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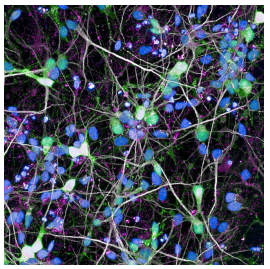
Immunofluorescence Staining for Assessing pUb(Ser65) Accumulation in iNeurons



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We use this protocol and it's working

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Abstract

Protocol for the imaging and analysis of iNeurons with anti-pUb(Ser65) immunofluorescence in order to measure PINK1-dependent mitophagy initiation.

Materials

- 10x Phosphate Buffered Saline (Fisher Scientific, 10649743)
- Heat-inactivated foetal bovine serum (FBS) (Gibco, A5256801)
- 10% w/v Triton-X-100 (Sigma, 93443)
- rabbit anti-pUb(Ser65) IgG (Cell Signaling Technology, Cat# 62802, RRID:AB_2799632)
- mouse anti-TOM20 IgG2a (Santa Cruz Biotechnology, Cat# sc-17764, RRID:AB_628381)
- chicken anti-MAP2 IgY (Millipore Cat# AB5543, RRID:AB_571049)
- goat anti-rabbit IgG (H+L) Alexa Fluor 488 (Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165)
- goat anti-mouse IgG (H+L) Alexa Fluor 568 (Thermo Fisher Scientific Cat# A-11004, RRID:AB_2534072)
- goat anti-chicken IgY (H+L) Alexa Fluor 647 (Thermo Fisher Scientific Cat# A-21449, RRID:AB_2535866).
- Hoechst 33342 Solution (20 mM) (Thermo Fisher Scientific Cat# 62249)

Immunofluorescence Staining

4h

1 iNeuron culture and formaldehyde fixation described in "iNeuron Differentiation and Culture in N2B27 vs BrainPhys for Immunofluorescence and Biochemistry Assessments of Mitophagy" (https://www.protocols.io/private/E5851436F8F111EF86990A58A9FEAC02).

1.1 Wash fixed iNeuron cultures in Revvity 96-well Phenoplates twice with 100 µL of PBS.

Note: cultures are very delicate and utmost care and steady liquid additions is required throughout the whole staining procedure in order to prevent detachment of iNeurons from culture dish en masse.

1.2 Incubate fixed iNeurons in blocking and permeabilisation solution ([M] 10 % (v/v) FBS, [M] 0.25 % (w/v) , 1x PBS) for 01:00:00 at Room temperature . 50 µL per well of Revvity Phenoplate 96-well.

1.3 Prepare primary antibody dilution in blocking solution ([M] 10 % (v/v) FBS, 1x PBS). 50 µL will be required for each well of a Revvity Phenoplate 96-well.
1:1000 rabbit anti-pUb(Ser65) IgG
1:1000 mouse anti-TOM20 IgG2a
1:5000 chicken anti-MAP2 IgY

1.4 Remove blocking and permeabilisation solution from cells and add 50 µL of primary antibody dilution prepared in go to step #1.6 to each well of fixed iNeurons. Incubate for 02:00:00 at Room temperature . 2h

1.5 Remove primary antibody dilution and wash wells 3x with 100 µL of PBS.

1.6 Prepare secondary antibody and Hoechst nuclear counterstain dilution in blocking solution ([M] 10 % (v/v) FBS, 1x PBS). 50 µL will be required for each well of a Revvity Phenoplate 96-well.
1:2000 goat anti-rabbit IgG (H+L) Alexa Fluor 488
1:2000 goat anti-mouse IgG (H+L) Alexa Fluor 568
1:2000 goat anti-chicken IgY (H+L) Alexa Fluor 647

10 µM Hoechst 33342

1.7 Remove PBS wash from cells and add 50 µL of secondary antibody with Hoechst dilution prepared in go to step #1.6 to each well of fixed iNeurons. Incubate for 02:00:00 at Room temperature. 2h

1.8 Remove secondary antibody with hoechst dilution and wash wells 3x with 100 µL of PBS.
Keep cells incubated in 100 µL of PBS for imaging.

Imaging

2 Imaging Parameters on Opera Phenix:
Use 40x objective
In confocal mode do a z-stack
1µm thick slices
Sequentially detect the 4x fluoroscent signals
Adjust pixel dwell time to give good signal but avoiding saturation
Hoechst 33342: Excitation 375nm laser, Emission 435-515nm
Alexa Fluor 488: Excitation 488nm laser, Emission 500-550nm
Alexa Fluor 568: Excitation 561nm laser, Emission 570-630nm
Alexa Fluor 647: Excitation 640nm laser, Emission 650-760nm
I usually do 6x fields per well and will take ~1.5hr to image 60-wells

Analysis

3 Using the columbus software the following is a useful analysis strategy for pUb(Ser65) accumulation in iNeurons:

1. Using max intensity project
2. Define MAP2+ area of each imaging field based on the intensity of Alexa Fluor 647 (MAP2 Secondary antibody)
3. Measure Alexa Fluor 488 (pUb(Ser65) secondary antibody) mean intensity in the MAP2+ area of each field.
4. Report the mean intensity of all fields within a well.
5. Average the mean well intensities across technical replicate wells

Protocol references

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