Oct 31, 2022

Microscopy-based mitochondrial morphology measurements in iNeurons

In 1 collection

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dx.doi.org/10.17504/protocols.io.4r3l274bqg1y/v1

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ABSTRACT

Protocol for microscopy-based mitochondrial morphology measurements in iNeurons

DOI

dx.doi.org/10.17504/protocols.io.4r3l274bqg1y/v1

PROTOCOL CITATION

Felix Kraus 2022. Microscopy-based mitochondrial morphology measurements in iNeurons. **protocols.io** https://dx.doi.org/10.17504/protocols.io.4r3l274bqg1y/v1

COLLECTIONS

Kraus et al., 2022 FBXO7 /Park15

CREATED

Oct 11, 2022

LAST MODIFIED

Oct 31, 2022

PROTOCOL INTEGER ID

71191

PARENT PROTOCOLS

Part of collection

Kraus et al., 2022 FBXO7 /Park15

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 · Doxycycline can be withdrawn on Day.

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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 AlexaFlour 488

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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/FBXO7).

24 Count number of nuclei for normalization.

25 Plot results in your tool of choice for graphing and statistical analysis.

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