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We are still developing and optimizing this protocol

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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 µM).
ND1 Medium:
DMEM/F12
N2 (100x) 1x
BDNF 10 ng/ml
NT3 10 ng/ml
NEAA (100X) 1x
Laminin 0.2 µg/ml
Doxycycline 2 µg/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 µg/ml

4 Day 4: Exchange 50% of the medium from each well.

5 Day 6: Treat the cells with Accutase and replate 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 · Doxycycline can be withdrawn on Day.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 AlexaFlour 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/FBXO7).

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