



Sep 06, 2021

Immunofluorescence of autophagic cargo receptors and p-TBK1 at LAMP1 lysosomes during lysophagy

Vinay V. Eapen¹, Sharan Swarup¹, Melissa Hoyer¹, Harper JW¹¹Department of Cell Biology, Harvard Medical School, Boston MA 02115

Harper JW: wade_harper@hms.harvard.edu

1 Works for me



Share

dx.doi.org/10.17504/protocols.io.bxghpjt6

Harper JW

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

ABSTRACT

Lysophagy-the selective elimination of damaged lysosomes by the autophagy pathway-is a critical housekeeping mechanism in cells. This pathway surveils lysosomes and selectively demarcates terminally damaged lysosomes for elimination. Among the most upstream signaling proteins in this pathway are the glycan binding proteins-Galectins-which recognize N and O linked glycan chains on the luminal side of transmembrane lysosomal proteins. These glycosyl modifications are only accessible to galectin proteins upon extensive lysosomal membrane rupture and serve as a sensitive measure of lysosomal damage and eventual clearance by selective autophagy. Indeed, prior work has shown that immunofluorescence of Galectin-3 serves as a convenient proxy for lysophagic flux in tissue culture cells (Aits et al., 2015; Maejima et al., 2013). Here we describe a method for monitoring protein recruitment to damaged lysosomes via immunofluorescence and confocal imaging.

ATTACHMENTS

[d7f2bhjdf.docx](#)

DOI

dx.doi.org/10.17504/protocols.io.bxghpjt6

PROTOCOL CITATION

Vinay V. Eapen, Sharan Swarup, Melissa Hoyer, Harper JW 2021. Immunofluorescence of autophagic cargo receptors and p-TBK1 at LAMP1 lysosomes during lysophagy. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.bxghpjt6>



FUNDERS ACKNOWLEDGEMENT

Aligning Science Across Parkinson's

Grant ID: ASAP-000282

NIH

Grant ID: NS110395

NIH

Grant ID: NS083524

KEYWORDS

Immunofluorescence, Autophagic cargo receptors , p-TBK1, LAMP1, Lysosomes, Lysophagy

LICENSE

———— This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

CREATED

Aug 18, 2021

LAST MODIFIED

Sep 06, 2021

OWNERSHIP HISTORY

Aug 18, 2021  Urmilas

Aug 23, 2021  Harper JW

PROTOCOL INTEGER ID

52457

MATERIALS TEXT

Materials:

A	B	C
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
LLoMe (L-Leucyl-L-Leucine methyl ester (hydrochloride))	Cayman Chemical	16008
Dulbecco's MEM (DMEM), high glucose, pyruvate	GIBCO / Invitrogen	11995
Phosphate Buffered Saline 1X	Corning	21-031-CV
Fetal Bovine Serum	Fisher	SH3008003
Bovine Serum Albumin	Gold biotechnology	A-420-250
paraformaldehyde	Electron Microscopy Sciences	15710
Triton-X	Sigma	T8787
Antibodies		
LAMP1 (D401S) Mouse mAb	Cell Signaling Technology	15665S
Anti-CALCOCO2 antibody produced in rabbit	Abcam	ab68588
Anti-OPTN antibody produced in rabbit	Sigma	HPA003279
Anti-TAX1BP1 antibody produced in rabbit	Sigma	HPA024432
phospho-TBK1/NAK (Ser172) (D52C2) Rabbit mAb	Cell Signaling Technology	5483S
Software		
Cell Profiler	CellProfiler v4.0.6	https://cellprofiler.org/
Fiji	ImageJ V.2.0.0	https://imagej.net/software/fiji/
Metamorph	Metamorph v	https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy#ref

Chemicals:

[L-Leucyl-L-Leucine methyl ester \(hydrochloride\)](#) **Cayman Chemical**

Company Catalog #16008

[DMEM, high glucose, pyruvate](#) **Thermo**

Fisher Catalog #11995065

[Phosphate Buffered Saline](#)

(PBS) **Corning Catalog #MT21-031-CV**

[Paraformaldehyde](#) **Electron Microscopy**

Sciences Catalog #15710

[Triton X-100](#) **Sigma**

Aldrich Catalog #T8787

Antibodies:

[LAMP1 \(D401S\) Mouse mAb #15665](#) **Cell Signaling**

Technology Catalog #15665S

[Anti-NDP52 antibody](#)


(ab68588) **Abcam Catalog #ab68588**

 Anti-OPTN antibody produced in rabbit **Sigma**

Aldrich Catalog #HPA003279

 Anti-TAX1BP1 antibody produced in rabbit **Sigma**

Aldrich Catalog #HPA024432

 Phospho-TBK1/NAK (Ser172) (D52C2) XP® Rabbit mAb #5483 **Cell Signaling**

Technology Catalog #5483S

DISCLAIMER:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Immunofluorescence of autophagic cargo receptors and p-TBK1 at LAMP1 lysosomes during lysophagy

4h 7m

- 1 Plate the cells (to be selected by the investigator) into 12 well glass bottom dishes (No. 1.5, 14 mm glass diameter, MatTek) are grown to 50-70% confluency in media.

For HeLa cells, we use Dulbecco's MEM (DMEM), high glucose (4500 mg/L), pyruvate (100 mg/L) supplemented with 10% fetal bovine serum

- 2 Treat the cells with **[M]500 Micromolar (μM)** – **[M]1 Milimolar (mM)** of LLoMe for **🕒01:00:00** .

1h

Note: The exact dosage varies with cell line and should be determined empirically depending on the line used. This dose range has been tested extensively in Hela cells, and routinely generated lysophagic flux.

- 3 Remove the LLoMe containing media from the cells and replace with fresh media not containing LLoMe.

- 4 

15m

After the indicated washout timepoint (4h for optimal receptor recruitment), wash the cells one time with phosphate buffered saline (PBS) and then fix with 4% paraformaldehyde in PBS for **🕒00:15:00** at **🌡 Room temperature** .

5  15m

Remove 4% paraformaldehyde in PBS, wash the cells once with PBS, and then solubilize cells with 0.1% triton-X in PBS for ⌚00:15:00 at 🌡 Room temperature .

6 Block the cells for ⌚00:30:00 at 🌡 Room temperature with sterile filtered blocking buffer (1% bovine serum albumin, 0.1% triton-X in PBS). 30m

7  1h 1m

Add primary antibodies for relevant cargo adaptors or TBK1 to blocking buffer at 1:300 and then spun down for ⌚00:01:00 at 🌀10000 x g . Remove the blocking buffer completely and then apply the antibody in blocking buffer to the cells (📄100 µl applied to the center of the well where the glass coverslip is attached) for ⌚01:00:00 at 🌡 Room temperature .

8 

Wash the cells 4 times with PBS (5min for each wash).

9  1h 1m

Add fluorescently conjugated secondary antibodies to blocking buffer at 1:300 and then spun down for ⌚00:01:00 at 🌀10000 x g . Remove the blocking buffer completely and then apply the antibody in blocking buffer to the cells (📄100 µl applied to the center of the well where the glass coverslip is attached) for ⌚01:00:00 at 🌡 Room temperature .

10  5m

Wash the cells 4 times with PBS (⌚00:05:00 for each wash) and left in PBS.

11 

Image the cells at 🌡 Room temperature using a Yokogawa CSU-X1 spinning disk confocal on a Nikon Ti-E inverted microscope at the Nikon Imaging Center in Harvard Medical School. Use the Nikon Perfect Focus System to maintain cell focus over time. Equip the microscope with a Nikon Plan Apo 40x/1.30 N.A or 100x/1.40 N.A objective lens.

445nm (75mW), 488nm (100mW), 561 nm (100mW) & 642nm (100mW) laser lines are controlled by AOTF. Collect all images with a Hamamatsu ORCA-ER cooled CCD camera (6.45 µm² photodiode) with MetaMorph image acquisition software.

12 Display Z series as maximum z-projections and save using Fiji software.

13 Perform the Mander's Overlap Correlation (MOC) in lysosomes in CellProfiler (see attached CellProfiler pipeline file).

Each field of view for every unique condition is thresholded in the same way. The "identify primary objects" tool is used to find puncta for both the lysosome channel and for the respective receptor or p-TBK1 stain. The "measure colocalization" module is used to compare the fluorescence intensities within the areas defined by the threshold. The MOC with Costes was reported for each field of view..

14 Each channel z series are brightness and adjust contrast equally and then convert to rgb for publication using FIJI software.