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Analysis of Lysophagic Flux in Cultured Induced Neurons using RFP-GFP-galectin3

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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 GFP positive RFP-GFP-galectin-3 GFP positive puncta clearance as a proxy for turnover of damaged lysosomes via immunofluorescence and confocal imaging.

ATTACHMENTS

Analysis of Lysophagic Flux in Cultured Induced Neurons using RFP-GFP-galectin3.cppipe

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PROTOCOL CITATION

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MATERIALS TEXT

A	B	C
REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
Chemicals		
LLoMe (L-Leucyl-L-Leucine methyl ester (hydrochloride))	Cayman Chemical	16008
Dulbecco's MEM (DMEM), F12	Thermo	11320033
Phosphate Buffered Saline 1X	Corning	21-031-CV
E8 components	See protocol dx.doi.org/10.17504/protocols.io.bsacnaaw	
ND1 and ND2 components	See protocol dx.doi.org/10.17504/protocols.io.br9em93e	
Hygromycin B	Life Technologies	10687-010
pAC150 GFP-RFP-LGALS3		Addgene #175778; RRID:Addgene_175778
pAC150 GFP-RFP-LGALS3 R186S		Addgene #175779; RRID:Addgene_175779
pCMV-hyPBase hyperactive piggyBac vector	Yusa et al 2011	Available upon request at the Sanger Institute Achives
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
Hardware		
ThermoFisher Neon Electroporator & Kit		

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Electroporation of PB vectors. Use ThermoFisher kit and ThermoFisher Neon Electroporator to electroporate ES cells with PB vector and PB helper vector.

1 

Add 10ml buffer R to a sterile 1.5ml tube. Add 0.5µg of pAC150 GFP-RFP-LGALS3 or pAC150 GFP-RFP-LGALS3 R186S vector and 0.5µg of pCMV-hyPBase hyperactive piggyBac vector. Pipet up and down to mix. Let it sit at RT for 10min. This is enough for 2 transfections (== one 6 well).

2 

Individualize cells with Accutase. Neutralize Accutase with 5 times volume E8 with Rock inhibitor.

3 Count cells. You will need $2x10^5$ for each transfection.

4 

Spin down cells. Let it sit for a while so all the residue media can go down to the bottom of the tube. If the residue media is too much, take it out with a P200 pipet.

5 

Resuspend cells to a concentration of $2x10^5$ per 5 ml (ie $4x10^7$ per ml) using buffer R. You don't have to take all the residue media off but you will need to take into account the volume of residue media so you are not too much off.

6 

Prepare a 6 well matrigel coated plate. Add 2mL of E8+ rock inhibitor (1:1000) to the wells you will use. Two transfections go into one well.

7 Wipe the Neon pipet station with EtOH and place it inside the hood.

8 Add 3ml of electrolytic buffer (buffer E) to the neon tube. Place the tube inside the station. You should feel a click before the tube is securely seated in the station.

9 Use program 13 from the optimization tab for electroporation parameter (Voltage: 1100. Pulse width: 20 Pulse number: 2). Program 9 should also work.

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10

When everything is ready, mix 10-11ml of resuspended cells with the plasmid containing R buffer. The final volume should be in the range of 21-22ml.

11

Take up a neon tip, pipet 10ml of the cell protein mix and electroporate with program 13. It is important to pipet slowly to avoid air bubble formation. It is also important to insert the pipet slowly into the station, especially during the end of the insertion when you will feel a click. Help the pipet down slowly during the clicking so there is no sudden movement of the tip, which might create tiny air bubbles.

12

If you see air bubble in the tip, take it out, push everything out of the tip and repipet the mixture.

13

If you see sparking during the electroporation, your efficiency will reduce significantly.

14

Once electroporation is complete, push everything into one well of a 6 well plate. Do not pipet up and down with Neon tip.

15

Repeat the same procedure with the same tip and the left over cell mixture. Place the second electroporated mixture into the same well.

16

Disperse cells evenly in the well and place cells in a low O2 incubator. 17. Put electroporated cells into low oxygen incubator for 2 days

17 Select cells with 50mg/mL hygromycin B 4 days post-electroporation. Grow cells in selection medium for 7-10 days until there is no longer any cell death and every cell has integration of the GFP-RFP-LGALS3 or GFP-RFP-LGALS3 R186S.

Differentiation of Stable Cell ES H9 line expressing RFP-GFP-galectin3 to induced neurons (iN)

18 Differentiation to induced neurons (iN) is done by following the protocol “Neural differentiation of AAVS1-TREG3-NGN2 pluripotent stem cells (dx.doi.org/10.17504/protocols.io.br9em93e)

Analysis of RFP-GFP-galectin3 clearance after LloMe damage via confocal microscopy

19

To help get spread out iNeurons for imaging, when cells get 90 percent confluent at any point in the day 5-7 range, cells are plated into onto 35 mm-glass bottom dishes (No. 1.5, 14 mm glass diameter, MatTek) at a low confluency (approximately 1x105 cells per 2mL dish).

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20

At day 12, 2h prior to LLoMe treatment, 0.75mL of media is removed to leave 0.75mL media. This 0.75mL media is saved as “condition media.” 0.75mL of fresh ND2 media is added so that the iN are in a fed state. Each well should contain 5mL media.

21

5mL of fresh ND2/LLoMe is added to a final concentration of 500 mM of LLoMe in 2mL. Cells are treated for 1h.

22

Fresh ND2 media is added to the condition media (every 0.75mL condition media gets 1.25mL of fresh ND2). The LLoMe-containing media is removed from the cells and replaced with the 2mL condition media/fresh ND2 media mixture that does not contain LLoMe.

23

After 12h, cells are washed one time with phosphate buffered saline (1xPBS) and then fixed with 4% paraformaldehyde in PBS for 15min at room temperature (RT). Then the fixation media is replaced with 1xPBS.

24

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. 488nm (100mW) and 561nm (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.

25 Z series are displayed as maximum z-projections and saved using Fiji software.

26

The GFP and RFP positive LGALS3 puncta are detected using Cell Profiler 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.

27 Each channel z series are brightness and contrast adjusted equally and then converted to RGB for publication using FIJI software.

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