Sep 06, 2021

Immunofluorescence of Galectin-3 Puncta after lysosomal damage with LLoMe

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

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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 our method for monitoring galectin-3 puncta clearance as a proxy for turnover of damaged lysosomes via immunofluorescence and confocal imaging.

ATTACHMENTS

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DOI

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

PROTOCOL CITATION

Vinay V. Eapen, Sharan Swarup, Melissa Hoyer, Harper JW 2021. Immunofluorescence of Galectin-3 Puncta after lysosomal damage with LLoMe . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bxgwpjxe

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FUNDERS ACKNOWLEDGEMENT

Aligning Science Across Parkinson's
Grant ID: ASAP-000282

NIH
Grant ID: NS083524

NIH
Grant ID: NS110395

KEYWORDS

Immunofluorescence, Galectin-3, Lysosomal damage, LLoMe

CREATED

Aug 18, 2021

LAST MODIFIED

Sep 06, 2021

OWNERSHIP HISTORY

Aug 18, 2021 Urmilas

Aug 23, 2021 Harper JW

PROTOCOL INTEGER ID

52470

MATERIALS TEXT

Materials:

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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
Galectin-3/ LGALS3 (M3/38) Rat mAb	Santa-Cruz	sc-23938
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#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 **Sigma Aldrich Catalog #T8787**

Antibodies:

☒ LAMP1 (D4O1S) Mouse mAb #15665 **Cell Signaling Technology Catalog #15665S**

☒ Anti-galectin-3 Antibody (M3/38): sc-23938 **Santa Cruz Biotechnology Catalog #sc-23938**

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Immunofluorescence of Galectin-3 Puncta after lysosomal damage with LLoMe

4h 12m

1 Plate the cells (selected by 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.

1h

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

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

15m

4

After 10h, 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 solubilize cells with 0.1% triton-X in PBS for **00:15:00** at **Room temperature** .

30m

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

1h 1m

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7

Add primary antibodies to blocking buffer at 1:300 and then spun down for **00:01:00** at **10000 x g** . Remove the blocking buffer is 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 5m

Wash the cells 4 times with PBS (**00:05:00** 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 is completely and then apply the antibody in blocking buffer to the cells (100µL 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 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), 561nm (100mW) & 642nm (100mW) laser lines are controlled by AOTF. All images are collected with a Hamamatsu ORCA-ER cooled CCD camera (6.45 µm2 photodiode) with MetaMorph image acquisition software.

12 Detect Galectin-3 puncta using CellProfiler with the same pipeline applied for each condition (see attached CellProfiler pipeline).

Each cell area is first defined using a “identify primary objects” module that included objects 200 to 1000 pixels units, and each puncta is marked using a “identify primary objects” module that included objects 2 to 20 pixels units both with an optimized “robust background” threshold. Each cell for each condition is thresholded in the same way with a consistent pipeline. Object size and shape is measured, and each punctum is related to its respective cell to yield a puncta per cell readout. Each channel z series are brightness and contrast adjusted equally and then converted to rgb for publication using FIJI software.

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