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Live imaging to investigate mitophagy kinetics and NEMO recruitment in HeLa-M cells (Provisional unformatted)

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ABSTRACT

There is no substitute for live cell imaging in the investigation of the kinetics of subcellular biology. Here, we enumerate a protocol to visualize fluorescently-tagged mitochondria, NEMO, OPTN, and p62 during the cellular response to mitochondrial depolarization. Because live cells are sensitive to photo-damage, we used low laser power and short exposure times in our confocal microscopy system. Even with minimally damaging parameters, we were able to collect high-content data that we subsequently analyzed. With this technique, we showed that NEMO and OPTN, despite containing highly similar domains, were recruited to damaged mitochondria with less correlation than NEMO and p62. Furthermore, live imaging of NEMO occupancy on damaged mitochondria was a necessary complement to our parallel fixation studies. Since fixation introduces artifacts, especially in samples with concentrated proteins like those in mitophagy, results from our live cell imaging corroborated our findings in fixation studies. Our reporting of these results would not have been possible without real-time, live cell imaging.

PROTOCOL CITATION

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1 - This protocol was developed with the HeLa subtype, HeLa-M. HeLa-M cells are flatter than standard HeLa cells, making them easier to image. They also uptake siRNA better than standard HeLa. Regardless, the protocol would be easily adaptable to standard HeLa cells or other cell culture lines.

2 - This protocol was created in order to investigate Parkin-dependent mitophagy. Parkin and several other fluorescently-tagged mitophagy components are intended for use in the protocol. Materials/Reagents

3 -

4 5 mL capped tubes (Millipore Sigma, EP022364120) Reagents

5 - FBS (HyClone)

6 - Leibovitz's L-15 Medium (Gibco, 11415064)

7 - Dimethyl Sulfoxide (DMSO) (Sigma, D650)

8 - Ethanol

9 - Antimycin A (Sigma-Aldrich, A8674) and

- Oligomycin A (Sigma-Aldrich, 75351) Equipment

- vacuum apparatus

-

C water bath

- confocal microscope with 60X objective, heated stage chamber, focus correction system, and associated software

o Note: our system is not equipped with a pressurized stage chamber to sustain 5% CO2 conditions. Thus, we use L-15 in order to buffer the samples in atmospheric CO2 conditions. Before start

- The start point for this protocol is after cells grown on 35-mm glass bottom dishes have been transfected with Mito-dsRed2, EGFP-NEMO, Halo-OPTN, and Parkin for 18-24 hours and tagged with Halo ligand.

- Prepare 45 mM stock of Antimycin A by suspending

mg solid AntA in 2 mL ethanol

- Prepare

mM stock Oligomycin A by suspending 5 mg solid OligA in 630 uL DMSO

21 - Prepare imaging media by making a 10% FBS solution in L-15 and warm in water bath

22 o Note: will use ~3 mL imaging media per dish

23 - Prepare working AntA/OligA solution by adding 1 uL 10 mM OligA and 4 mL 45 mM AntA to .5 mL Imaging media in a 1.5 mL tube. Keep warm in bath or imaging chamber.

24 - Heat microscope imaging chamber to 37 C Step-by-step Replace Standard Media with Imaging Media

25 - Wait until imaging chamber is heated to 37 C

26 - Aspirate media from sample

27 - Add 200 uL Imaging media gently to dish

28 - Aspirate

29 - Repeat above two steps for a total of 2 washes

30 - Add 1.5 mL Imaging media and place dish in 37 C imaging chamber

31 - Incubate sample in imaging conditions for at least 10 min before imaging Imaging set-up

32 - Place dish on microscope stage, moving stage adjusters if necessary to fit the dish

33 o Note: Remove the lid of the dish to maneuver it more easily without spilling its contents. Replace the lid after dish is firmly secured on the stage

34 - raise objective so that there is an oil interface between the objective lens and glass bottom of the dish

35 - Allow dish to settle at least 5 min in this position before imaging to minimize drift during imaging

36 - Using 60X objective and RFP epifluorescence, find the focal plane of fixed cells by looking for dsRed2-labeled mitochondria

37 o Note: We find that 60X is sufficient magnification to collect several cells in each field of view with enough resolution to perform analysis and quantification. Other magnifications may be appropriate for various applications.

38 o Note: Note the health of cells, transfection efficiency of the constructs, and brightness of fluorescence. If you will image multiple samples with varying conditions, you may want to observe all dishes before confirming the imaging parameters so that no images are overexposed or too dim.

39 - Configure 488, 561, and 647 lasers and accompanying exposure times

40 o Note: Set parameters as low as possible while still detecting the signals in order to avoid phototoxicity and bleaching. For a benchmark, we use <10% power and <200 ms exposure for each channel. Save settings in order to maintain consistency among experiments.

41 Option 1: Imaging confocal (single z section) timelapse sequence during mito depolarization

Note: this option is used to collect information in single sections of the sample over the course of 1-2 hr. Data can be used to reconstruct recruitment kinetics, however single section imaging is not ideal for assays that require a high signal-to-noise ratio.

42 - Choose a field of view with multiple cells that look healthy and express all three fluorescent constructs. Set focus on a confocal section approximately between 5 and 7, where 0 is the bottom, attached part of the cell and 10 is the top of the cell.

43 o Note: If scope is equipped with multi-stage position feature, choose several fields that fulfill these requirements and add to sequence

44 - Begin imaging at acquisition rate of 1 frame per min

45 - After 5-10 frames (5-10 min), remove the lid of the imaging dish and add the working AntA/OligA solution for a total of 2 mL (5 uM AntA/10 uM OligA)

46 o Note: Record the frame number at which AntA/OligA was added

47 - Replace the lid to the dish and continue imaging for as long as desired

48 o Note: OPTN recruitment is visible between 30 and 60 min

49 o Note: after 1 hr imaging at 1 frame per minute, even low laser power settings will damage the cells. Take caution in analyzing data from samples after 1 hr imaging. Option 2: Imaging timepoint Z stacks before and after mito depolarization Note: this option is used to collect information in ~2 um volumes of the sample at several timepoints. Data can be used to characterize the extent of recruitment of proteins of interest on mitochondria, however Z stack imaging will produce phototoxicity; the same cells should not be imaged at each timepoint.

50 - Choose a field of view with multiple cells that look healthy and express all three fluorescent constructs. Set Z stack to collect volume from the 50th to 75th percentile of the cell (see Figure 1).

51 - Collect images in order to record 10-20 cells, depending on the quality of the transfection and cells. Collect images from diverse areas of the coverslip.

52 - Add the working AntA/OligA solution for a total of 2 mL (5 uM AntA/10 uM OligA).

53 - Incubate ~50 min or to desired timepoint

54 - Reposition dish to a new field of view with cells that demonstrate OPTN recruitment

55 - Collect images in order to record 10-20 treated cells, again collecting images from diverse areas of the coverslip.

56 o Note: Work quickly to gather data within ~10-15 min of the desired time point (5 min each side) Figure SEQ Figure * ARABIC 1. Schematic of HeLa cell volume. Dotted lines indicate target volume to image. End of imaging

57 - Samples may be saved for biochemical analysis by scraping cells into 1.5 mL tube and snap freezing in liquid nitrogen after aspirating any media