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Sample Preparation and 3D-SIM fixed-cell imaging of FTH1 and Filipin in NPC1 and NPC2 mutants

Forked from Evaluation of pUb kinetics using 3D-SIM

In 1 collection

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Abstract

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

Seeding of HeLa cells

1 Wash HeLa TMEM192-3xHA control and NPC1, NPC2 mutants with 1xPBS

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 growth medium

Staining

8 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).
PFA is toxic and discard in the appropriate waste streams.

9 Stain cholesterol with Filipin (0.05mg/ml in PBS / 10% FBS) for 2h at RT.
Wash cells after staining with PBST for 2 min.

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 over night at 4°C with gentle shaking.
a. anti-FTH1 (rabbit)

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.
a. Goat anti-rabbit AlexaFluor 568

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

18 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.

Fixed-cell 3D-SIM microscopy

19 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).

20 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).

3D-redendering in ChimeraX

21 Open .dv composite image stacks in Fiji. Separate channels and save them as separate .tiff stacks for import into ChimeraX

22 Open ChimeraX and open one of the two image stacks into a new project.

23 While not moving the scene, drag the second tiff stack into ChimeraX. The two channels should thus keep their previous alignment.

24 Go Tools > Volume Data > Volume Viewer.
Change the representation of both channels to surface or mesh (or what fits your visualization needs best).
Adjust color and levels of the image on the histogram.

25 In the Volume Viewer tab, use the "Hide Dust" function to remove unspecific background specs / spots from the render. Repeat for both channels.

26 Move the render into an adequate position and export the image.

27 Import the resulting image into illustrator for making your figure.