PROCEDURE TO ISOLATE AND CULTURE NEURONS FROM EMBRYONIC MOUSE CORTEX

In 1 collection

Jul 31, 2021

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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

dx.doi.org/10.17504/protocols.io.bsr6nd9e

PROTOCOL CITATION

Odetta Antico, Miratul M. Muqit 2021. PROCEDURE TO ISOLATE AND CULTURE NEURONS FROM EMBRYONIC MOUSE CORTEX . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bsr6nd9e

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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Feb 25, 2021 Jesintha Maniraja

Feb 25, 2021 Urmilas

Mar 01, 2021 Urmilas

May 05, 2021 m.muqit

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47646

PARENT PROTOCOLS

Part of collection

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

MATERIALS TEXT

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, calcium, magnesium, no phenol red **Gibco - Thermo**

Fisher Catalog #14025050

3. **Digestion medium**:

Trypsin-EDTA **Gibco - Thermo**

Fisher Catalog #25300054

DNase I **Merck Millipore**

Sigma Catalog #11284932001 in HBSS

A	B
Trypsin-EDTA	0.025%
DNase I	0.125 mg/mL

4. **Dissociation medium**:

Neurobasal™ Medium **Gibco - Thermo**

Fisher Catalog #21103049

Fetal Bovine Serum qualified heat inactivated Brazil **Gibco - Thermo**

Fisher Catalog #10500064

B-27™ Supplement (50X) serum free **Gibco - Thermo**

Fisher Catalog #17504044

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GlutaMAX™ Supplement **Gibco - Thermo Fisher Catalog #35050061**

A	B
Neurobasal medium	
Foetal Bovine Serum (FBS) heat-inactivated	10%
B27 supplement, serum free	1X
GlutaMAX supplement	1%

5. **Culturing medium:**

Neurobasal™ Medium **Gibco - Thermo Fisher Catalog #21103049**

B-27™ Supplement (50X) serum free **Gibco - Thermo Fisher Catalog #17504044**

GlutaMAX™ Supplement **Gibco - Thermo Fisher Catalog #35050061**

A	B
Neurobasal medium	
B27 supplement, serum free	1X
GlutaMAX supplement	1%

Poly-L-lysine

6. hydrobromide **Merck Catalog #P2636**

Water sterile-filtered BioReagent suitable for cell

7. culture **Merck Catalog #W3500**

Trypan Blue solution **Sigma –**

8. **Aldrich Catalog #T8154**

For biochemistry

1. **Mitochondrial depolarisation**:

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

10 µM **Aldrich Catalog #A8674**

Oligomycin A **Sigma –**

1 µM **Aldrich Catalog #75351** in DMSO

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Dimethyl sulfoxide **Sigma – Aldrich Catalog #D2650**

2. **Lysis Buffer:**

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

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

A	B
Tris-HCl (pH 7.5)	50 mM
Sucrose	250 mM
EDTA	1 mM
EGTA	1 mM
Sodium orthovanadate	1 mM
Sodium β-glycerophosphate	10 mM
Sodium fluoride	50 mM
Sodium pyrophosphate	5 mM
TritonX	1% (w/v)
Protease inhibitor cocktail supplemented with 200 mM chloroacetamide	

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

3. **Fischer Catalog #14190094**

Coomassie Protein Assay Reagent **Thermo**

4. **Scientific Catalog #1856209**

NuPAGE™ LDS Sample Buffer (4X) **Invitrogen - Thermo**

5. **Fisher Catalog #NP0008**

2-Mercaptoethanol **Sigma –**

6. **Aldrich Catalog #M6250**

PageRuler™ Prestained Protein Ladder 10 to 180 kDa **Thermo Fisher**

7. **Scientific Catalog #26616**

Immobilon-P PVDF

8. Membrane **Merck Catalog #IPVH00010**

Amersham™ Protran® Western blotting membranes

9. nitrocellulose **Merck Catalog #GE10600041**

10.

NuPAGE™ 4 to 12% Bis-Tris 1.0 mm Mini Protein Gel 10-well **Invitrogen - Thermo Fisher Catalog #NP0321BOX**

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NuPAGE™ 4 to 12% Bis-Tris 1.0 mm Midi Protein Gel 20-well **Invitrogen - Thermo Fisher Catalog #WG1402BOX**

NuPAGE™ MOPS SDS Running Buffer (20X) **Invitrogen - Thermo**

11. **Fisher Catalog #NP000102**

12. 1 X Towbin transfer buffer: **25 mM** Tris, **192 mM** Glycine, 20% methanol

13. 1X Tris Buffered-Saline (TBS): **500 mM** Tris, **150 mM** 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

Bovine Serum Albumin Fraction V **Sigma –**

16. 5% **Aldrich Catalog #10735094001**

17. **Primary antibodies**:

Phospho-Ubiquitin (Ser65) (E2J6T) Rabbit mAb **Cell Signaling Technology Catalog #62802**

Purified anti-Ubiquitin Antibody **BioLegend Catalog #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 Catalog #32282**

GAPDH Antibody (6C5) **Santa Cruz Biotechnology Catalog #32233**

18. **Secondary Antibodies**:

Goat anti-Rabbit IgG (H L) Secondary Antibody HRP **Invitrogen - Thermo Fisher Catalog #31460**

Rabbit anti-Mouse IgG (H L) Secondary Antibody HRP **Invitrogen - Thermo Fisher Catalog #31450**

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

ECL™ Western Blotting Reagents **Merck Catalog #RPN2106**

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

Hyperfilm™ ECL™ **Merck Catalog #28906837**

STOCK SOLUTION PREPARATION:

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- **Poly-L-lysine solution**: Dissolve [M]**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 [M]**100 mg/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 **50 mM** 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

- Dumont #5 Forceps Biologie Inox (Fine Science Tool #11252-20)

Dumont #5 Forceps
Forceps
Fine Science Tools **11252-20**

- Dumont #5XL Forceps Standard Inox (Fine Science Tool #11253-10)

Dumont #5XL Forceps
Forceps
Fine Science Tools **11253-10**

- Dumont #7 Fine Forceps Biologie Inox (Fine Science Tool #11274-20)

Dumont #7 - Fine Forceps
Forceps
Fine Science Tools **11274-20**

- Dumont #5 45 Forceps Standard Dumoxel (Fine Science Tool #11251-35)

Dumont #5/45 Forceps
Forceps
Fine Science Tools **11251-35**

- Fine Scissors ToughCut Straight 9cm (Fine Science Tool #14058-09)

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Fine Scissors - ToughCut®
Scissor
Fine Science Tools **14058-09**

- Dissecting microscope Nikon (P-PS32 Plain Stand) with fiber light illumination unit
- Cell Counter-DeNovix CellDrop^{TM}
- 37 °C water bath
- Laminar flow cell culture hood
- Cell culture incubator 5% CO2, 95% humidity HERAcell®CO2 incubator (150 L)
- Microcentrifuges, Micro Star 17R (VWR #521-1647)

Microcentrifuges, ventilated/refrigerated, Micro Star 17 / 17R
Microcentrifuges
VWR **521-1647**

- Probe sonicator, Branson Digital Sonifier.
- XCell SureLock™ 4Midi-Cell running tank (Invitrogen™ #WR0100)

XCell4 SureLock™ Midi-Cell
Midi-Cell running tank
Invitrogen **WR0100**

- XCell SureLock™ Mini-Cell running tank (Invitrogen™ #EI0001)

XCell SureLock Mini-Cell Electrophoresis System
Electrophoresis System
Invitrogen **EI0001**

- Mini Trans-Blot®Cell transfer tank (BIORAD # 1703930)

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Mini Trans-Blot Electrophoretic Transfer Cell
Electrophoresis System
Mini Trans-Blot **1703930**

- Trans-Blot®Cell transfer tank (BIORAD # 1703939)

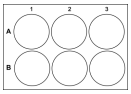
Trans-Blot Cell With Plate Electrodes and Super Cooling Coil
Trans-Blot **1703939**

- ChemiDoc MP Imaging System (BIORAD)
- ECOMAX™ X-ray Processor

CONSUMABLES

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

Nunc 6-Well Plate, Round
cell culture plate
Thermo Fisher Scientific **140675**
Nunc 6-Well Plate, Round



- Cell strainer 40µM (Greiner Bio-one #5420400)

EASYSTRAINER 40 µM, FOR 50 ML TUBES, FOR TUBES 227XXX/210XXX, GREEN, STERILE, SINGLE PACKED
Cell strainer
Greiner Bio-one **542040**

- Stericups 0.22um, 250 mL and 500 mL (Merck #SCGPU02RE, #SCGPU05RE)

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Stericup-GP Sterile Vacuum Filtration System
Sterile Vacuum Filtration System
Merck **SCGPU05RE**

Stericup-GP Sterile Vacuum Filtration System
Sterile Vacuum Filtration System
Merck **SCGPU02RE**

- 50mL Stripette® Serological Pipets (Corning #4490)

Stripette™ Serological Pipets
Serological Pipet
Stripette **4490**

- 25mL Stripette® Serological Pipets (Corning #4489)

25 mL Stripette™ Serological Pipets, Polystyrene, Individually Paper/Plastic Wrapped, Sterile, 25/Bag, 200/Case
Serological Pipet
Stripette **4489**

- 10mL Stripette® Serological Pipets (Corning #4488)

Stripette™ Serological Pipets
Serological Pipet
Stripette **4488**

- 5mL Stripette® Serological Pipets (Corning #4487)

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5 mL Stripette™ Serological Pipets, Polystyrene, Individually Paper/Plastic Wrapped, Sterile, 50/Bag, 200/Case
Serological Pipet
Stripette **4487**

- 15ml CELLSTAR® tubes (Greiner bio-one. Catalog# 188271)

15 ml CELLSTAR® Polypropylene Tube
Polypropylene Tube
CELLSTAR **188271**

- 50ml CELLSTAR® tubes (Greiner bio-one. Catalog# 227261)

50 ml CELLSTAR® Polypropylene Tube
Polypropylene Tube
CELLSTAR **227261**

- Standard 1ml and 200µl Pipette tips (Greiner bio-one # 686271, #685261)

100 - 200 µl Pipette Tips
Pipette Tip
Greiner bio-one **685261**

1000 µl Pipette Tips
Pipette Tip
Greiner bio-one **686271**

- Syringe filter (0.22µm. Sartorius, Item # ST16541-Q)

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Minisart® Syringe Filter, Polyethersulfone (PES), Pore Size 0.22 µm, Non-Sterile, Female Luer Lock, Male Luer Slip, Pack Size 500
Syringe Filter
Sartorius **ST16541-Q**

- Syringes (50ml) (Terumo™# 8SS50L1)

Terumo™ 3-Part 50mL Luer Lock Syringes
Luer Lock Syringe
Terumo **8SS50L1**

- 1.5ml Eppendorf tubes (Eppendorf™ # 0030120086)

Eppendorf® Safe-Lock micro test tubes
Micro test tube
Eppendorf **0030120086**

- Disposable Cell Lifter (FisherBrand #08100240)

Fisherbrand™ Cell Lifters
Cell Lifter
Fisherbrand **08100240**

Coating of multiwell dishes with poly-L-lysine

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1

◊TIMING **00:30:00** , 1 d before culture in this section.

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

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

4

◊TIMING **01:00:00** , day of culture in this section.

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

7

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◊TIMING 01:00:00 , 00:02:00 – 00:05:00 for each embryo in this section.

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 15 mL tubes with 1 mL of dissection medium. If you are culturing pooled embryos, prepare 15 mL 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.

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.

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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 an angled forceps scoop out the striatum and cortex can also be dissected out and processed for cortical cultures.

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 **15 mL** tube containing **1 mL** of dissection medium. For pooled cultures, put cortices from two embryos into one **15 mL** tube containing **5 mL** of dissection medium.

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 42m

18

◊TIMING ~ **01:00:00** - **01:30:00** in this section.

Prepare digestion medium by adding **125 µl** of DNase I (stock solution **10 mg/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 **1 mL** of digestion media, for pooled mouse culture add **5 mL** of digestion media.

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Note. It is common to use HBSS Ca2+ and Mg2+ free buffer. In our hands, we have noticed that using HBSS plus Ca2+ and Mg2+ 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 

30m

Incubate at **37 °C** in a water bath for **00:30:00** .

It is important to ensure that this incubation does not proceed for longer than **00:30:00**

21 

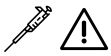
Inactivate trypsin digestion by adding dissociation medium, **2 mL** for individual mouse culture or **5 mL** for pooled mouse culture.

22 

5m

Centrifuge at **1200 rpm** for **00:05:00** .

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.

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 

7m

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Centrifuge at **700 rpm** for **00:07:00** 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.

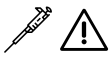
27 Resuspend the dissociated cells in **2 mL** (for individual embryos) or **4 mL** (for pooled embryos) of culturing medium.

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 x10^6/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

30 

◊TIMING **72:00:00** Up to **504:00:00** in this section.

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.

It is important to not replace the entire medium, neurons secrete factors that promote growth and survival.

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