Sep 05, 2021 Version 1

Immunofluorescence of autophagic cargo receptors and p-TBK1 at LAMP1 lysosomes during lysophagy V.1

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

Vinay V. Eapen[1], Sharan Swarup[1], Melissa Hoyer[1], Harper JW[1]

[1]Department of Cell Biology, Harvard Medical School, Boston MA 02115

Harper JW: wade_harper@hms.harvard.edu

Harper JW
harvard university

DOI: https://dx.doi.org/10.17504/protocols.io.bxghpjt6

External link: https://doi.org/10.7554/eLife.72328

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**

Manuscript citation:
Eapen VV, Swarup S, Hoyer MJ, Paulo JA, Harper JW, Quantitative proteomics reveals the selectivity of ubiquitin-binding autophagy receptors in the turnover of damaged lysosomes by lysophagy. eLife doi: 10.7554/eLife.72328

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

Created: August 18, 2021

Last Modified: May 31, 2024

Protocol Integer ID: 52457

Keywords: Immunofluorescence, Autophagic cargo receptors, p-TBK1, LAMP1, Lysosomes, Lysophagy, ASAPCRN, lysosomes by the autophagy pathway, accessible to galectin protein, galectin protein, lysosomes via immunofluorescence, protein recruitment to damaged lysosome, damaged lysosome, lysosomes for elimination, sensitive measure of lysosomal damage, transmembrane lysosomal protein, lysosomal damage, extensive lysosomal membrane rupture, galectin, autophagy pathway, selective autophagy, signaling protein, eventual clearance by selective autophagy, proteins in this pathway, glycosyl modification, glycan chains on the luminal side, immunofluorescence, lysophagy lysophagy, lysophagy, glycan chain, protein, autophagic cargo receptor, glycan, binding protein, critical housekeeping mechanism in cell, monitoring protein recruitment, lysophagic flux, convenient proxy for lysophagic flux

Funders Acknowledgements:
Aligning Science Across Parkinson's
Grant ID: ASAP-000282
NIH
Grant ID: NS110395
NIH
Grant ID: NS083524

Disclaimer

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

DOCX

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22KB

Icon for Immunofl

Immunofluorescence o...

139KB

Materials

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.molecular

	A	B	C
			devices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy#gref

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 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T8787

Antibodies:

- LAMP1 (D4O1S) Mouse mAb #15665 **Cell Signaling Technology** **Catalog #**15665S
- Anti-NDP52 antibody (ab68588) **Abcam** **Catalog #**ab68588
- Anti-OPTN antibody produced in rabbit **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**HPA003279
- Anti-TAX1BP1 antibody produced in rabbit **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**HPA024432
- Phospho-TBK1/NAK (Ser172) (D52C2) XP® Rabbit mAb #5483 **Cell Signaling Technology** **Catalog #**5483S

Troubleshooting

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.

Note

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 millimolar (mM) of LLoMe for 01:00:00 .

1h

Note

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 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 .

15m

5 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 .

15m

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 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 .

1h 1m

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

9 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 .

1h 1m

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

5m

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.

Note

445nm (75mW), 488nm (100mW), 561nm (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).

Note

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.