Oct 31, 2022

Evaluation of pUb kinetics using 3D-SIM

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 20 815, USA

dx.doi.org/10.17504/protocols.io.kqdg396yqg25/v1

Felix Kraus

ABSTRACT

Protocol for the evaluation of pUb kinetics using 3D-SIM

DOI

dx.doi.org/10.17504/protocols.io.kqdg396yqg25/v1

PROTOCOL CITATION

Felix Kraus 2022. Evaluation of pUb kinetics using 3D-SIM. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kqdg396yqg25/v1

COLLECTIONS

Kraus et al., 2022 FBXO7 /Park15

CREATED

Oct 11, 2022

LAST MODIFIED

Oct 31, 2022

PROTOCOL INTEGER ID

71192

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 onto 18x18mm Marienfeld Precision cover glasses thickness No. 1.5H (tol. ± 5 µm).

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

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:

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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 18x18mm Marienfeld Precision cover glasses thickness No. 1.5H (tol. ± 5 µm).

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 · Doxycycline can be withdrawn on Day.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 DMEM and fix cells in warm paraformaldehyde 3% Glutaraldehyde 0.35% in 0.1M Sodium Cacodylate, pH 7.4Aspirate PFA solution and wash wells 3x with PBST (1x PBS, 0.02% Tween 20)

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

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

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

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

21 Incubate with primary antibodies in 3% BSA - 1x PBS over night at 4°C with gentle shaking.
a. Anti-HSP60 (mouse)
b. Anti-pUb (rabbit)

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

23 Incubate with secondary antibodies in 3% BSA - 1x PBS for 45 min – 1h.
a. Goat anti-mouse AlexaFlour 488
b. Goat anti-rabbit AlexaFluor 568

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

25 Add DAPI 1:2000 to wells for 5 min with gentle shaking.

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

27 Wash coverslips with 1x PBS and mount in Vectashield (Vector Laboratories, H-1000-10) on glass slides.Exchange PBST with 1x PBS and keep cells at 4°C until imaging. Image within the next few days.

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Fixed-cell 3D-SIM microscopy

28 Image cells on DeltaVision OMX v4 using an Olympus 60x / 1.42 Plan Apo oil objective (Olympus, Japan). Record 405, 488 and 568 channels using a front-illuminated sCMOS (PCO Photonics, USA) in 512x512px image size mode, 1x binning, 125 nm z-stepping and with 15 raw images taken per z-plane (5 phase-shifts, 3 angles).

29 Reconstruct raw images using CUDA-accelerated 3D-SIM reconstruction code (https://github.com/scopetools/cudasirecon) based on Gustafsson et al. (2008[FK1]). The Optimal optical transfer function (OTF) was determined via an in-house build software, developed by Talley Lambert from the NIC / CBMF (GitHub: https://github.com/tlambert03/otfsearch, all channels were registered to the 528nm output channel, Wiener filter: 0.002, background: 90).

Evaluation

30 Import composite .dv stacks into Imaris (v9.7) and convert into native .ims files.

31 Import into Imaris Arena and perform global background subtraction.

32 Segment pUb and mitochondrial objects from seeds (XY starting diameter: 0.08µm == pixel size of images), segmented based on automatic thresholding with local background subtraction and splitting of touching objects (0.4µm).

33 Pipe segmented objects into Imaris Vantage module for further analysis.

34 In Vantage, compute nearest neighbour distances of pUb to pUb and between pUb and mitochondria, as well as volume of segmented objects.

35 Test pipeline on WT control cells and then applied for batch processing on all other genotype to allow for unbiased segmentation and analysis.

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

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