# Cataloguing information

### Title

Treatment and staining of iPSC-derived neurons for lysosomal phenotype analysis.

### Description (min 150 words)

This protocol describes the preparation and treatment of neuronal cultures to be imaged for its analysis using the Opera Phenix high-content screening system. This includes the preparation of the cultures and its treatment to stain Lysosomes using a lysosome staining reagent, the treatment with DQ-red BSA to analyse lysosomal activity and the fixation and staining of the autophagic markers P62 and LC3 in the presence and absence of the autophagy-lysosomal pathway inhibitor Bafilomycin A1. Quantification of autophagy measures or autophagy flux in the presence and absence of bafilomycin A1 treatment offers a dynamic readout of the autophagy state that cannot be captured otherwise in immunostaining and western blot experiments. The aim of this protocol is to provide a guideline for stain and image any cell line for its analysis using a high content imaging system, allowing the process of large number of conditions/cell lines for the measurement of lysosomal and autophagosomal phenotypes.

### Has this output been funded by ASAP?

Yes

### Has this output been used in a publication?

No

### Keywords (minimum of 5)

Immunocytochemistry, iPSC, neurons, Live imaging, Autophagy, Lysosome

### DOI (if applicable)

### Usage notes (i.e. to access will you need to create an account with a particular provider?)

### Contributors

1. ASAP Teams (e.g. Kirik, Alessi, Scherzer, Lee)
	1. Kirik
2. Labs (e.g. Kirik, Parish, Thompson, Halliday, Sue, Johnston)
	1. Halliday
3. Authors (e.g. Dad Abu-bonsrah, Louise Cottle, Gautam Wali, Adahir Labrador-Garrido)
	1. Jessica Chedid, Adahir Labrador-Garrido, Nicolas Dzamko

# Treatment and staining of iPSC-derived neurons for lysosomal phenotype analysis Protocol

### Key equipment/consumables/reagents/solutions

### Consumables

### 96 well-plates from Perkin Elmer: CELLCARRIER-96 ULTRA Black with clear bottom, TC treated, sterile with lid (catalogue no. 6055300)

### Reagents

* **For live cells:**

Cell culture media\* (refer to ‘material input’ section for details)

DQ-red BSA (ThermoFisher D12051)

Lysosomal Staining Reagent- Orange-Cytopainter (abcam ab176827)

Mitotracker deep red (ThermoFisher M22426)

Cell proliferation staining reagent – Green fluorescence – Cytopainter (abcam ab176735)

PUREBLUTM Hoesht 33342 (Bio-Rad #1351304)

* **For fixed cells**:

Triton X100 (Sigma #T8787-100ml)

Bovine serum albumin (Bovostar BSAS-AU 500g)

Paraformaldehyde (Sigma #ACR416780010)

Bafilomycin A1 (B1793-10UG)

DAPI nuclear stain

Antibodies:

|  |  |  |  |
| --- | --- | --- | --- |
| Antibodies | Species | Source | Cat n# |
| MAP2\* | Chicken | Thermo Fisher | PA1-10005 |
| TH\* | Sheep | Thermo Fisher | PA1-4679 |
| TH\* | Rabbit | Thermo Fisher | OPA1-04050 |
| LC3 | Rabbit | Abcam | ab192890 |
| P62 | Mouse | Abcam | ab56416 |

\* These antibodies are included to identify desired cell populations and can be substituted as required for different differentiation protocols.

### solutions

fixing solution: 4% PFA in 1xPBS

Blocking buffer: 3% BSA + 0.1% Triton X-100 in 1 x PBS

Permeabilization buffer: 0.3% Triton X-100 in 1 x PBS

Antibodies dilution solution: antibodies are prepared in blocking buffer

Washing solution: 1x PBS

### Material input (animal, cell, tissue, fraction details)

This protocol can be applied to different cell types for the assessment of lysosomal functions. Here we apply it to induced pluripotent stem cells differentiated into Ventral Medial Dopaminergic neurons (protocol available at [10.17504/protocols.io.bu7ynzpw](https://protect-au.mimecast.com/s/8ywjC2xMQziklG67AH13Nut?domain=doi.org)) or cortical neurons (protocol available at [10.17504/protocols.io.bu6znzf6](https://protect-au.mimecast.com/s/mvfcC3QNPBimAMxZVU2pt4-?domain=doi.org)). Cortical neurons were cultured until DIV50 and ventral medial dopaminergic neurons cultured until DIV40.

### Experimental Outline

Cells are seeded at 30-50k cells/well and maintained in cell culture media until the desired experimental endpoint. Initial plating densities should be optimized for each cell type or cell line to provide optimal survival rates, morphology, and differentiation at final timepoint.

When cells are ready to be stained, the protocol diverges into 2 separate series of steps:

1. **Probing and imaging live cells**
2. **Treating, staining, and imaging fixed cells.**
3. **Live cells experimental outline:**
* Prepare: DQ-red-BSA 1:100, Cytopainter green cell proliferation reagent 1:500 and Hoechst 1:100 in complete cell culture media.
* Alternatively prepare: Mitotracker 1:10.000, Lysosomal staining 1:500, Cytopainter 1:500 and Hoechst 1:100 in complete cell culture media.
* Gently replace cell culture media on the cells with the prepared solution (100ul/well)
* Cells are imaged 15, 45 and 90 minutes after adding the probes using the Opera Phenix high-content screening system. Hoechst, Alexa488 and Alexa561 Laser/filter pairs are used for DQ-red BSA treatment imaging. Hoechst, Alexa488, Alexa561 and Alexa647 laser/filter pairs are used to image Mitotracker/Lysosomal probes.
* Suggested Imaging conditions:

 40x water objective, 3 z-steps, at least 25 fields of view, imaging done in cell culture conditions (37°C, 5% CO2).

Note: 40x objective is needed to obtain enough detail for accurate Lysosome-Mitophagy analysis. Z-step and fields of view are selected to obtain enough images without compromising the time it takes to finish a round of imaging.

1. **Fixed cells experimental outline:**

Step 1: (bafilomycin treatment and fixation)

* To treat the cells, cell culture media is replaced with 150ml media containing 400nM bafilomycin A1, and incubated at 37°C, 5%CO2 for 4 hours.
* After 4h cells are fixed in 2 steps to avoid detachment:
* Remove 75µl of the culture media and replace with the same volume of 4% PFA, incubate at room temperature in the dark for 10 minutes.
* Remove mixture of cell culture media and PFA gently and replace with 70 µl of 4% PFA and incubate for 15 minutes.
* Remove PFA solution and gently wash with 1x PBS.

Cells can be stored in PBS at 4°C before commencing staining. (At this point plates can be used for later steps of permeabilization, blocking and staining or can be stored at 4°C in the dark for several days).

Step 2 staining with primary antibodies:

* Discard 1x PBS solution from wells and add permeabilization buffer (100 µl per well), incubate for 20 minutes.
* Discard permeabilization buffer and add blocking buffer (100 µl per well), incubate for 1 hour.
* Prepare antibody combinations to desired final concentrations in blocking buffer, discard blocking buffer from plates and replace with primary antibody dilutions, incubate overnight at 4°C.
* Wash cells with 1x PBS for 5 minutes (3 times)
* Add secondary antibodies diluted (1:500) in blocking buffer to cells (100 µl per well), incubate for 1 hour at room temperature.
* Wash cells with 1x PBS for 5 minutes (2 times).
* Add 1x PBS with DAPI, incubate for at least 7 minutes.
* Wash cells with 1x PBS, leave in 200 µl of 1x PBS per well to avoid drying out.
* Plates are now ready to be imaged.
* Suggested Imaging conditions:

40x water objective, 10 z-steps (0.5mm step size as recommended by the manufacturer), at least 46 fields of view per well (covering 16% of the well’s area).

Note: Imaging conditions are selected taking into consideration the detail needed (analysis of organelles need higher magnification), and the minimum number of cells needed to obtain a robust result (if the culture has very little number of cells, more fields of view could be needed). Please refer to the Harmony software manual (<https://www.perkinelmer.com/uk/product/harmony-4-9-office-license-hh17000010>) for assistance in setting imaging parameters.

## Methods section 2

### Key equipment/consumables/reagents/solutions

### Experimental Outline

## Methods section 3

### Key equipment/consumables/reagents/solutions

### Experimental Outline