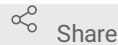




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Microscopy-based mitochondrial morphology measurements in iNeurons

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ABSTRACT

Protocol for microscopy-based mitochondrial morphology measurements in iNeurons

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COLLECTIONS

 **Kraus et al., 2022 FBX07 /Park15**

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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 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Doxycycline can be withdrawn on Day.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 medium 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.
 - a. 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.
 - b. Goat anti-mouse AlexaFluor 488

- 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 488/568/647 fluorophores in sequential manner with a Nikon LUN-F XL solid state laser combiner ([laser line – laser power]: 488 - 80mW, 561 - 65mW, 640nm - 60mW]) using a Semrock Di01-T405/488/568/647 dichroic mirror. Fluorescence emissions were collected with 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 Count number of nuclei for normalization.
- 25 Plot results in your tool of choice for graphing and statistical analysis.

