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Protocol status: In development
We are still developing and optimizing this protocol

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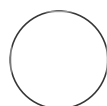
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Microscopy-based pUb-coverage measurements of mitochondria in iNeurons

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ABSTRACT

Microscopy-based pUb-coverage measurements of mitochondria in iNeurons

Differentiation of iNeurons

- 1** Day 0: Treat AAVS1-TRE3G-NGN2 cells with Accutase and plate the dissociated cells in matrigel-coated 6-well plates (2x10⁵ cells/well) in ND1 Medium supplemented with Y27632 (10 μ M).
ND1 Medium:
DMEM/F12
N2 (100x) 1x
BDNF 10 ng/ml
NT3 10 ng/ml
NEAA (100X) 1x
Laminin 0.2 μ g/ml
Doxycycline 2 μ g/ml
- 2** Day 1: Replace the medium with ND1 Medium.
- 3** Day 2: Replace the medium with ND2 Medium.
ND2 Medium
Neurobasal medium
B27 (50x) 1x
GlutaMax (100x) 1x
BDNF 10 ng/ml
NT3 10 ng/ml
Doxycycline 2 μ g/ml
- 4** Day 4: Exchange 50% of the medium from each well.
- 5** Day 6: Treat the cells with Accutase and replat the dissociated cells in matrigel-coated 6-/12-well glass bottom plates (2-4x10⁵ cells/well for 6 wells) in ND2 Medium.
- 6** Day 8 and thereafter: Exchange 50% of the medium from each well every other day.
Doxycycline can be withdrawn on Day.
- 7** Induce mitophagy using Antimycin A / Oligomycin A for the desired time.

Staining

- 8 Aspirate ND2 and fix cells in 1 ml pre-warmed 4% PFA for 30 min.
- 9 Aspirate PFA solution and wash wells 3x with PBST (1x PBS, 0.02% Tween 20)
- 10 Permeabilize the cells by adding 0.2% Triton X-100 in PBS.
- 11 Remove the detergent solution by aspiration. Wash wells 3x with PBST (1x PBS, 0.02% Tween 20). Drain well.
- 12 Block cells for 10 min with 3% BSA – 1x PBS.
- 13 Remove BSA solution by aspiration. Wash wells 3x with PBST (1x PBS, 0.02% Tween 20). Drain well.
- 14 Incubate with primary antibodies in 3% BSA - 1x PBS for 3h at RT with gentle shaking.

14.1 Anti-pUb (rabbit)

14.2 Anti-HSP60 (mouse)

15 Wash wells 3x with PBST (1x PBS, 0.02% Tween 20). Drain well.

16 Incubate with secondary antibodies in 3% BSA - 1x PBS for 45 min – 1h.

16.1 Goat anti-rabbit AlexaFluor 568

16.2 Goat anti-mouse AlexaFluor 647

17 Wash wells 3x with PBST (1x PBS, 0.02% Tween 20). Drain well.

18 Add Hoechst33342 or DAPI 1:2000 to wells for 5 min with gentle shaking.

19 Wash wells 3x with PBST (1x PBS, 0.02% Tween 20). Drain well.

- 20 Exchange PBST with 1x PBS and keep cells at 4°C until imaging. Image within the next few days.

Fixed-cell microscopy

- 21 Mount glass bottom plate on Yokogawa CSU-W1 spinning disk confocal on a Nikon Eclipse Ti-E motorized microscope equipped with a Nikon Apochromat 60×/1.42 N.A oil-objective lens. Image signals of 405/488/568/647 fluorophores in sequential manner with a Nikon LUN-F XL solid state laser combiner ([laser line – laser power]: 405 - 80mW, 488 - 80mW, 561 - 65mW, 640nm - 60mW]) using a Semrock Di01-T405/488/568/647 dichroic mirror. Fluorescence emissions were collected with Chroma ET455/50m [405 nm], 488 Chroma ET525/50m [488 nm], 568 Chroma ET605/52m [561 nm], 633 Chroma ET705/72m [640 nm] filters, respectively (Chroma Technologies) using NIS-Elements image acquisition software. Consistent laser intensity and exposure times must be maintained for all samples. Acquire 8 μm z-stacks for each image.
- 22 Image adequate number of cells per repeat in each condition.

Evaluation

- 23 Perform image quantification was in your tool of choice. Here we will use ImageJ/Fiji and custom-written batch-macros (<https://github.com/harperlaboratory/FBX07>).
- 24 Filter mitochondrial signal (Gaussian Blur, sigma=2) and converted images into binary files and holes in the resulting mask filled.
- 25 Threshold pUb channel into a binary file (Triangle method) and measure binary file these masks were using the "Analyze Particles..." command (pixel size exclusion: 0.5-∞, exclude edge objects).
- 26 Save results for each image-stack (both mito and pUb) as .csv files, together with the original

overlay .tiff file for QC purposes.

- 27** Count number of nuclei for normalization.
- 28** Plot results in your tool of choice for graphing and statistical analysis.
- 29** Calculate the % of mitochondrial pUb coverage was and normalized to [t]=6h AO