Apr 30, 2025

Immunofluorescence-based Assay for Monitoring pUb in Pink1-Parkin-Mediated Mitophagy in iPSC-Derived Dopaminergic Neurons

DOI
https://dx.doi.org/10.17504/protocols.io.yxmvm95jol3p/v1

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Protocol Citation: Roxanne Larivière, Edward A. Fon 2025. Immunofluorescence-based Assay for Monitoring pUb in Pink1-Parkin-Mediated Mitophagy in iPSC-Derived Dopaminergic Neurons. **protocols.io** https://dx.doi.org/10.17504/protocols.io.yxmvm95jol3p/v1

Protocol status: Working
We use this protocol and it is working

Created: January 08, 2025

Last Modified: April 30, 2025

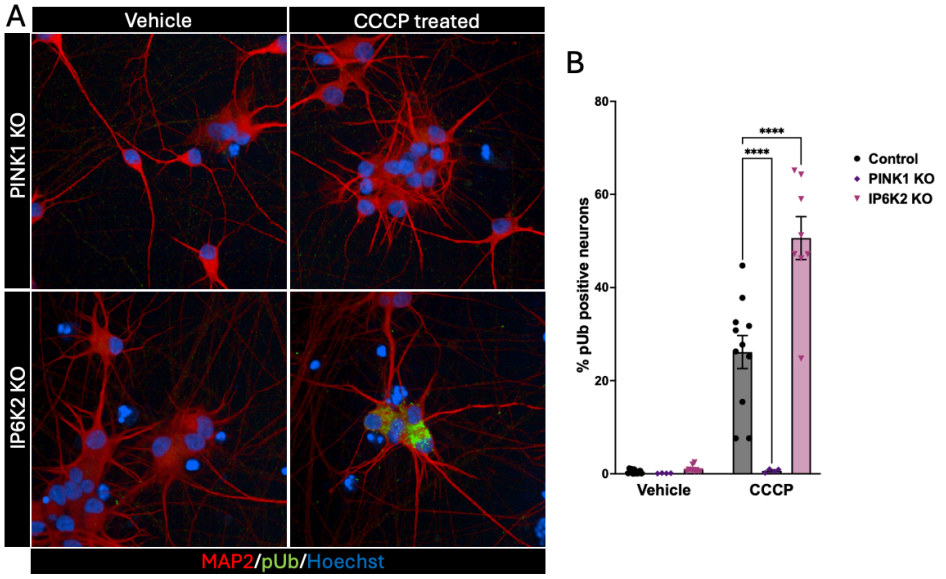
Protocol Integer ID: 117936

Keywords: IF, immunofluorescence, MAP2, mitophagy, iPSC, dopaminergic neurons, dopaminergic neuron, derived dopaminergic neuron, derived dopaminergic neurons this protocol, mediated mitophagy, mitophagy in ipsc, induced pluripotent stem cell, pluripotent stem cell, using immunofluorescence, immunofluorescence, based assay

Funders Acknowledgements:
Michael J. Fox Foundation for Parkinson's research
Grant ID: MJFF-020696

Abstract

This protocol outlines the procedure for assaying pUb in Pink1-Parkin-mediated mitophagy in induced pluripotent stem cell (iPSC)-derived dopaminergic neurons using immunofluorescence.



(A) Immunofluorescence labelling of 6 weeks differentiated PINK1 KO and IP6K2 KO iPSC-derived dopaminergic neurons treated with or without CCCP 20µM using pUb and MAP2 antibodies. (B) Quantification of pUb positive neurons in PINK1 KO, IP6K2 KO and isogenic control dopaminergic neurons.

Materials

Reagents	Company	Cat. Number
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Selleckchem	S6494
Phospho-Ubiquitin (Ser65) (E2J6T) Rabbit mAb	Cell Signaling	62802
mouse Lamp2 antibody	Santa Cruz	sc-18822
Chicken MAP2 antibody	Encor Biotech	CPCA-MAP2
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 555	ThermoFisher Scientific	A21437
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher Scientific	A21235
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher Scientific	A11008
Hoechst 33342	SIGMA	H3570
16% Formaldehyde solution (w/v) Methanol-free	ThermoScientific	28908
Normal goat serum	SIGMA	G9023
Triton X-100	SIGMA	X-100

Before start

iPSC-derived dopaminergic neurons are cultured on 96-well plates for up to 4 to 6 weeks of differentiation. We use black Costar 96-well assay plates (Corning cat. no. 3904), which are compatible with imaging using the Opera Phenix high-content screening system.

Be extremely gentle when dispensing into the wells, as dopaminergic neurons are prone to detaching with forceful pipetting.

CCCP treatment

4h

1 - Treat neurons (at least 5 columns of one 96 well plate) with CCCP 20µM for 04:00:00

4h

Fixation of dopaminergic neurons in 96 well plates

20m

2 - Leave 50 µL of media and gently add 50 µL of 8% PFA per well.
- Incubate at room temperature 00:20:00 .
- Remove PFA and wash 3 times with 1X PBS, 75 µL per well, 00:05:00 each

20m

Permeabilization

25m

3 - Prepare permeabilization solution: 0.2% TX-100; 1X PBS.
- Incubate in permeabilization solution 00:10:00 , 60 µL per well
- Remove permeabilization solution and wash 3 times with 1X PBS, 75 µL per well, 00:05:00 each

25m

Blocking

1h

4 - Prepare blocking buffer: 5% normal goat serum; 0.02% TX-100; 1X PBS
- Incubate in blocking solution 01:00:00 , 60 µL per well

1h

Incubation with primary antibodies

16h

5 - Prepare primary antibody mix in blocking buffer, 60 µL per well

- rabbit pUb antibody 1:1250
- chicken MAP2 antibody 1:1500

- Incubate Overnight at 4 °C with gentle shaking

16h

Washes

20m

6 - Wash 4 times with 75 µL 1X PBS, 00:05:00 each 20m

Incubation with secondary antibodies 2h

7 - Prepare secondary antibody mix in 1% normal goat serum; 1X PBS. Dispense 60 µL per well. 2h

- anti-Rabbit Alexa Fluor 488 1:1000
- anti-Chicken Alexa Fluor 555 1:1000
- Hoechst 33342 1:5000

- Incubate 02:00:00 at Room temperature with gentle shaking

Washes 20m

8 - Wash 4 times with 75 µL 1X PBS, 00:05:00 each 20m

- Leave 100 µL of 1X PBS into wells

Imaging

9 - Samples are ready for imaging