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

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ABSTRACT

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.

ATTACHMENTS

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GUIDELINES

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.

MATERIALS TEXT

Consumables:

96 well-plates from Perkin Elmer: CELLCARRIER-96 ULTRA Black with clear bottom TC treated sterile **Perkin Elmer Catalog #6055300**

Reagents

For live cells:

- Cell culture media* (refer to 'material input' section for details)
  DQ™ Red BSA - Special Packaging **Thermo**
- **Fisher Catalog #D12051**
  Lysosomal Staining Reagent- Orange-Cytopainter
- **Abcam Catalog #ab176827**
  MitoTracker™ Deep Red FM - Special Packaging **Thermo**
- **Fisher Catalog #M22426**
  Cell Proliferation Staining Reagent - Green Fluorescence - Cytopainter
- **Abcam Catalog #176735**
  PureBlu™ Hoechst 33342 Nuclear Staining Dye **BIO-**
- **RAD Catalog #1351304**

For fixed cells:

Triton™ X-100 **Sigma**

- **Aldrich Catalog #T8787-100ML**
- Bovine serum albumin (Bovostar BSAS-AU 500g)
  Paraformaldehyde **Sigma**
- **Aldrich Catalog #416780010**
  Bafilomycin A1 from Streptomyces griseus **Sigma**
- **Aldrich Catalog #B1793-10UG**
- DAPI nuclear stain

Antibodies:

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A	B	C	D
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.

☒ MAP2 Polyclonal Antibody **Thermo Fisher Scientific Catalog #PA1-10005**

☒ Tyrosine Hydroxylase Polyclonal Antibody **Thermo Fisher Scientific Catalog #PA1-4679**

☒ Tyrosine Hydroxylase Polyclonal Antibody **Thermo Fisher Scientific Catalog #OPA1-04050**

☒ Recombinant Anti-LC3B antibody [EPR18709] - Autophagosome Marker (ab192890) **Abcam Catalog #ab192890**

☒ Anti-SQSTM1 / p62 antibody [2C11] - BSA and Azide free **Abcam Catalog #ab56416**

Solutions:

A	B
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) or cortical neurons (protocol available at 10.17504/protocols.io.bu6znzf6). Cortical neurons were cultured until DIV50 and ventral medial dopaminergic neurons cultured until DIV40.

Live cells experimental outline

1 Prepare: DQ-red-BSA 1:100, Cytopainter green cell proliferation reagent 1:500 and Hoechst 1:100 in complete cell culture media.

2 Alternatively prepare: Mitotracker 1:10.000, Lysosomal staining 1:500, Cytopainter 1:500 and Hoechst 1:100 in complete cell culture media.

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3

Gently replace cell culture media on the cells with the prepared solution (**100 µL** /well).

4 2h 30m

Image the cells for **00:15:00** , **00:45:00** and **01:30:00** 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% CO_2).
- 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.

Fixed cells experimental outline-Bafilomycin treatment and fixation 12h 2m

5 4h

To treat the cells, replace the cell culture media with **150 µL** media containing **400 nanomolar (nM)** bafilomycin A1, and incubate at **37 °C** , 5% CO_2 for **04:00:00** .

6 4h

Fix the cells after **04:00:00** in 2 steps to avoid detachment.

7 10m

Remove **75 µL** of the culture media and replace with the same volume of 4% PFA, incubate at **Room temperature** in the dark for **00:10:00** .

8 15m

Remove mixture of cell culture media and PFA gently and replace with **70 µL** of 4% PFA and incubate for **00:15:00** .

9 Remove PFA solution and gently wash with 1x PBS.

10 Store the cells 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.

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Fixed cells experimental outline-Staining with primary antibodies 12h 2m

11 20m

Discard 1x PBS solution from wells and add permeabilization buffer (**100 µL** per well), incubate for **00:20:00** .

12 1h

Discard permeabilization buffer and add blocking buffer (**100 µL** per well), incubate for **01:00:00** .

13 1h

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** .

14

Wash cells with 1x PBS for 5 min (3 times).

14.1 Wash cells with 1x PBS for **00:05:00** (1/3). 5m

14.2 Wash cells with 1x PBS for **00:05:00** (2/3). 5m

14.3 Wash cells with 1x PBS for **00:05:00** (3/3). 5m

15 1h

Add secondary antibodies diluted (1:500) in blocking buffer to cells (**100 µL** per well), incubate for **01:00:00** at **Room temperature** .

16

Wash cells with 1x PBS for 5 min (2 times).

16.1 Wash cells with 1x PBS for **00:05:00** (1/2). 5m

16.2 Wash cells with 1x PBS for **00:05:00** (2/2). 5m

17 7m

Add 1x PBS with DAPI, incubate for at least **00:07:00** .

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18

Wash cells with 1x PBS, leave in **200 µL** of 1x PBS per well to avoid drying out.

19 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.

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