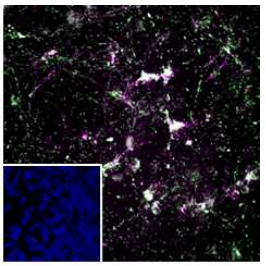
Mar 12, 2025

Detection of Lysosomal Delivery of Mitochondria in iNeurons Using MitoSRAI Reporter



bioRxiv

DOI
https://dx.doi.org/10.17504/protocols.io.261ged7qjv47/v1

Benjamin O'Callaghan[1,2], Helene Plun-Favreau[1,2]

[1]Department of Neurodegenerative Diseases, UCL Queen Square Institute of Neurology, London, UK;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, USA, 20815

Benjamin O'Callaghan
UCL Queen Square Institute of Neurology

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.261ged7qjv47/v1

External link: https://doi.org/10.1101/2025.04.01.646648

Protocol Citation: Benjamin O'Callaghan, Helene Plun-Favreau 2025. Detection of Lysosomal Delivery of Mitochondria in iNeurons Using MitoSRAI Reporter. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261ged7qjv47/v1

Manuscript citation:
Benjamin O'Callaghan, Daniela Melandri, Darija Soltic, Katharina Cosker, Marc P.M. Soutar, Helene Plun-Favreau (2025) iNeurons are sweet, maybe too sweet? Exploring the impact of media composition on PINK1-dependent mitophagy.bioRxiv doi: 10.1101/2025.04.01.646648

Protocol status: Working
We use this protocol and it's working

Created: January 22, 2024

Last Modified: March 12, 2025

Protocol Integer ID: 93878

Keywords: ASAPCRN, mitophagy, mitoSRAI, Opera Phenix, iNeuron, mitochondria in ineuron, assessment of lysosomal mitochondrial delivery, lysosomal mitochondrial delivery, ineuron hpsc line, detection of lysosomal delivery, mitochondria, ineuron, lysosomal delivery, using mitosrai reporter protocol outlining establishment, mitosrai construct

Funders Acknowledgements:
Aligning Science Across Parkinson's
Grant ID: ASAP 000478

Abstract

Protocol outlining establishment of a iNeuron hPSC line which stably expresses the mitoSRAI construct and the differentiation, and assessment of lysosomal mitochondrial delivery in iNeurons.

Materials

- iNeuron hPSC Line (iNeuron hPSC Line established on WTC11 hPSC background with TET-ON system at the AAVS1 safe-harbour locus for overexpression of murine Ngn2 and dCas9-KRAB at the CLYBL safe harbour locus was a kind gift from the lab of Michael Ward)
- A23_pLVX_mitoSRAI (10.5281/zenodo.14966995)
- pMD2.G (Addgene plasmid no. 12259, RRID:Addgene_12259)
- pCMVR8.74 (Addgene plasmid #22036, RRID:Addgene_22036)
- mTeSR Plus (STEMCELL Technologies, 100-0276)
- Ca^{2+}/Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, 14190169)
- 0.5M EDTA (Invitrogen, 15575020)
- Geltrex (Gibco, A1413302)
- TrypLE Express (Gibco, 12604021)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 11995-065)
- Heat-inactivated foetal bovine serum (FBS) (Gibco, A5256801)
- Y27632 ROCKi (MedChem, HY-10071)
- Polybrene (Sigma, H9268)
- DMEM/F12 - HEPES (Gibco, 11330032)
- Neurobasal (Gibco, 21103049)
- Glutamax Supplement (Gibco, 35050061)
- Non-essential amino acids (NEAA) (Gibco, 35050061)
- N2 supplement (Gibco, 17502048)
- B27 Supplement (Gibco, 17504044)
- Insulin (~10mg/ml, Sigma, I9278)
- B-mercaptoethanol (50mM, Gibco, 31350010)
- Accutase (Sigma, A6964)
- BrainPhys (STEMCELL Technologies, 5790)
- BDNF (Peprotech, 450-02)
- NT-3 (Peprotech, 450-03)
- Doxycycline (Dox) (Sigma, D5207)
- Puromycin (M P BIOMEDICALS UK, 0210055225)
- 10x Phosphate Buffered Saline (Fisher Scientific, 10649743)
- DRAQ5 (abcam, AB108410-1001)

Buffers/Stocks:

- Geltrex is diluted 1:100 in cold serum free DMEM for coating of culture surfaces
- 0.5mM EDTA = 1:1000 dilution of 0.5M EDTA in DPBS
- 5mM Y27632 ROCKi = powder dissolved in DPBS
- 5mg/ml polybrene = powder dissolved in DPBS
- 2mg/ml doxycycline = powder dissolved in DPBS
- 10ug/ml BDNF = powder dissolved in DPBS
- 10ug/ml NT-3 = powder dissolved in DPBS
- 1x PBS = 1:10 dilution of 10x PBS in dH2O

- 24% formaldehyde in 1x PBS = 37% formaldehyde diluted with dH2O and 10xPBS

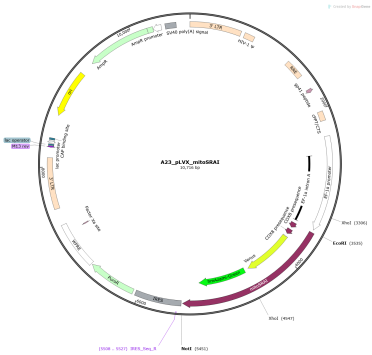
Safety warnings

- Oligomycin and Antimycin are mitochondrial poisons which should be handled with appropriate PPE and disposed of through safe disposal routes
- Formaldehyde is systemic poison which should be handled with appropriate PPE and disposed of through safe disposal routes

MitoSRAI iNeuron hPSC Line

1 **Establishment of mitoSRAI iNeuron hPSC Line**

Stable expression of mitoSRAI was established through reverse transduction of iNeuron hPSCs with lentivirus encoding mitoSRAI.



A23_pLVX_mitoSRAI_IRES_puro Plasmid Map

FILE A23_pLVX_mitoSRAI.dna

1.1 **Generation of mitoSRAI Lentivirus**

Lenti-X 293 T HEK cells in 6-well dish were cultured to ~90% confluency in DMEM 10% FBS media.

90% confluent Lenti-X 293 T HEK cells were then cotransfected with with pMD2.G, pCMVR8.74 and pLVX-EF1α-mitoSRAI-IRES-Puro at a 1:1:2 molar mass ratio (2ug final plasmid DNA total) using 12ul of p3000 regant and 6ul Lipofectamine 3000.

The next day, a full media change was performed with 2 mL mTeSR Plus and cells cultured for further 24 h.

The lentivirus containing media was collected and diluted 1:2 with fresh mTeSR Plus before filtering through 0.44 µm PES filters.

1.2 **iNeuron hPSC Transduction**

1×10^6 iNeuron hPSCs were reverse transduced with 750 µL of the lentivirus supernatant prepared in go to step #1.1 in 1.5 mL final volume of mTeSR Plus medium supplemented with [M] 5 µg/ml polybrene and [M] 10 micromolar (µM) ROCKi onto a geltrex coated 6-well dish.

Stably expressing cells were selected with [M] 1 µg/ml puromycin for >3 weeks prior to any iNeuron inductions.

Puromycin was maintained during routine culture but withdrawn when seeding for iNeuron differentiation.

iNeuron hPSC Maintenance

4d 0h 8m

2 **Maintenance of mitoSRAI iNeuron hPSCs and iNeuron Differentiation**
Conducted as described in protocol hPSC Culture, "iNeuron Differentiation and Culture in N2B27 vs BrainPhys for Immunofluorescence and Biochemistry Assessments of Mitophagy" (https://www.protocols.io/private/E5851436F8F111EF86990A58A9FEAC02). Specifics of the seeding and culture of mitoSRAI iNeurons for the mitophagy assay are described below in

iNeuron Culture

3h

3 **Seeding of d3 mitoSRAI iNeurons**
3h
Before beginning make sure you have Geltrex coated required wells of Revvity 96-well Phenoplates (Generally aim to do Overnight but > 03:00:00 will be fine).

3.1 Harvest d3 iNeurons by accutase as already described in "iNeuron Differentiation and Culture in N2B27 vs BrainPhys for Immunofluorescence and Biochemistry Assessments of Mitophagy".

Prepare 6×10^5 cells per ml suspension in N2B27 or BrainPhys media. 100ul of this suspension (=6×10^4 cells) will be required for every Revvity Phenoplate 96-well seeded.

3.2 Remove geltrex from plates, add cells as prepared above in go to step #3.1 to all wells except the perimeter wells (only use central 6 rows x 10 columns).

Note: I have found edge effects associated with extended neuron cultures in 96-well plate format. Therefore tend to only seed the internal 60-wells of a 96-well, and instead fill the perimeter wells with sterile water or PBS.

Treatment and Collection

20m

4 **Differentiation Stage:**
Collection of cells on a variety of differentiation stages might be interesting.
For assessing differences in mitophagy between i3N-Neurons cultured in N2B27 and BrainPhys, we typically begin the treatment time-course on d23 with collection on d24.

Mitophagy Induction:
In order to depolarise the mitochondrial membrane potential and initiate PINK1-dependent mitophagy we generally use an equimolar combination of oligomycin and

antimycin (O/A) at a [M] 1 micromolar (µM) final concentration. Unlike cell line models overexpressing Parkin, O/A induced increases in the lysosomal delivery of mitochondria are more subtle. Progressive increases in the lysosomal delivery of mitochondria are observed across 36h time-course for i3N-neurons cultured in N2B27. Unfortunately i3N-neurons cultured in BrainPhys show substantial cell death at timepoints >12h.

Treatment Strategy:
In order to gain some insight into the kinetics of lysosomal mitochondrial delivery, at least 3 different O/A time-points are typically assessed through a reverse time-course treatment strategy, alongside DMSO control treated wells. Wherever possible >3 technical replicate wells are assessed for each treatment, and we generally aim for 5 replicate wells wherever possible.

4.1 Perform half media change at least 1hr prior to first treatment time point.

4.2 **Reverse O/A Time Course:**

First prepare sufficient [M] 6 micromolar (µM) O/A dilution in appropriate media, for treatment of all experimental wells in O/A time-course. Also make appropriate 6x DMSO control.

Add appropriate volume of prepared 6x DMSO and [M] 6 micromolar (µM) O/A to have 1x final desired concentration (e.g. 30 µL for cells currently cultured in 150 µL media).
As an example see below for treatment plans of 24h O/A reverse time-course.

D1 9pm: Add 30 µL DMSO (24h DMSO) or 30 µL [M] 6 micromolar (µM) O/A (24h OA) to each appropriate well
D2 9am: Add 30 µL [M] 6 micromolar (µM) (12h OA) to each appropriate well
D2 12pm: Add 30 µL [M] 6 micromolar (µM) (9h OA) to each appropriate well
D2 3pm: Add 30 µL [M] 6 micromolar (µM) (6h OA) to each appropriate well

4.3 **Fixation:** 20m

Following the experimental end-point being reached, add [M] 24 % (w/v) formaldehyde prepared in 1x PBS to each experimental well to achieve [M] 4 % (w/v) final concentration.
E.g. for cells treated as in go to step #4.2 currently in 180 µL final volume, add 36 µL of [M] 24 % (w/v) formaldehyde.

Incubate cells for 00:20:00 in formaldehyde containing media

4.4 Following the 20min incubation, remove the formaldehyde containing media and dispose.

Add 200ul of DPBS

NOTE: Formaldehyde and O/A are toxic and must be disposed of through appropriate safe disposal routes

Imaging

5 While mitoSRAI does permit immunofluorescence costaining with Hoechst/DAPI, Alexa568 and Alexa647 it is not necessary for detection of mitoSRAI mitophagy readout.

Note: we see strong bleedthrough of Hoechst channel into the TOLLES signal which hampers mitophagy detection/quantification. It is recommended that Hoechst is not used prior to TOLLES/YPet detection. If nuclear stain is required DRAQ5 could be used instead

5.1 Imaging Parameters on Opera Phenix:
Use 63x objective
In confocal mode do a z-stack
1µm thick slices
Sequentially detect the 2x fluorophores
Adjust pixel dwell time to give good signal but avoiding saturation

TOLLES: Excitation 425nm laser, Emission 535-515nm
YPet: Excitation 488nm laser, Emission 500-550nm

I usually do 6x fields per well and will take ~1hr to image 60-wells

Analysis

6 Using the columbus software the below analysis strategy gives a robust mitophagy index readout:

1. Using max intensity project
2. Detect total mitochondria by finding TOLLES positive spots (note, methods which give small mitochondrial units are better here as will allow more granular separation of lysosomal vs non-lysosomal mitochondrial areas)
3. Perform image calculation dividing the TOLLES image by the YPet Image (TOLLES/YPet Ratio Image)
4. Select TOLLES positive spot population that has TOLLES/YPet ratio intensity >1 = YPet negative lysosomal mitochondria

5. Select TOLLES positive spot population that has TOLLES/YPet ratio intensity <1 = non-lysosomal mitochondria
6. Mitophagy index is proportion of total mitochondrial area (TOLLES total spot area) which are YPet negative lysosomal mitochondria (i.e. TOLLES Spots <1 ratio total spot area / TOLLES spots total spot area)
7. By using the sum of total mitochondrial area and lysosomal mitochondria area for each well, the value reported will represent the proportions across all fields of a well.
8. Average the technical replicate wells to get the average for that biological N

Note: it may be necessary to adjust the ratio intensity measures used to define lysosomal vs non-lysosomal mitochondria.

Protocol references

Soutar MPM, Melandri D, O'Callaghan B, Annuario E, Monaghan AE, Welsh NJ, D'Sa K, Guelfi S, Zhang D, Pittman A, Trabzuni D, Verboven AHA, Pan KS, Kia DA, Bictash M, Gandhi S, Houlden H, Cookson MR, Kasri NN, Wood NW, Singleton AB, Hardy J, Whiting PJ, Blauwendraat C, Whitworth AJ, Manzoni C, Ryten M, Lewis PA, Plun-Favreau H. Regulation of mitophagy by the NSL complex underlies genetic risk for Parkinson's disease at 16q11.2 and MAPT H1 loci. Brain. 2022 Dec 19;145(12):4349-4367. doi: **10.1093/brain/awac325**. PMID: 36074904; PMCID: PMC9762952.

Tian R, Gachechiladze MA, Ludwig CH, Laurie MT, Hong JY, Nathaniel D, Prabhu AV, Fernandopulle MS, Patel R, Abshari M, Ward ME, Kampmann M. CRISPR Interference-Based Platform for Multimodal Genetic Screens in Human iPSC-Derived Neurons. Neuron. 2019 Oct 23;104(2):239-255.e12. doi: **10.1016/j.neuron.2019.07.014**. Epub 2019 Aug 15. PMID: 31422865; PMCID: PMC6813890.

Fernandopulle MS, Prestil R, Grunseich C, Wang C, Gan L, Ward ME. Transcription Factor-Mediated Differentiation of Human iPSCs into Neurons. Curr Protoc Cell Biol. 2018 Jun;79(1):e51. doi: **10.1002/cpcb.51**. Epub 2018 May 18. PMID: 29924488; PMCID: PMC6993937.

Katayama H, Hama H, Nagasawa K, Kurokawa H, Sugiyama M, Ando R, Funata M, Yoshida N, Homma M, Nishimura T, Takahashi M, Ishida Y, Hioki H, Tsujihata Y, Miyawaki A. Visualizing and Modulating Mitophagy for Therapeutic Studies of Neurodegeneration. Cell. 2020 May 28;181(5):1176-1187.e16. doi: 10.1016/j.cell.2020.04.025. Epub 2020 May 20. PMID: 32437660.

de Talhouët C, Esteras N, Soutar MPM, O'Callaghan B, Plun-Favreau H. KAT8 compound inhibition inhibits the initial steps of PINK1-dependant mitophagy. Sci Rep. 2024 May 22;14(1):11721. doi: 10.1038/s41598-024-60602-9. PMID: 38777823; PMCID: PMC11111795.