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Microscopy-based measurements of p62 recruitment in HeLa

In 1 collection

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ABSTRACT

Protocol for microscopy-based measurements of p62 recruitment in HeLa

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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. Staining

Staining

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

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

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

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

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

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

15 Incubate with primary antibodies in 3% BSA - 1x PBS for 3h at RT with gentle shaking.
a. Anti-p62 (mouse)
b. Anti-FIP200 (rabbit)

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

17 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

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

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

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

21 Exchange PBST with 1x PBS and keep cells at 4°C until imaging. Image within the next few days. Fixed-cell microscopy

22 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.

23 Image adequate number of cells per repeat in each condition. Evaluation

Evaluation

24 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).

25 Filter p62 signal (Gaussian Blur, sigma=2) and converted images into binary files using the “Intermodes thresholding” method.

26 Measure binary file these masks were using the "Analyze Particles..." command (pixel size exclusion: 0.1-30, exclude edge objects).

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

28 Count number of nuclei for normalization.

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

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