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Image_processing_to_investigate_mitophagy_in_HelaM_and_neurons

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ABSTRACT

This is a method for imaging cells and quantifying subcellular structures in the resulting data. We developed this protocol for assessing clearance of mitochondria involving OPTN and TBK1, however the method could be used for other applications.

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GUIDELINES

Intensity measurements must only be carried out on non-deconvolved images. For this reason, always save an original image identical to the deconvolved image so that any ROIs generated from the deconvolved image can be projected to the original .tif.

Imaging

1 Collect images of cells with a confocal microscope at 100X.

1.1 Refer to https://svi.nl/NyquistCalculator in order to determine optimal collection parameters

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1.2 Depending on the analysis, you may collect 3D samples (XYZ or XYT)

1.3 Try to maximize the number of cells in the field of view

Image deconvolution

2 Use a software such as Huygens to deconvolve the images

2.1 When there are multiple cells in one field of view, crop the image prior to deconvolution and save the pre-deconvolved cropped images as .tifs.

Quantifying mitophagy rings (density, intensity, diameter)

3 Open each image in ImageJ/FIJI

4 If applicable, maximally project deconvolved images

4.1 Maintain a constant depth of projection, i.e. 4 um for each image.

5 Using channels collected for mitochondria and TBK1, manually draw regions of interest (ROIs) around mitophagy events and add each ROI to the manager

5.1 The criteria for a mitophagy event is:
-ring must envelop a rounded mitochondria
-ring must be visible around at least half of the mitochondria
-if applicable, Parkin must also be present as a ring

5.2 These steps may also be applied to quantification of OPTN rings.

6 Draw an ROI around the whole cell area using TBK1 channel to delineate cell outline.

7 Save the set of ROIs for each image

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8 Open the corresponding pre-deconvolved image in ImageJ/FIJI

9 Maximally project this image identically to the deconvolved image

10 Project the ROI set onto the pre-deconvolved image in the TBK1 channel

11 In the ROI manager, highlight all ROIs, then select Multi-measure and generate measurements for the single channel.

12 Copy and paste the ROI measurements into an Excel document

13 To determine TBK1 recruitment extent, calculate the number of rings divided by the total area of the cell.

14 To determine average intensity, calculate the average of the average gray value of all rings in the TBK1 channel

15 To determine average diameter, calculate the average of the Feret diameter of all rings

16 Proceed to repeat for all cells.

Ring tracking in live cells

17 Assess the deconvolved timelapse images to identify events that are visible over the timelapse and remain in focus

18 Use the Parkin and mitochondria channels to draw ROIs around the forming ring for each timepoint. Save each ROI to the manager.

18.1 It is easiest to start at the end of the timelapse (or the end of the sequence when the ring is still visible) when the event is brightest, then move backward through the steps.

19 Save each event's ROI's as its own set.

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20 Open the corresponding pre-deconvolved timelapse image.

21 Project each saved ROI onto its corresponding timepoint and measure the intensity within the ROI for Parkin and TBK1 channels.

21.1 The timepoint information should be saved in the ROI's data.

22 Go to timepoint zero and draw an ROI around an area of the cell with no mitochondria, puncta, or aberrations. Save the average gray value measurements of the Parkin and TBK1 channels as the background.

23 Copy and paste the intensity data from the events and the background to a new Excel document

24 Subtract the average gray value of the background ROI from each of the mitophagy event ROIs in the respective channels.

25 Determine the maximum value of Parkin intensity for each event and divide by two for Parkin half-max.

26 Align all values to Parkin half-max, setting Parkin half-max as zero.

Quantifying % mitochondria with LC3

27 Import deconvolved images of mitochondria to Ilastik or other segmentation software.

27.1 If using ilastik, develop a segmentation algorithm to identify mitochondria from non-mitochondria. Export segmented images as .tifs

28 Repeat steps 5-15 for LC3 channel.

29 Open segmented mitochondria image in ImageJ/FIJI and perform particle analysis to determine the number of discrete mitochondria

30 Calculate % mitochondria that are LC3 positive by dividing the number of LC3 events by total number of mitochondria.

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