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

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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 a method for monitoring protein recruitment to 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 |
| 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#gref |

1. Cells (to be selected by the 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 the indicated washout timepoint (4h for optimal receptor recruitment), 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 then 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 for relevant cargo adaptors or TBK1 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. Z series are displayed as maximum z-projections and saved using Fiji software.
11. Mander’s Overlap Correlation (MOC) in lysosomes is performed 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.
12. Each channel z series are brightness and contrast adjusted equally and then converted to rgb for publication using FIJI software.