**Analysis of Lysophagic Flux in Cultured Cells using Lyso-Keima**

Vinay V. Eapen, Sharan Swarup, Melisa Hoyer and J. Wade Harper

**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 facile method for monitoring lysophagy using the acid sensitive fluorophore mKeima, affixed onto Galectin-3, which allows for the monitoring of lysophagic flux by Flow cytometry, Western blotting or Confocal imaging. This method, which we have termed Lyso-Keima, serves as a facile and quantitative assay for monitoring lysophagy in tissue culture cells.

**Materials**

|  |  |  |
| --- | --- | --- |
| **REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER** |
| **Chemicals** |
| LLoMe (L-Leucyl-L-Leucine methyl ester (hydrochloride))  | Cayman Chemical | 16008 |
| Bafilomycin A1 | Cayman Chemical | 88899-55-2 |
| Dulbecco’s MEM (DMEM), high glucose, pyruvate  | GIBCO / Invitrogen  | 11995 |
| Puromycin  | Gold Biotechnology  | Gold Biotechnology |
| Phosphate Buffered Saline 1X  | Corning | 21-031-CV |
| Fetal Bovine Serum | Fisher | SH3008003 |
| Protease inhibitor cocktail  | Sigma-Aldrich  | P8340 |
| PEI | Polysciences | 23966-2 |
| FluoroBrite DMEM | ThermoScientific | A1896701 |
| Anti-Keima-Red mAb | MBL international | M182-3M |
| **Recombinant DNA**  |
| pHAGE-mKeima-LGALS3 | Addgene |  |
| pPAX2 | Addgene | 12260 |
| pMD2 | Addgene | 12259 |
| **Critical Commercial Assays** |
| Bio-Rad Protein Assay Dye Reagent Concentrate | Bio-Rad | 5000006 |
| **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 |
| Flowjo | Flowjo, v10.7 | https://www.flowjo.com |
| **Other**  |
| 35 mm-glass bottom dishes No. 1.5, 14 mm glass diameter | MatTek | P35G-1.5-14-C |
| FACS Tubes  | Corning | 352235 |

**Step1: Generation of Stable Cell line expressing mKeima-Galectin-3**

1. mKeima tagged Galectin 3 Lentiviral vector were packaged in HEK293T by cotransfection of pPAX2, pMD2 and the vector of interest in a 4:2:1 ratio using polyethelenimine (PEI).
2. Virus containing supernatant was collected 2 days after transfection and filtered through a .22 micron syringe filter. Polybrene was added at 8 mg/ml to the viral supernatant.
3. After infecting target cells with varying amounts of relevant viruses, cells were selected in puromycin (1mg/ml for Hela cells, will vary for other cell lines).

**Note:** stably selected mKeima tagged Galectin-3 cells usually expresses well, and to sufficient amounts for downstream applications however, expression level should be checked by population based measurements such as Flow Cytometry or Confocal imaging. If the levels are low, consider sorting Keima positive cells by FACS to obtain a homogenous and high expressing population (See flow cytometry tab).

**Step2: Analysis of Lyso-Keima using Fluorescent activated cell sorting (FACS)**

1. Cells stably expressing Keima-Galectin-3 are grown to 50-70% confluency in 6-well plates in triplicates.
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. At this point, Bafilomycin A1 (BafA) can be added at 20nM to one set of well to serve as a negative control, as BafA blocks lysosomal acidification and autophagic flux.
2. At the time of harvesting, cells were trypsinized, pelleted at 1000 rpm for 3 min, and then resuspended in FACS buffer (1X PBS, 2% FBS). The resuspended cells were filtered through cell strainer caps into FACS tubes and placed on ice. The cells (~10,000 per replicate) were then analyzed by flow cytometry the data was exported into Flowjo.
3. ­­**Gating Strategy:**
4. Live cells are selected by gating Side Scatter (Area-SSC-A) with Forward Scatter (Area-FSC-A)
5. Singlets are selected by gating SSC-A with SSC-W(width)
6. Keima positive cells are selected from the singlets by plotting the Acidic Keima (Texas Red A) to the

Neutral Keima (BB630-A). These setting will vary on the instrument used and reflect the settings on a BD FACS Symphony Cell sorter.

1. The ratio of Acidic Keima to Neutral is calculated in flowjo by dividing the mean of acidic keima signal to neutral keima signal. Successful lysophagic flux can be visually seen as a shift in in the Acidic population (blue) relative to the untreated sample (Red).

**Step3: Analysis of Lyso-Keima using Western Blotting of processed Keima**

1. Treat cells as in 2. Steps 1-3 are the same. If doing a time course, cells can be collected at the required timepoints.
2. For western blotting, cell pellets were collected and resuspended in 8M Urea buffer (8M Urea, 150 mM TRIS pH 7.4, 50 mM NaCl) supplemented with Protease and Phosphatase Inhibitors.
3. The resuspended pellets were sonicated, and the lysate was spun at 13,000 RPM for 10 min. Bradford or BCA assay was performed on clarified lysate and equal amounts of lysate were boiled in 1X SDS containing Laemmeli buffer. **Note:** Cells can also be lysed by other methods, such as with RIPA buffer. Whichever method is used a minimum of 10-15mg of protein lysate is sufficient for Keima Immunoblotting
4. **** Lysates were run on 4-20% Tris Glycine gels (BioRad) and transferred via Wet transfer onto PVDF membranes for immunoblotting with the indicated antibodies. For Anti Keima Immunoblotting use α-Keima antibody at 1:1000 dilution in 5% Milk TBST (Tris buffered saline -Tween 1%).
5. Images of blots can be acquired using Enhanced-Chemi luminescence via film or digital imaging. Processed Keima runs at ~25 KDa and full length Keima-Galectin-3 runs at ~50 kDa.

**Step4: Analysis of Lyso-Keima using Live Cell Fluorescent Microscopy (LC-FM)**

1. Cells stably expressing Keima-Galectin-3 are plated onto 35 mm-glass bottom dishes (No. 1.5, 14 mm glass diameter, MatTek) and grown to 50-70% confluency in media (Dulbecco’s MEM (DMEM), high glucose, pyruvate 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 and devoid of phenol red (FluoroBrite DMEM supplemented with 10% FBS). At this point, Bafilomycin A1 (BafA) can be added at 20nM to one set of well to serve as a negative control, as BafA blocks lysosomal acidification and autophagic flux.
2. After the indicated washout timepoint, cells are imaged at 37°C 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. Pairs of images for ratiometric analysis of mKeima fluorescence are collected sequentially using 100 mW 442 nm and 100 mW 561 solid state lasers and emission is collected with a 620/60 nm filter (Chroma Technologies). All images are collected with a Hamamatsu ORCA-ER cooled CCD camera (6.45 µm2 photodiode) with MetaMorph image acquisition software.
3. Z series are displayed as maximum z-projections and saved using Fiji software.
4. Analysis of acidic Keima-LGALS3 puncta at 12h washout is done in CellProfiler using the same pipeline for each condition (see attached CellProfiler pipeline). Using the “image math” module divide the 561-excitation channel image by the 442-excitation channel image. The acidic puncta in the resulting image are then marked using the “identify primary objects” tool by applying an Otsu threshold for puncta 5-20 pixels in diameter. Each resulting puncta is matched to its respective cell and counted.
5. Each channel z series are brightness and contrast adjusted equally and then converted to rgb for publication using FIJI software.
6. A “Fire” look up table in Fiji is applied to the exported “image math” image to show the acidic signal (561/442) hotspots and then converted to rgb for publication using Fiji software.
7. The image of the acidic puncta identified (also exported from CellProfiler) is also converted to rgb for publication using Fiji software.

Aits, S., Kricker, J., Liu, B., Ellegaard, A.M., Hämälistö, S., Tvingsholm, S., Corcelle-Termeau, E., Høgh, S., Farkas, T., Jonassen, A.H., et al. (2015). Sensitive detection of lysosomal membrane permeabilization by lysosomal galectin puncta assay. Autophagy *11*, 1408–1424.

Maejima, I., Takahashi, A., Omori, H., Kimura, T., Takabatake, Y., Saitoh, T., Yamamoto, A., Hamasaki, M., Noda, T., Isaka, Y., et al. (2013). Autophagy sequesters damaged lysosomes to control lysosomal biogenesis and kidney injury. EMBO Journal *32*, 2336–2347.

 McQuin, C., Goodman, A., Chernyshev, V., Kamentsky, L., Cimini, B.A., Karhohs, K.W., Doan, M., Ding, L., Rafelski, S.M., Thirstrup, D., et al. (2018). CellProfiler 3.0: Next-generation image processing for biology. PLoS Biol. 16(7):e2005970.