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Image processing to investigate NEMO recruitment and involvement in mitophagy and inflammatory signaling (Provisional unformatted)

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ABSTRACT

Beautiful images are not sufficient to robustly characterize and interrogate a cellular mechanism. In order to show recruitment of NEMO and its relationship to OPTN and other mitophagy factors, we processed hundreds of still and timelapse images and extracted quantifiable data in order to perform statistical analysis comparing different conditions. In many cases, we used software to deconvolve confocal fluorescent images, allowing us to algorithmically surpass the resolution limit. This was especially useful for live cell images that had been collected with low power settings to preserve cell health. We also employed machine learning software to generate binary segmentations and carry out particle analysis on putatively overlapping structures. Finally, in some cases we simply identified fluorescent structures by hand and measured their fluorescent intensities. We approached image analysis in a multitude of creative, effective ways, and importantly we maintained consistency of analysis within experiments in order to present the results with integrity and reproducibility.

PROTOCOL CITATION

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1 - This protocol addresses multiple image analysis pipelines used in the corresponding manuscript to investigate NEMO and its role in mitophagy

2 - We developed several methods for quantitative analysis with our varying experiments Equipment/software

3 - ImageJ/FIJI

4 - Ilastik

5 - Deconvolution software such as Huygens

6 - Excel Before start

7 - For each experiment, collect images for at least 10 cells from at least three biological replicates

8 - Maintain consistent imaging parameters by saving and/or recording laser powers and exposure times Step-by-step Blinded whole-cell recruitment assessment This method was used to assess NEMO recruitment in cells before and after AntA/OligA treatment, in the presence or absence of Parkin or p62

9 - Start with Z stack images of live cells collected with a 60X objective

10 - Max project the Z stack fields of view by ~2 um to capture the center to upper half of the cell

11 - Crop each cell and save only the NEMO channel with a file name to identify the conditions

12 - Copy cropped NEMO channel images to a new folder

13 - Use a random number generator such as Random.org/strings/ to generate a number of strings equivalent to the total number of cells

14 - Copy the strings to a document and save

15 - Rename the copied images in the new folder as the random strings

16 - Arrange files in the folder by file name in order to randomly mix images

17 - Open newly named files and judge whether there is NEMO recruitment or not, recording YES or NO for each image by its random string file name

18 - After categorizing every image, unblind the results and determine the number of cells marked YES for each condition compared to the total for that condition. Mitochondrial recruitment assay This method was used to assess percentages of mitochondria that had recruited NEMO, OPTN, GABARAPs, and/or LC3B in various conditions.

19 - Start with Z stack images of cells collected with 60X objective

20 - Crop and save each cell visible in the field of view

21 - Deconvolve images with Huygens or similar deconvolution software

22 - Max project each cell ~2 um

23 - Split channels and save each channel

24 - Import 5-7 channels displaying mitochondria into the Ilastik software for segmentation

25 - Train algorithm to recognize “mitochondria” or “not mitochondria”

26 o Note: if there are cells with poor expression or labeling of the mitochondria, these may be discarded

27 - Generate binary images for all mitochondria channels

28 o Import segmented Ilastik results to FIJI

29 o Use the original image file to draw an ROI outlining the cell

30 o Clear Outside the cell ROI on the segmented image

31 o Run the threshold function with threshold set to (255,255)

32 o Save binary image

33 - Repeat previous 3 steps for NEMO puncta and OPTN rings/puncta

34 - GABARAPs and LC3B antibodies do not produce high enough signal-to-noise to segment with Ilastik. For these experiments, only segment NEMO and mitochondria. In FIJI, draw ROI's around GABARAPs- or LC3B-positive mitochondria by hand. Save ROIs.

35 - To determine the % of mitochondria that recruited NEMO,

36 o open the binary mitochondria image in FIJI

37 o Use the Analyze Particles function to generate a mask of binary image, filtering particles to exclude those fewer than 5 pixels.

38 o Summarize and add to manager

39 o Record the total number of mitochondria in Excel

40 o Open binary image of NEMO channel

41 o Use the Analyze Particles function to generate a mask of binary image, filtering particles to exclude those fewer than 5 pixels, and add particles to manager.

42 o Project NEMO particles to Mito binary channel and multi-measure

43 o Copy results to Excel

44 o The count of particles with an average intensity of >127.5 is the number of mitochondria positive for NEMO

45 o Divide this number by the total number of mitochondria to calculate the proportion

46 - To determine the % of mitochondria that recruited NEMO and/or OPTN,

47 o Repeat the first four steps above to record total number of mitochondria.

48 o Load the binary OPTN channel to FIJI and Fill Holes

49 o Project mitochondrial particle ROIs onto OPTN binary image and multi-measure

50 o Record results in Excel

51 o The count of particles with an average intensity of >127.5 is the number of mitochondria positive for OPTN

52 o Delete mitochondria particles from the manager and add NEMO particles

53 o Project NEMO particles onto mitochondria and perform the same calculation

54 o Use the image calculator to add the mitochondria channel to the OPTN channel with Filled Holes.

55 o Use Math > Subtract to subtract 255 and float the resulting image

56 o Convert to an 8-bit image

57 o Project NEMO particles to this new image and multi-measure

58 o Record results in Excel

59 o The count of particles with an average intensity greater or equal to 64 is the count of mitochondria that are positive for both NEMO and OPTN

60 - To determine the % of mitochondria that recruited NEMO and/or GABARAPs or LC3B,

61 o Use the Image Calculator to add NEMO particles to Mitochondria particles

62 o Use Math > Subtract to subtract 255 and float the resulting image

63 o Convert to an 8-bit image

64 o Project hand-drawn ROIs to resulting image and multi-measure

65 o Record results in Excel

66 o The count of particles with an average intensity greater or equal to 64 is the count of mitochondria that are positive for both NEMO and GABARAPs or LC3B

67 - Use the R code library(eulerr) to generate Euler diagrams of the resulting data Recruitment assay This method was used to quantify the extent of NEMO, OPTN, and p62 recruitment over time.

68 - Start with confocal timelapse images in which the timepoint with added AntA/OligA is known

69 - Choose several events per cell in which the mitochondria stays in the field of view for a majority of the timecourse and the protein of interest is recruited

70 - For each time point, use the mitochondria channel to draw a generous ROI around the mitochondria

71 o This will capture rings or puncta that are recruited

72 - Measure the intensity of NEMO and/or OPTN or p62 for every ROI and record in Excel

73 - Calculate the 5-frame moving average across the timecourse for each channel

74 - Calculate the background intensity by averaging the moving average intensity of the first ten frames of the timecourse

75 - Subtract the background from every frame

76 - Determine the maximum intensity for the event

77 - Normalize the timecourse intensities to the max intensity so that the intensity of each frame is a percentage of the max intensity

78 - Calculate the half-max timepoint based on when the normalized intensity surpasses 50%