Jul 31, 2023

Live imaging to investigate mitophagy kinetics and NEMO recruitment in HeLa-M cells

DOI
dx.doi.org/10.17504/protocols.io.rm7vzy9w5lx1/v1

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

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OLIVIA HARDING
Children's Hospital of Philadelphia

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Protocol Citation: OLIVIA HARDING, Erika L.F. Holzbaur 2023. Live imaging to investigate mitophagy kinetics and NEMO recruitment in HeLa-M cells. **protocols.io https://dx.doi.org/10.17504/protocols.io.rm7vzy9w5lx1/v1**

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

Created: June 22, 2022

Last Modified: May 31, 2024

Protocol Integer ID: 65074

Keywords: Tissue culture, Mitophagy, Fluorescent ligands, Confocal microscopy, Live cell imaging, ASAPCRN, damaged mitochondria, live cell imaging, tagged mitochondria, mitochondrial depolarization, substitute for live cell imaging, mitochondria with less correlation, live imaging of nemo occupancy, microscopy, p62 during the cellular response, mitophagy kinetic, subcellular biology, live imaging, confocal microscopy system, imaging, live cell, cell, cellular response

Funders Acknowledgements:
Aligning Science Across Parkinson's
Grant ID: Mechanisms of mitochondrial damage control by PINK1 and Parkin (ASAP-000350)

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.

Attachments

PDF

470-983.pdf

251KB

Guidelines

- 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.
- 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

Materials/Reagents

1.5 mL capped tubes **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP022364120

Reagents

- FBS (HyClone)
- Leibovitz's L-15 Medium **Thermo Fisher Catalog #**11415064
- Dimethyl sulfoxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D2650
- Ethanol
- Antimycin A from Streptomyces sp. **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A8674
- Oligomycin A **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**75351

Equipment:

- Vacuum apparatus
- 37 °C water bath
- Confocal microscope with 60X objective, heated stage chamber, focus correction system, and associated software

Note

Our system is not equipped with a pressurized stage chamber to sustain 5% CO_2 conditions. Thus, we use L-15 in order to buffer the samples in atmospheric CO_2 conditions.

Troubleshooting

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, HaloOPTN, and Parkin for 18:00:00 - 24:00:00 and tagged with Halo ligand.
- Prepare [M] 45 millimolar (mM) stock of Antimycin A by suspending 50 mg solid AntA in 2 mL ethanol.
- Prepare [M] 10 millimolar (mM) stock Oligomycin A by suspending 5 mg solid OligA in 630 µL DMSO.
- Prepare imaging media by making a 10% FBS solution in L-15 and warm in water bath.

Note

Will use ~ 3 mL imaging media per dish.

- Prepare working AntA/OligA solution by adding 1 µL [M] 10 millimolar (mM) OligA and 4 mL [M] 45 millimolar (mM) AntA to 0.5 mL Imaging media in a 1.5 mL tube. Keep warm in bath or imaging chamber.
- Heat microscope imaging chamber to 37 °C .

Replace Standard Media with Imaging Media

10m

1 Wait until imaging chamber is heated to 37 °C.

2 Aspirate media from sample.

3 Repeat the following two steps for a total of 2 washes.

- Add 200 µL Imaging media gently to dish.
- Aspirate.

4 Add 1.5 mL Imaging media and place dish in 37 °C imaging chamber.

5 Incubate sample in imaging conditions for at least 00:10:00 before imaging.

10m

Imaging set-up

15m

6 Place dish on microscope stage, moving stage adjusters if necessary to fit the dish.

> 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.

7 Raise objective so that there is an oil interface between the objective lens and glass bottom of the dish.

8 Allow dish to settle at least 00:05:00 in this position before imaging to minimize drift during imaging.

5m

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

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.

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.

10 Configure 488, 561, and 647 lasers and accompanying exposure times.

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.

STEP CASE

Imaging confocal (single z section) 4 steps

11

Note

This option is used to collect information in single sections of the sample over the course of 1- 02:00:00 . 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.

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.

Note

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

12 Begin imaging at acquisition rate of 1 frame per min.

13 After 5-10 frames (00:05:00 - 00:10:00), remove the lid of the imaging dish and add the working AntA/OligA solution for a total of 2 mL ([M] 5 micromolar (µM) AntA/ [M] 10 micromolar (µM) OligA). 15m

Note

Record the frame number at which AntA/OligA was added.

14 Replace the lid to the dish and continue imaging for as long as desired.

Note

- OPTN recruitment is visible between 30 and 01:00:00 .
- After 01:00:00 imaging at 1 frame per minute, even low laser power settings will damage the cells. Take caution in analyzing data from samples after 01:00:00 imaging.