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Analysis of Lysophagic Flux in Cultured Cells using Lyso-Keima V.2

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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 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.

ATTACHMENTS

d62cbhjdf.docx Analysis of Lysophagic Flux in Cultured Cells using Lyso-Keima.cpproj

DOI

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PROTOCOL CITATION

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KEYWORDS

Lysophagic Flux, Lyso-Keima, Cultured cells

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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
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

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**

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Protease Inhibitor Cocktail **Sigma Aldrich Catalog #P8340**

FluoroBrite™ DMEM **Thermo Fisher Catalog #A1896701**

psPAX2 **addgene Catalog #12260**

pMD2.G **addgene Catalog #12259**

Bio-Rad Protein Assay Dye Reagent Concentrate **Bio-rad Laboratories Catalog #5000006**

Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap **Corning Catalog #352235**

Anti-Keima-Red mAb (Monoclonal Antibody) **Mbl International Catalog #M182-3M**

35 mm Dish | No. 1.5 Coverslip | 14 mm Glass Diameter | Uncoated **MatTek Corporation Catalog #P35G-1.5-14-C**

Generation of Stable Cell line expressing mKeima-Galectin-3

1 Pack mKeima tagged Galectin 3 Lentiviral vector in HEK293T by cotransfection of pPAX2, pMD2 and the vector of interest in a 4:2:1 ratio using polyethelenimine (PEI).

2 

Collect virus containing supernatant 2 days after transfection and filter through a .22 micron syringe filter. Add polybrene at [M]**8 mg/ml** to the viral supernatant.

3 After infecting target cells with varying amounts of relevant viruses, select cells in puromycin ([M]**1 mg/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).

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Analysis of Lyso-Keima using Fluorescent activated cell sorting (FACS) 1h 3m

4 Grow cells stably expressing Keima-Galectin-3 to 50-70% confluency in 6-well plates in triplicates.

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

> **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.

6 Remove the LLoMe containing media from the cells and replace 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.

7  3m

At the time of harvesting, trypsinize cells, pellet at **1000 rpm** for **00:03:00** , and then resuspend in FACS buffer (1X PBS, 2% FBS). Filter the resuspended cells through cell strainer caps into FACS tubes and place them **On ice** . Analyze the cells (~10,000 per replicate) by flow cytometry the data was exported into Flowjo.

Gating Strategy

8 Select the live cells by gating Side Scatter (Area-SSC-A) with Forward Scatter (Area-FSC-A).

9 Select the singlets by gating SSC-A with SSC-W(width).

10 Select the Keima positive cells 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.

11 Calculate the ratio of Acidic Keima to Neutral in flowjo by dividing the mean of acidic keima signal to neutral keima signal.

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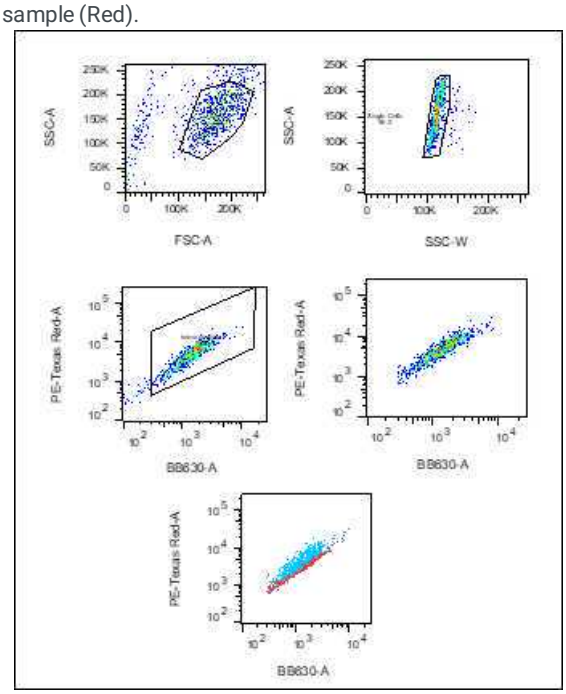
Successful lysophagic flux can be visually seen as a shift in the Acidic population (blue) relative to the untreated sample (Red).



Analysis of Lyso-Keima using Western Blotting of processed Keima

12 Treat the cells as in section 2 (Analysis of Lyso-Keima using Fluorescent activated cell sorting (FACS)).

Sections 1-3 are the same. If doing a time course, collectt the cells at the required timepoints.

13 For western blotting, collect the cell pellets and resuspend in [M]**8 Molarity (M)** Urea buffer ([M]**8 Molarity (M)** Urea, [M]**150 Milimolar (mM)** TRIS pH**7.4** , [M]**50 Milimolar (mM)** NaCl) supplemented with Protease and Phosphatase Inhibitors.

14 10m

Sonicate the resuspended pellets, and spin the lysate at **13000 rpm** for **00:10:00** . Perform the Bradford or BCA assay on clarified lysate and boil the equal amounts of lysate 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 10-15μg of protein lysate is sufficient for Keima Immunoblotting.

15 Run the lysates on 4-20% Tris Glycine gels (BioRad) and transfer via Wet transfer onto PVDF membranes for

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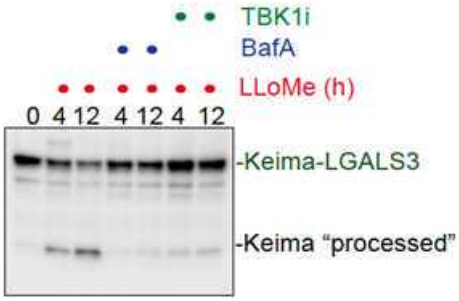
immunoblotting with the indicated antibodies. For Anti Keima Immunoblotting use α-Keima antibody at 1:1000 dilution in 5% Milk TBST (Tris buffered saline -Tween 1%).

16 Acquire the images of blots 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.



Analysis of Lyso-Keima using Live Cell Fluorescent Microscopy (LC-FM) 1h

17 Plate the cells stably expressing Keima-Galectin-3 onto **35 mm** -glass bottom dishes (No. 1.5, **14 mm** glass diameter, MatTek) and grow to 50-70% confluency in media (Dulbecco's MEM (DMEM), high glucose, pyruvate supplemented with 10% fetal bovine serum).

18 Treat the cells with **500 Micromolar (µM)** – **1 Milimolar (mM)** of LLoMe for **01:00:00** . 1h

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.

19 Remove the LLoMe containing media from the cells and replace 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.

20

After the indicated washout timepoint, image the cells 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. 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. Collect the pairs of images for ratiometric analysis of mKeima fluorescence sequentially using 100 mW 442 nm and 100 mW 561 solid state lasers and collect the emission with a 620/60 nm filter (Chroma Technologies). Collect all images with a Hamamatsu ORCA-ER cooled CCD camera (6.45 µm2 photodiode) with MetaMorph image acquisition software.

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21 Display the Z series as maximum z-projections and save using Fiji software.

22 Analyze acidic Keima-LGALS3 puncta at 12h washout 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.

23 Each channel z series are brightness and adjust contrast equally and then convert to rgb for publication using FIJI software.

24 Apply a “Fire” look up table in Fiji to the exported “image math” image to show the acidic signal (561/442) hotspots and then convert to rgb for publication using Fiji software.

25 Convert the image of the acidic puncta which is identified (also exported from CellProfiler) to rgb for publication using Fiji software.

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