST MODIFIED

Jul 31, 2021

OWNERSHIP HISTORY

Feb 28, 2021 Jesintha Maniraja

Mar 01, 2021 Urmilas

May 05, 2021 m.muqit

PROTOCOL INTEGER ID

47748

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

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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 ~

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02:00:00 .

11 1h 30m

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.

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.

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