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Microscopy-based mtDNA turnover measurements in HeLa and iNeurons

In 1 collection

Felix Kraus[1,2]

[1]Department of Cell Biology, Blavatnik Institute, Harvard Medical School, 240 Longwood Ave, Boston MA 02115, USA;

[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20815, USA

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Felix Kraus

ABSTRACT

Protocol for microscopy-based mtDNA turnover measurements in HeLa and iNeurons

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COLLECTIONS

Kraus et al., 2022 FBXO7 /Park15

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Seeding of Hela cells

1 Wash HeLa cells expressing doxycycline-inducible Parkin with 1x PBS

2 Add Trypsin to cells for 5 min and incubate at 37°C to dissociate cells from plastic well

3 Resuspend cells in 1 mL DMEM media

4 Count cells

5 Seed appropriate number of cells into 24-well glass bottom dish

6 Top up glass bottom dish with either 1 mL DMEM and place cells back into incubator

7 The next day exchange DMEM with DMEM + 2µg/ml doxycycline for 18h to induce Parkin expression.

8 Induce mitophagy using Antimycin A / Oligomycin A for the desired time.

Differentiation of iNeurons

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9 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

10 Day 1: Replace the medium with ND1 Medium.

11 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

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

13 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.

14 Day 8 and thereafter: Exchange 50% of the medium from each well every other day. · Doxycycline can be withdrawn on Day.

15 Induce mitophagy using Antimycin A / Oligomycin A for the desired time.

Staining

16 Aspirate media and fix cells in 1 ml pre-warmed 4% PFA for 30 min.

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17 Aspirate PFA solution and wash wells 3x with PBST (1x PBS, 0.02% Tween 20)

18 Permeabilize the cells by adding 0.2% Triton X-100 in PBS.

19 Remove the detergent solution by aspiration. Wash wells 3x with PBST (1x PBS, 0.02% Tween 20). Drain well.

20 Block cells for 10 min with 3% BSA – 1x PBS.

21 Remove BSA solution by aspiration. Wash wells 3x with PBST (1x PBS, 0.02% Tween 20). Drain well.

22 Incubate with primary antibodies in 3% BSA - 1x PBS for 3h at RT with gentle shaking.
a. Anti-DNA (mouse)

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

24 Incubate with secondary antibodies in 3% BSA - 1x PBS for 45 min – 1h. a. Goat anti-mouse AlexaFlour 488

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

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

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

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

Fixed-cell microscopy

29 Mount glass bottom plate on Yokogawa CSU-W1 spinning disk confocal on a Nikon Eclipse Ti-E motorized microscope equipped with a Nikon Plan Apo 100×/1.45 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.

30 Image adequate number of cells per repeat in each condition.

Evaluation

31 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).

32 Filter nuclear signal (Gaussian Blur, sigma=2) and converted images into binary files.

33 Convert aDNA into binary files.

34 Subtract the nuclear signal from the aDNA, resulting in an image containing only the mtDNA intensities.

35 Measure binary file these masks were using the "Analyze Particles..." command (pixel size exclusion: 0.05-3, exclude edge objects)).

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36 Save results image stacks as .csv files, together with the original overlay.tiff file for QC purposes.

37 Count number of nuclei for normalization.

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

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