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

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# Live imaging and analysis to investigate phase separation properties of NEMO during mitophagy

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

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## ABSTRACT

Phase condensation of biomolecular particles is increasingly examined for its relevance in physiological contexts. When we observed formation of NEMO puncta associated with the outer mitochondrial membrane of damaged mitochondria, we wondered whether these puncta were incorporated into phase separated particles. Three standards of the field for determining phase separation are: (A) particles undergo fission and fusion like droplets of water, (B) individual puncta recover fluorescence after photobleaching, and (C) particles dissipate upon exposure to the organic compound 1,6-Hexanediol. Our system wherein NEMO puncta appeared to be attached to fragmented mitochondria precluded a systematic assessment of standard A. I.e., one NEMO punctum would so rarely encounter another, nor would it be prompted to split, that we could not confirm or rule out the potential for fission and fusion of droplets. This protocol describes our approach to investigating standards B and C.

## 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.

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**Keywords:** Tissue culture, Mitophagy, Fluorescent ligands, Confocal microscopy, Live cell imaging, ASAPCRN

## 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.

## BEFORE START INSTRUCTIONS

- The start point for this protocol is after cells grown on 35 mm glass bottom dishes have been transfected with the relevant constructs for 18:00:00 - 24:00:00 .
- Prepare [M] 45 millimolar (mM) stock of Antimycin A by suspending 50 mg solid AntA in 2 mL ethanol. On day of use, prepare a secondary dilution of [M] 0.45 millimolar (mM)
- 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 ~ 1 mL imaging media per dish.

- Prepare working AntA/OligA solution by adding 0.5 µL [M] 10 millimolar (mM) OligA and 22.5 mL [M] 045. millimolar (mM) AntA to 0.25 mL Imaging media in a 1.5 mL tube. Keep warm in bath or imaging chamber.
- Prepare 2X (10%) 1,6-Hexanediol in imaging media and warm to 37 °C
- Heat microscope imaging chamber to 37 °C .
- Calibrate microscope FRAP capability.

## Replace Standard Media with Imaging Media and Induce Mi...

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 .75 mL Imaging media and place dish in 37 °C imaging chamber.

5 00:45:00 prior to imaging, add the .25 mL working solution of AntA/OligA to the dish making a total of 1 mL

45m

Note

Samples should equilibrate to imaging media and microscope chamber for at least 10 min prior to imaging.

## 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 100X objective and GFP or RFP epifluorescence, find the focal plane.

Note

100X is necessary to achieve the desired resolution for these 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 10 includes a Step case.
**FRAP assay**
**1,6-Hexanediol assay**

## Imaging set-up and FRAP

2h 10m

step case

### FRAP assay

Cells expressing Parkin (no tag), Mito-sBFP2, EGFP-NEMO, and mCherry-p62 are treated with AntA/OligA for at least 45 min to induce mitophagy and NEMO puncta recruitment. Photobleaching is accomplished with the use of a high-powered laser. This assay should be carried out between 50 min and 1 hr 20 min post-AntA/OligA addition.

11 Set FRAP and timelapse imaging parameters and engage auto-focus to maintain Z plane.

Note

For FRAP, I used 18 ms/pixel, 1 cycle of photobleaching. For timelapse, I set acquisition to 1 frame/5 sec. Program acquired 5 frames before commencing photobleach, then acquisition continued for a total of 10 min.

12 Choose a field of view with cell(s) that look healthy and express all three fluorescent constructs, and exhibit visible NEMO/p62 puncta recruitment to mitochondria. 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.

13 Choose several puncta to photobleach and draw box-shaped ROIs targeting them. Choose NEMO-only and NEMO-p62 puncta. Save the ROI set.

14 Commence imaging. Repeat FRAP assay as many times as possible within the 30 min span.

## FRAP data analysis

15 Load timelapse image and saved ROI set to ImageJ.

16 Draw a new ROI to encompass a background area that exhibits no puncta for the whole video. Save to the ROI manager.

17 Measure average fluorescence for NEMO channel and p62 channel for each ROI (including background) for every timepoint. Paste fluorescence values to a new spreadsheet in Excel.

> **Note**
>
> Exclude data from puncta that do not stay in the z frame.

> **Note**
>
> Note whether the puncta is positive for only EGFP-NEMO, or EGFP-NEMO and mCherry-p62.

18 Subtract the respective background value from each puncta ROI measurement for each timepoint to calculate "Sub-bg", i.e. NEMO puncta value at $t_1$ minus NEMO background value at $t_1$ equal Sub-bg.

> **Note**
>
> This step accounts for whole-cell bleaching effects.

19 Identify the minimum intensity value for each channel for each puncta, then subtract the minimum from Sub-bg's to calculate "Sub-min".

Note

At least one timepoint will be recorded as 0.

20 Identify the maximum intensity Sub-min values for each channel for each puncta, then divide each Sub-min value by the maximum to calculate "Norm-to-max".

Note

At least one timepoint will be recorded as 0, and one will be recorded as 1.

21 Plot these Norm-to-max values in Prism using the XY format with replicate values in side-by-side columns where NEMO values populate group 1 and p62 values populate group 2.

Note

NEMO data can be further divided by whether puncta are NEMO-only or NEMO-p62.