Image processing to investigate NEMO recruitment and involvement in mitophagy and inflammatory signaling

OLIVIA HARDING[1,2], Erika L.F. Holzbaur[1,2]

[1]Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815

JUL 31, 2023

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.n2bvj61xxlk5/v1

Protocol Citation: OLIVIA HARDING, Erika L.F. Holzbaur 2023. Image processing to investigate NEMO recruitment and involvement in mitophagy and inflammatory signaling. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n2bvj61xxlk5/v1

Protocol status: Working
We use this protocol and it's working

Liv

OLIVIA HARDING

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.

ATTACHMENTS

470-985.pdf

GUIDELINES

- This protocol addresses multiple image analysis pipelines used in the corresponding manuscript to investigate NEMO and its role in mitophagy.
- We developed several methods for quantitative analysis with our varying experiments.

Created: Jun 23, 2022

Last Modified: Jul 31, 2023

PROTOCOL integer ID: 65163

Keywords: Confocal imaging, Deconvolution, Image processing, Image analysis, Fluorescent intensity analysis, Subcellular imaging, Organelle morphology, Segmentation, ASAPCRN

MATERIALS

Equipment/software

- ImageJ/FIJI
- Ilastik
- Deconvolution software such as Huygens
- Excel

BEFORE START INSTRUCTIONS

- For each experiment, collect images for at least 10 cells from at least three biological replicates.
- Maintain consistent imaging parameters by saving and/or recording laser powers and exposure times.

Blinded whole-cell recruitment assessment

1

Note

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.

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

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

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

4 Copy cropped NEMO channel images to a new folder.

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

6 Copy the strings to a document and save.

7 Rename the copied images in the new folder as the random strings.

8 Arrange files in the folder by file name in order to randomly mix images.

9 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.

10 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

11

Note

This method was used to assess percentages of mitochondria that had recruited NEMO, OPTN, GABARAPs, and/or LC3B in various conditions.

Start with Z stack images of cells collected with 60X objective.

Crop and save each cell visible in the field of view.

12

13 Deconvolve images with Huygens or similar deconvolution software.

14 Max project each cell ~2 um.

15 Split channels and save each channel.

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

17 Train algorithm to recognize “mitochondria” or “not mitochondria”.

Note

If there are cells with poor expression or labeling of the mitochondria, these may be discarded.

18 Generate binary images for all mitochondria channels.

18.1 Import segmented Ilastik results to FIJI.

18.2 Use the original image file to draw an ROI outlining the cell.

18.3 Clear Outside the cell ROI on the segmented image.

18.4 Run the threshold function with threshold set to (255,255).

18.5 Save binary image.

19 Repeat previous 3 steps for NEMO puncta and OPTN rings/puncta.

20 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.

21 To determine the % of mitochondria that recruited NEMO,

21.1 Open the binary mitochondria image in FIJI.

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

21.3 Summarize and add to manager.

21.4 Record the total number of mitochondria in Excel.

21.5 Open binary image of NEMO channel.

21.6 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.

21.7 Project NEMO particles to Mito binary channel and multimeasure.

21.8 Copy results to Excel.

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

21.10 Divide this number by the total number of mitochondria to calculate the proportion.

22 To determine the % of mitochondria that recruited NEMO and/or OPTN.

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

22.2 Load the binary OPTN channel to FIJI and Fill Holes.

22.3 Project mitochondrial particle ROIs onto OPTN binary image and multi-measure.

22.4 Record results in Excel.

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

22.6 Delete mitochondria particles from the manager and add NEMO particles.

22.7 Project NEMO particles onto mitochondria and perform the same calculation.

Use the image calculator to add the mitochondria channel to the OPTN channel with Filled

22.8 Holes.

22.9 Use Math > Subtract to subtract 255 and float the resulting image.

22.10 Convert to an 8-bit image.

22.11 Project NEMO particles to this new image and multi measure.

22.12 Record results in Excel.

22.13 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.

23 To determine the % of mitochondria that recruited NEMO and/or GABARAPs or LC3B.

23.1 Use the Image Calculator to add NEMO particles to Mitochondria particles.

23.2 Use Math > Subtract to subtract 255 and float the resulting image.

23.3 Convert to an 8-bit image.

23.4 Project hand-drawn ROIs to resulting image and multi measure.

23.5 Record results in Excel.

23.6 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.

24 Use the R code library(eulerr) to generate Euler diagrams of the resulting data.

Recruitment assay

25

> Note
>
> This method was used to quantify the extent of NEMO, OPTN, and p62 recruitment over time.

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

26 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.

27 For each time point, use the mitochondria channel to draw a generous ROI around the mitochondria.

27.1 This will capture rings or puncta that are recruited.

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

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

30 Calculate the background intensity by averaging the moving average intensity of the first ten frames of the timecourse.

31 Subtract the background from every frame.

32 Determine the maximum intensity for the event.

33 Normalize the timecourse intensities to the max intensity so that the intensity of each frame is a percentage of the max intensity.

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