**Immunofluorescence of Galectin-3 Puncta after lysosomal damage with LLoMe**

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**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.

**Materials**

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| **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 |

1. Cells (selected by investigator) are plated 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. Cells are treated with 500 μM – 1 mM of LLoMe for one hour.

**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.

1. LLoMe containing media is removed from the cells and replaced with fresh media not containing LLoMe.
2. After 10h, cells are washed one time with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS for 15min at room temperature (RT).
3. 4% paraformaldehyde in PBS is removed, the cells are washed once with PBS, and cells are solubilized with 0.1% triton-X in PBS for 15 min at RT.
4. Cells are then blocked for 30 min at RT with sterile filtered blocking buffer (1% bovine serum albumin, 0.1% triton-X in PBS)
5. Primary antibodies are added to blocking buffer at 1:300 and then spun down for 1min at 10,000 x g. The blocking buffer is completely removed and then the antibody in blocking buffer is applied to the cells (100μL applied to the center of the well where the glass coverslip is attached) for 1h at RT.
6. Cells are washed 4 times with PBS (5min for each wash).
7. Fluorescently conjugated secondary antibodies are added to blocking buffer at 1:300 and then spun down for 1min at 10,000 x g. The blocking buffer is completely removed and then the antibody in blocking buffer is applied to the cells (100μL applied to the center of the well where the glass coverslip is attached) for 1h at RT.
8. Cells are washed 4 times with PBS (5min for each wash) and left in PBS.
9. Cells are imaged at RT using a Yokogawa CSU-X1 spinning disk confocal on a Nikon Ti-E inverted microscope at the Nikon Imaging Center in Harvard Medical School. Nikon Perfect Focus System was used to maintain cell focus over time. The microscope is equipped 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.
10. Galectin-3 puncta are detected 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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