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

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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.

KEYWORDS

Neurons, PINK1, Parkin, Mitochondrial stress, ubiquitin

MATERIALS

For culture

1. E16.5 mouse embryos (8–10 embryos, either sex; we routinely use C57BL/6j mice or PINK1 wild-type and knockout mice)
   ! CRITICAL All experiments must be conducted in accordance with the relevant institutional and governmental guidelines and regulations.

2. **Dissection medium**: HBSS (Gibco™, #14025050)

3. **Digestion medium**: 0.025% Trypsin-EDTA (Gibco™ #25300054); 0.125mg/mL DNase I (Merck #11284932001) in HBSS

4. **Dissociation medium**: Neurobasal medium (Gibco™ #21103049); 10% Foetal Bovine Serum (FBS) heat-inactivated (Gibco™ #10500064); 1X B27 supplement, serum free (Gibco™ #17504044); 1% GlutaMAX supplement (Gibco™ #35050061)

5. **Culturing medium:** Neurobasal medium (Gibco™ #21103049); 1X B27 supplement, serum free (Gibco™ #17504044); 1% GlutaMAX supplement (Gibco™ #35050061)

6. Poly-L-lysine hydrobromide (Merck #P2636)
7. Water sterile-filtered, suitable for cell culture (Merck #W3500)
8. Trypan Blue solution (Sigma-Aldrich #T8154)

For biochemistry

1. **Mitochondrial depolarisation:** 10 µM Antimycin A (Sigma-Aldrich #A8674); 1 µM Oligomycin (Sigma-Aldrich #75351) in DMSO (Sigma-Aldrich #D2650)
2. **Lysis Buffer**: 50 mM Tris-HCl (pH7.5), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (w/v) TritonX and protease inhibitor cocktail (Roche 11873580001) supplemented with 200 mM chloroacetamide (Sigma-Aldrich #C0267)**.**
3. DPBS, no calcium, no magnesium (Gibco™ #14190094)
4. Coomassie Protein Assay (Thermo Scientific™ #1856209)
5. 4X NuPAGE™ LDS Sample Buffer (Invitrogen™ #NP0008)
6. 2-Mercaptoethanol (Sigma-Aldrich M6250)
7. PageRuler™ Prestained Protein Ladder (Thermo Scientific™ #26616)
8. Immobilon-P PVDF Membrane (Merck #IPVH00010)
9. Amersham™ Protran® Nitrocellulose membranes (Merck #GE10600041)
10. NuPAGE™ 4 to 12%, Bis-Tris Mini Protein Gel, 10-well or 20-well (Invitrogen™ #NP0321BOX, #WG1402BOX).
11. NuPAGE™ MOPS SDS Running Buffer-20X(Invitrogen™ #NP000102)
12. 1 X Towbin transfer buffer :25mM Tris, 192 mM Glycine, 20% methanol
13. 1X Tris Buffered-Saline (TBS): 500mM Tris, 150mM sodium chloride, pH 7.6, at 25 °C.
14. 1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent (TBST)

15. 5 % non-fat milk in TBST

16. 5% bovine serum albumin (BSA) in TBST (Sigma-Aldrich #10735094001)

17. **Primary antibodies**: Phospho-Ubiquitin (Ser65) (E2J6T) Rabbit mAb (Cell signalling technology #62802), Ubiquitin Antibody P4D1 (Biolegend #646302), Anti-Parkin phospho-Ser65 Rabbit mAb by Epitomics in collaboration with the Michael J Fox Foundation for Research, Parkin Antibody PRK8 (Santa Cruz Biotechnology #sc-32282), GAPDH Antibody 6C5 (Santa Cruz Biotechnology # sc-32233)

18. **Secondary Antibodies**: Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate (Invitrogen™ #31460), Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP conjugate (Invitrogen™ #31450)

19. ECL™ Western Blotting Reagents (Merck, Cytiva, #RPN2106)

20. Amersham Hyperfilm ECL (Merck, Cytiva, #28906837)

STOCK SOLUTION PREPARATION

- **Poly-L-lysine solution:** Dissolve 10 mg /mL of poly-l-lysine in sterile water; filter, aliquot and store at − 20 °C. The solution is stable for 2–3 months.

- **DNaseI:** Dissolve 100mg/mL (wt/vol) DNase in sterile double-distilled water; filter, aliquot and store at − 20 °C. The solution is stable for 2–3 months

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

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

EQUIPMENT

1. Dumont #5 Forceps Biologie Inox (Fine Science Tool #11252-20)
2. Dumont #5XL Forceps Standard Inox (Fine Science Tool #11253-10)
3. Dumont #7 Fine Forceps Biologie Inox (Fine Science Tool #11274-20)
4. Dumont #5 45 Forceps Standard Dumoxel (Fine Science Tool #11251-35)
5. Fine Scissors ToughCut Straight 9cm (Fine Science Tool #14058-09)
6. Dissecting microscope Nikon (P-PS32 Plain Stand) with fiber light illumination unit

7. Cell Counter-DeNovix CellDrop™
8. 37 °C water bath
9. Laminar flow cell culture hood
10. Cell culture incubator 5% CO2, 95% humidity HERAcell®CO2 incubator (150 L)
11. Microcentrifuges, Micro Star 17R (VWR #521-1647)
12. Probe sonicator, Branson Digital Sonifier.
13. XCell *SureLock*™ 4Midi-Cell running tank (Invitrogen™ #WR0100)
14. XCell *SureLock*™ Mini-Cell running tank (Invitrogen™ #EI0001)
15. Mini Trans-Blot® Cell transfer tank (BIORAD # 1703930)
16. Trans-Blot® Cell transfer tank (BIORAD # 1703939)
17. ChemiDoc MP Imaging System (BIORAD)
18. ECOMAX™ X-ray Processor

CONSUMABLES

1. Cell culture multidishes, 6 well (Thermo Scientific™ #140675)
2. Cell strainer 40µM (Greiner Bio-one #5420400)
3. Stericups 0.22um, 250 mL and 500 mL (Merck #SCGPU02RE, #SCGPU05RE)
4. 50mL Stripette® Serological Pipets (Corning #4490)
5. 25mL Stripette® Serological Pipets (Corning #4489)
6. 10mL Stripette® Serological Pipets (Corning #4488)
7. 5mL Stripette® Serological Pipets (Corning #4487)
8. 15ml CELLSTAR® tubes (Greiner bio-one. Catalog# 188271)
9. 50ml CELLSTAR® tubes (Greiner bio-one. Catalog# 227261)
10. Standard 1ml and 200µl Pipette tips (Greiner bio-one # 686271, #685261)
11. Syringe filter (0.22µm. Sartorius, Item # ST16541-Q)
12. Syringes (50ml) (Terumo™# 8SS50L1)
13. 1.5ml Eppendorf tubes (Eppendorf™ # 0030120086)

14. Disposable Cell Lifter (FisherBrand #08100240)

PROCEDURE TO ISOLATE AND CULTURE NEURONS FROM EMBRYONIC MOUSE CORTEX

Coating of multiwell dishes with poly-L-lysine ◊TIMING 30 min, 1 d before culture

1. In a sterile laminar hood, prepare **poly-L-lysine 1 mg/ml in sterile** from 10mg/ml stock solution.

2. Add enough poly-l-lysine solution to cover the bottom of the well, ensure that the volume of poly-l-lysine covers the bottom of the dish entirely, usually 1 mL for 1 single well.

3. Wrap the dish in aluminum foil to prevent evaporation and leave it overnight at room temperature.

CRITICAL STEP! It is important to make sure that the poly-l-lysine does not dry out during incubation. If you are working in the laminar hood, be sure to turn the blower off during the incubation.

Washing of dishes after coating ◊TIMING 1 h, day of culture

4. Aspirate the poly-l-lysine carefully.
5. Add 1 ml of sterile water into each well and aspirate; repeat this step three times.
6. Aspirate water and leave dishes under the hood until completely dry.

Dissection of cortex from E16.5 mouse embryos ◊TIMING 1 h, 2–5 min for each embryo

7. Use sterilized instruments by autoclave or washing them with 70% (vol/vol) ethanol. Dry thoroughly if ethanol is used.

8. Prepare 60-mm dishes with dissection medium. If you are culturing from individual embryos, prepare 15mL tubes with 1 ml of dissection medium. If you are culturing pooled embryos, prepare 15mL tubes with 5 ml of dissection medium.

9. Euthanize the embryos pup by decapitation and separate the head from the body.

10. Place the head on a dish with dissection medium and hold down the sides with forceps.

11. Under a dissecting microscope, dissect the skin on the top of the head and hold down the skin on either side with the forceps.

12. Cut open the skull by making an incision at the base of the brain. Separate the two halves of the skull and remove carefully. Take care to not cut through the brain tissue when removing the skull bone.

13. By using forceps, pinch off the brain from the base and peel off the meninges carefully, ideally as a single piece. Check for the remaining pieces of meninges and remove them completely.

CRITICAL STEP! The meninges can be sticky and difficult to remove, it is important to ensure that the meninges are completely removed, so that they do not contribute any non-neuronal cells to the culture.

14. Separate the two halves of the brain by making a sagittal cut along the midline.

15. Orient the tissue so that the hippocampus is on the top. The hippocampus can be identified by its C-shaped structure and opacity, which differ from the neighboring cortical tissue. Cut away hippocampus and flat the cortex to expose the striatum. Using and angled forceps scoop out the striatum and cortex can also be dissected out and processed for cortical cultures.
CRITICAL STEP! it is important to ensure that the hippocampus and striatum are completely removed, so that they do not affect cortical distribution of neurons and contribute to an increase of glia cells.

16. To culture neurons from individual embryo, put each brain in a separate 15mL tube containing 1mL of dissection medium. For pooled cultures, put cortices from two embryos into one 15 mL tube containing 5 mL of dissection medium.
CRITICAL STEP! Ensure that the cortices are submerged in the medium and do not let them dry out at any point. It is important that the dissection be done as quickly as possible to ensure cell viability and health.
17. For individual cultures, collect a piece of tail for genotyping.

Cell dissociation and plating ◊TIMING ~1-1.5 h

18. Prepare digestion medium by adding 125 µL of DNase I (stock solution 10mg/mL) to 10 mL of Trypsin 0,05%.

19. Add 1:1 digestion medium to dissection medium containing the cortices, for individual mouse culture add 1mL of digestion media, for pooled mouse culture add 5mL of digestion media. Note. It is common to use HBSS Ca^{2+} and Mg^{2+} free buffer. In our hands, we have noticed that using HBSS plus Ca^{2+} and Mg^{2+} ensure a milder but optimal concentration of trypsin that allows to reduce excessive number of dead cells and higher yield of cells. At the same time, it stimulates DNase I activity.
20. Incubate at 37 °C in a water bath for 30 min.
CRITICAL STEP! It is important to ensure that this incubation does not proceed for longer than 30min.

21. Inactivate trypsin digestion by adding dissociation medium, 2mL for individual mouse culture or 5 mL for pooled mouse culture.

22. Centrifuge at 1200 rpm for 5 min

23. Resuspend cortices in 2 mL (for individual embryos) or 4 mL (for pooled embryos) of dissociation medium.

24. Carefully triturate the tissue 20 times to dissociate the cells gently and obtain a homogenous cell suspension, by using a p1000 pipette.
CRITICAL STEP! The trituration of cells should be done slowly and carefully to minimize damage to cells. It is best to avoid any bubbling during the procedure.
25. Remove any chunks of tissue by using a cell strainer and distribute drop by drop the cell suspension.

26. Centrifuge at 700 rpm for 7 min, to pellet down the cells and resuspend them in the culturing medium. Note. It is important to remove FBS from the medium to reduce the proliferation of glial cells.

27. Resuspend the dissociated cells in 2 mL (for individual embryos) or 4 mL (for pooled embryos) of culturing medium.

28. Take a 15 µL aliquot, add 1:1 ratio Trypan Blu and determine the density of cells and cell viability to the cell counter. Typical yields are ~2 x 10^6/mL and viability >80 %.

29. 5.0 × 10^5 cells/well plates are plated out on 6-well multidishes, containing 2 mL of pre-warmed culturing media.

Maintenance of neurons ◊TIMING 3 Up to 21 days

30. Every 5 days aspirate 1/3 of the media from each well and replace it with fresh culturing medium warmed to 37 °C. These neurons can be maintained in culture for up to DIV 28 and be used anytime during this period (DIV0-28). The culture can be used for examining PINK1 activation at 21DIV, since at this stage they express functional activity of Parkin and PINK1.
    CRITICAL STEP! It is important to not replace the entire medium, neurons secrete factors that promote growth and survival.

PROCEDURE TO INDUCE MITOCHONDRIAL DEPOLARISATION AND LYSING OF MOUSE CORTICAL NEURONS

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

1. To depolarize or uncouple mitochondrial membrane potential in neurons, cultures were treated for 3 to 9 hours with a combination of 10 µM Antimycin A and 1 µM Oligomycin dissolved in DMSO at 37°C.

Lysing of mouse neuronal cultures ◊TIMING 1-1.5h

2. 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 200mM Chloroacetamide. Note. Chloracetamide 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. Sonicate the cell lysate with a probe sonicator 5 seconds 20% amplitude and incubate for 30 minutes on ice.

6. Centrifuge the cell lysate at 17000*g* in a refrigerated centrifuge for 30 min. 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 ◊TIMING 5h-2d

8. 40µg of cell lysates are diluted 4X into 4X LDS loading buffer supplemented with fresh 10% 2-mercaptoethanol.

9. Samples are boiled for 3 min at 97°C or for 20 min at 37°C.
CRITICAL STEP! do not boil the sample for an extended time, ubiquitin can refold on membrane and obscure epitope.

10. Analyse samples by running on Nu-page Bis-Tris 4-12% gels for a better resolution of ubiquitin chains, at 120 V for ~2h.

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 1.5 h 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. Incubate membrane with blocking buffer 5% milk in 0.1% TBS-Tween for 1 h at Room Temperature.

13. 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 phosphor-Ubiquitin (1:2000), Ubiquitin (1:1000), GAPDH (1:5000) and Parkin (1:1000) in 5% BSA (TBS-Tween). Prepare phospho-Parkin (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. Remove primary antibody and wash 3 times with 0.1%TBS-Tween for 10 min.

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

16. Remove secondary antibody and wash 3 times with 0.1%TBS-Tween for 10 min.

17. Develop signal using ECL western Blotting reagents and analysing with Chemidoc. Note. Depending on signal, film can be best for sensitivity.