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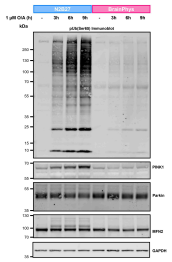
Immunoblot Assessments of PINK1-Dependent Mitophagy in iNeurons



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We use this protocol and it's working

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Abstract

Protocol outlining the use of immunoblots for assessment of the PINK1-dependent mitophagy process in iNeurons.

Materials

- Trizma hydrochloride (Tris-HCl) (Sigma, T3253)
- Sodium Hydroxide (Sigma, S5881)
- Sodium Chloride (NaCl) (Sigma, S9625)
- 10% w/v Triton-X-100 (Sigma, 93443)
- Sodium Deoxycholate (Sigma, D6750)
- Sodium Dodecyl Sulphate (SDS) (Sigma, L4509)
- PhosSTOP Phosphatase Inhibitors (Sigma, PHOSS-RO)
- cOmplete mini, EDTA-free Protease Inhibitors (Sigma, 11836170001)
- 10x Phosphate Buffered Saline (Fisher Scientific, 10649743)
- NuPAGE 4x LDS Sample Buffer (Invitrogen, NP0008)
- Dithiothreitol (DTT) (Sigma, D0632)
- NuPAGE Bis-Tris Mini Protein Gels, 4–12%, 10-well (Invitrogen, NP0335)
- NuPAGE Bis-Tris Mini Protein Gels, 4–12%, 12-well (Invitrogen, NP0322)
- NuPAGE Bis-Tris Mini Protein Gels, 4–12%, 15-well (Invitrogen, NP0336)
- NuPAGE Bis-Tris Midi Protein Gels, 4–12%, 20-well (Invitrogen, WG1402)
- NuPAGE Bis-Tris Midi Protein Gels, 4–12%, 26-well (Invitrogen, WG1403)
- rabbit anti-pUb(Ser65) IgG (Cell Signaling Technology Cat# 62802, RRID:AB_2799632)
- rabbit anti-PINK1 (Takeda, RRID:AB_3661827)
- rabbit anti-Parkin (Invitrogen Cat# 702785, RRID:AB_2942251)
- rabbit anti-MFN2 (Cell Signaling Technology Cat# 9482, RRID:AB_2716838)
- mouse anti-TIM23 IgG2a (BD Biosciences Cat# 611223, RRID:AB_398755)
- mouse anti-GAPDH IgG2b (Abcam Cat# ab110305, RRID:AB_10861081)
- mouse anti-β3-Tubulin Mouse IgG2a (BioLegend Cat# 801201, RRID:AB_2313773)
- donkey anti-mouse IgG (H+L) IRDye 680LT (LI-COR Biosciences Cat# 926-68022, RRID:AB_10715072)
- donkey anti-rabbit IgG (H+L) IRDye 800CW (LI-COR Biosciences Cat# 926-32213, RRID:AB_621848)

Buffers/Stocks

- 1M DTT = powder dissolved in dH2O
- 1M Tris-HCl (pH=7.4) = powder dissolved in dH2O and pH to 7.4 with sodium hydroxide
- 2M NaCl = solid dissolved in dH2O
- 10% w/v sodium deoxycholate = solid dissolved in dH2O
- 2% w/v SDS = powder dissolved in dH2O
- RIPA Lysis Buffer = 50mM Tris-HCl (pH=7.4), 150mM NaCl, 1% w/v triton-X-1OO, 0.5% sodium deoxycholate, 0.1% w/v SDS, 1x phosphatase inhibitors, 1x protease inhibitors, prepared in dH2O

Whole-Cell Lysate Preparation

30m

1 iNeuron culture and initial collection of whole-cell lysates desribed in "iNeuron Differentiation and Culture in N2B27 vs BrainPhys for Immunofluorescence and Biochemistry Assessments of Mitophagy" (https://www.protocols.io/private/E5851436F8F111EF86990A58A9FEAC02).

1.1 Thaw iNeuron samples frozen in RIPA lysis buffer on ice.

1.2 Use a cell scraper to collect iNeuron lysis preparation and transfer to prechilled eppendorf tube.

1.3 Incubate lysis preparations on ice for at least 1-2h with intermittent vigorous vortexing in order ensure complete lysis.

1.4 Centrifuge lysate preparation 16000 x g, 4°C, 00:20:00 20m

1.5 Transfer supernatant (soluble fraction) to a new pre-chilled eppendorf tube.

1.6 Quantify protein content of soluble fraction using DC Protein assay or similar quantification method.

1.7 Prepare equal amounts of lysate in 1x LDS Sample buffer supplemented with 10mM DTT by equalising with RIPA lysis buffer. We usually aim for a loading preparation > [M] 0.5 µg/µL .

1.8 Heat denature LDS sample preparation 70 °C 00:10:00 10m

SDS-PAGE and Transfer

20m

2 Load equal amounts of LDS sample alongside molecular weight protein ladder (e.g. Chameleon Duo, PageRuler Plus) into NuPAGE 4-12% BIS-Tris Mini or Midi gels. We most commonly aim for > 10 µg , but > 7 µg appears sufficient for detection of most desired protein targets.

2.1 Separate proteins in MOPS-SDS running buffer by gel electrophoresis. Run at 80V for 00:20:00 before increasing voltage to 120-160V until the dye front (~5kDa) has reached the bottom of the gel. 20m

2.2 We most commonly perform wet transfer to PVDF membrane, however nitrocellulose membranes and semi-dry transfer systems have been successfully used also.

Wet transfer proteins from gel to a pre-activated Immobilon-FL PVDF Membrane in Tris/Glycine transfer buffer supplemented with 20% v/v methanol. We typically use the Bio-Rad Mini Trans-Blot Cell at 80V for 2h for mini gels, or SureLock Tandem Midi Blot Module at 30V for 1h for midi gels.

It is important to activate PVDF membranes by incubating in methanol prior to transfer. If using near infra-red secondary antibody immunoblot detection use low fluorescence membranes such as Immobilon-FL.

Immunoblotting 3h 15m

3 Wash membrane with transferred proteins using PBS supplemented with [M] 0.1 % (v/v) Tween-20 (PBS-T)
Check homogenous protein transfer using Ponceau S Staining solution and then wash again with PBS-T.

3.1 Incubate membranes in blocking solution consisting of [M] 5 % (w/v) skimmed milk powder in PBS-T for 01:00:00 Room temperature .

3.2 Prepare primary antibody dilutions in [M] 3 % (w/v) skimmed milk powder in PBS-T supplemented with [M] 0.02 % (w/v) sodium azide.

1:1000 rabbit anti-pUb(Ser65) IgG
1:1000 rabbit anti-PINK1
1:500 rabbit anti-Parkin
1:1000 rabbit anti-MFN2
1:1000 mouse anti-TIM23 IgG2a
1:10000 mouse anti-GAPDH IgG2b
1:10000 mouse anti-β3-Tubulin Mouse IgG2a

3.3 Incubate blocked membranes with primary antibody dilutions at 4 °C Overnight 1h

3.4 Wash membranes 3x with PBS-T

3.5 Prepare secondary antibody dilutions in PBS-T additionally supplemented with [M] 0.02 % (w/v) SDS.

1:20000 donkey anti-mouse IgG (H+L) IRDye 680LT
1:20000 donkey anti-rabbit IgG (H+L) IRDye 800CW

3.6 Incubate immunoblots with secondary antibody dilutions at Room temperature 02:00:00 2h

3.7 Wash immunoblots 3x 00:10:00 with PBS-T, and 1x 00:05:00 with PBS 15m

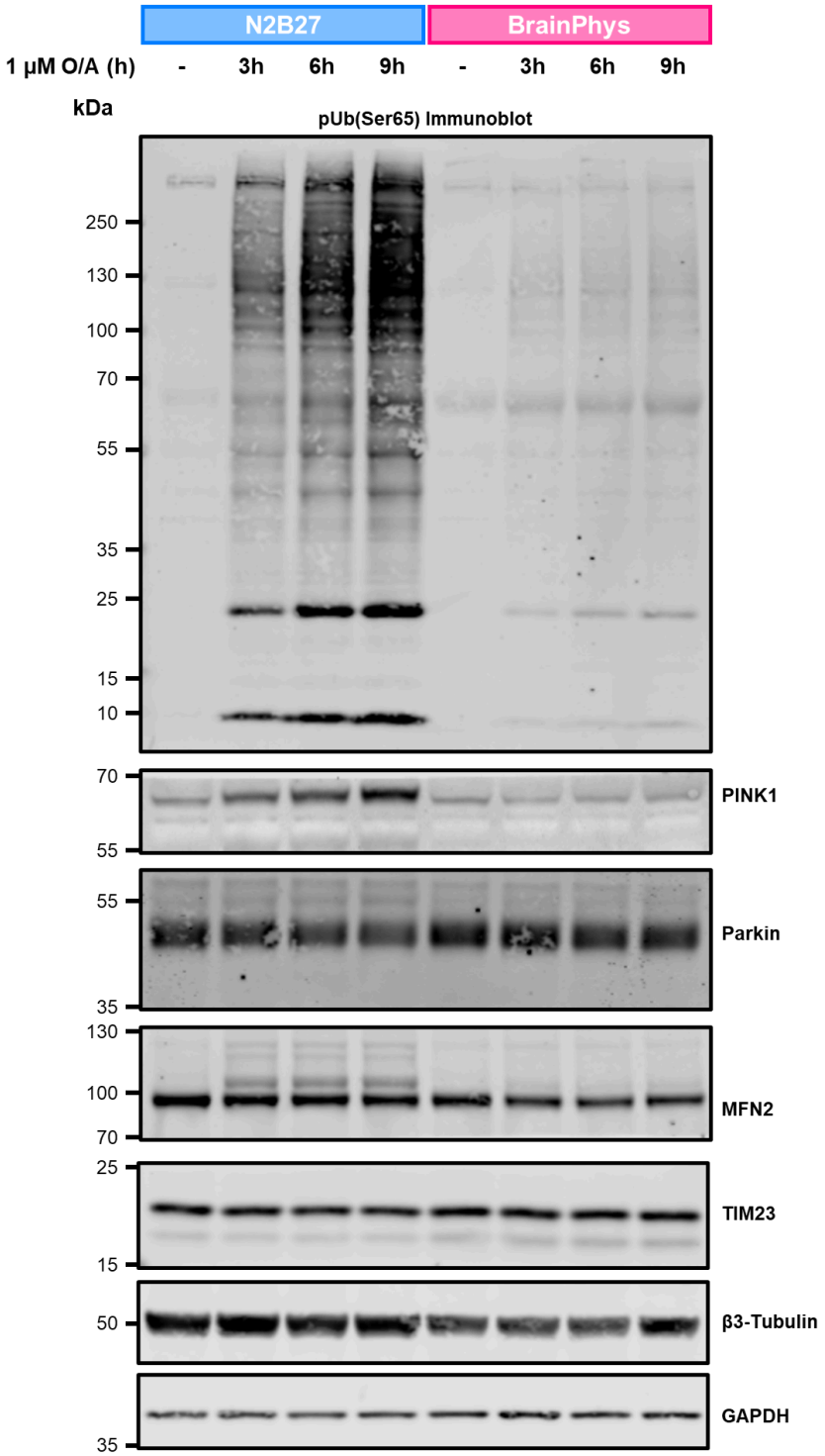
3.8 Detect near-infrared immunofluorescence using an Odyssey CLx imager. We typically find the automatic intensity detection to be appropriate in the majority of cases.

3.9 In most cases it is possible to probe for a housekeeping protein (e.g. GAPDH) of on all immunoblots for quantification normalisation purposes. For work-flow ease, membranes can be cut in order to probe for proteins with different molecular weight simultaneously.

Expected Immunoblot Detection and Analysis

4 pUb(Ser65) is low under basal culture conditions but appears as a smeared signal across molecular weights representing all the pUb(Ser65) following mitochondrial depolarisation.
PINK1 is often difficult to detect in samples from *in vitro* cultures at basal conditions. Following mitochondrial depolarisation full-length PINK1 is stabilised and detected at approximately 60kDa. Following proteosomal inhibition a cleaved PINK1 product is stabilised and detected at approximately 50kDa.
Parkin is detected at approximately 50kDa. Parkin is often reduced following mitochondrial depolarisation.
MFN2 is detected at approximately 80kDa. Following mitochondrial depolarisation higher molecular weight bands representing MFN2 ubiquitinated by activated Parkin are detected.
TIM23 is detected at approximately 20kDa and represents a useful mitochondrial marker.
GAPDH is detected at approximately 35kDa
β3-Tubulin is detected at 50kDa

4.1 Signal integrated densities are measured using Li-Cor Image Studio Lite v5.2 analysis software.
Band/smear intensities are background corrected using the median intensity of pixels surrounding the region of interest.
Band/smear intensities are normalised to the background corrected intensity of GAPDH for each protein sample.



Immunoblots from d24 iNeurons cultured in N2B27 or BrainPhys across 9h of mitochondrial depolarisation by 1uM Oligomycin-Antimycin to trigger PINK1-dependent mitiophagy.