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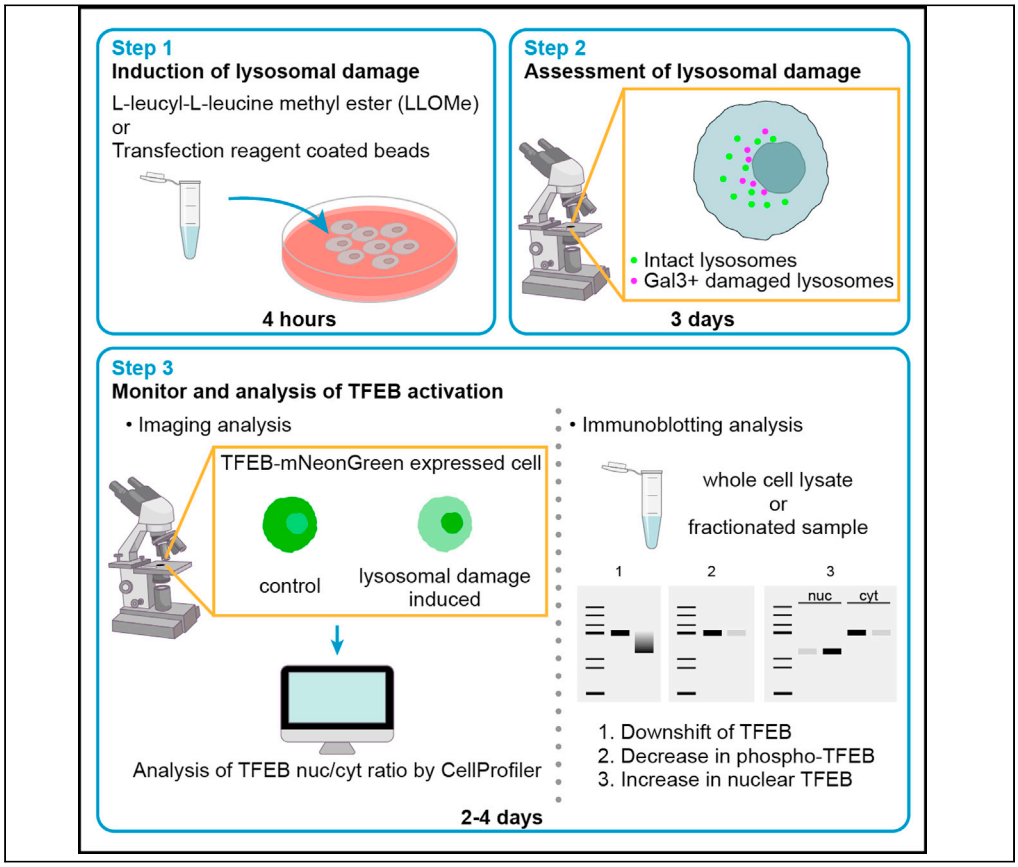
Protocols to monitor TFEB activation following lysosomal damage in cultured cells using microscopy and immunoblotting



Following lysosomal damage, activation and nuclear translocation of transcription factor EB (TFEB) is the key event to maintain lysosomal homeostasis. Here, we describe steps to induce lysosomal damage in HeLa cells. This can be followed by monitoring the changes in TFEB localization using widefield fluorescence microscopy. As a complementary approach, we describe the use of immunoblotting to follow the activation and localization of TFEB in cell lysates. These protocols enable quantitative analysis of TFEB.

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Highlights

Detailed protocol to induce lysosomal damage in cultured cells

Microscopy-based analysis of changes in TFEB localization in response to damage

Immunoblotting approach to follow the activation and localization of TFEB in cell lysates

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Protocols to monitor TFEB activation following lysosomal damage in cultured cells using microscopy and immunoblotting

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SUMMARY

Following lysosomal damage, activation and nuclear translocation of transcription factor EB (TFEB) is the key event to maintain lysosomal homeostasis. Here, we describe steps to induce lysosomal damage in HeLa cells. This can be followed by monitoring the changes in TFEB localization using widefield fluorescence microscopy. As a complementary approach, we describe the use of immunoblotting to follow the activation and localization of TFEB in cell lysates. These protocols enable quantitative analysis of TFEB.

For complete details on the use and execution of this protocol, please refer to Nakamura et al. (2020).

BEFORE YOU BEGIN

The protocols below describe how to monitor subcellular localization and phosphorylation state of TFEB, both are key factors defining the activation state of TFEB. TFEB shuttles between cytosol and nucleus in response to the cellular condition. Under nutrient-rich conditions, TFEB mainly localizes to cytosol, and it is inactive. Under stress conditions such as starvation or lysosomal damage, TFEB translocates to the nucleus, which leads to the activation of target genes. The subcellular localization of TFEB is strictly regulated through its phosphorylation by mTORC1. In particular, Ser211 residues in the TFEB protein are important for its subcellular localization. When these Ser211 residues are dephosphorylated, TFEB rapidly translocates from the cytosol to the nucleus (Napolitano and Ballabio, 2016).

This protocol consists of three sections: induction of lysosomal damage, assessment of the lysosomal damage using microscopy, monitor and analysis of TFEB. Figure 1 is a flowchart of this protocol. The section "assessment of the lysosomal damage using microscopy" is optional and not necessary at every experiment. However, before proceeding monitor and analysis step, we recommend taking this step once to confirm whether the experimental condition is optimized.

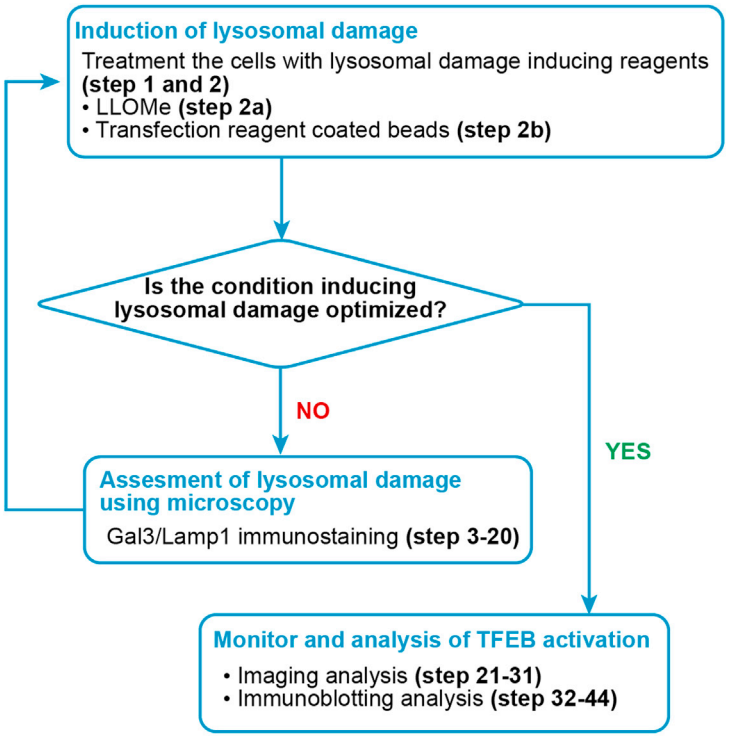

Figure 1. Flowchart of this protocol

The entire flow of this protocol. Steps indicated here correspond to steps in the section "step-by-step method details".

Cell culture and passage

Timing: 10 min

Culture cells in an incubator with 5% CO_2 at 37°C. In our experiment, we use DMEM with high glucose to avoid glucose starvation. Cells are passaged about 3 times in a week.

Note: Cells are used for experiments within 2–15 passages.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-Gal-3 (rat, 1:1,000)	Santa Cruz Biotechnology	Cat# sc-23938
anti-LAMP1 (mouse, 1:1,000)	Santa Cruz Biotechnology	Cat# sc-19992
anti-TFEB (rabbit, 1:1,000)	Cell Signaling Technology	Cat# 4240
anti-phospho Ser211 TFEB (rabbit, 1:1,000)	Cell Signaling Technology	Cat# 37681
anti-GAPDH (rabbit, 1:20,000)	Cell Signaling Technology	Cat# 2118
anti-Lamin B (goat, 1:1,000)	Santa Cruz Biotechnology	Cat# sc-6217
HRP-conjugated goat anti-rabbit IgG (1:5,000)	Jackson ImmunoResearch	Cat# 111-035-003
HRP-conjugated goat anti-rat IgG (1:5,000)	Jackson ImmunoResearch	Cat# 112-035-003

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HRP-conjugated goat anti-mouse IgG (1:5,000)	Jackson ImmunoResearch	Cat# 115-035-003
goat anti-rabbit Alexa Fluor 488 pre-absorbed (1:2,000)	Abcam	Cat# ab150085
goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 568 (1:2,000)	Invitrogen	Cat# A11004
goat anti-rat Alexa Fluor 647 pre-absorbed (1:2,000)	Abcam	Cat# ab150167
Chemicals, peptides, and recombinant proteins		
Leu-Leu methyl ester hydrobromide (LLOMe)	Sigma-Aldrich	Cat# L7393-500MG
4% Paraformaldehyde Phosphate Buffer Solution (PFA)	Nacalai Tesque	Cat# 09154-85
VECTASHIELD Mounting Medium with DAPI	VECTOR LABORATORIES	Cat# H-1200
Gelatin, from Bovine Bone	Wako	Cat# 073-06295
Digitonin	Wako	Cat# 044-02121
Ethanol (99.5)	Wako	Cat# 052-07221
Dulbecco's Modified Eagle's Medium – high glucose (DMEM)	Sigma-Aldrich	Cat# D6429-500ML
Fetal Bovine Serum	Sigma-Aldrich	Cat# F7524
Effectene transfection reagent	Qiagen	Cat# 301425
Skim Milk for immunoassay	Nacalai Tesque	Cat# 31149-75
Trypsin-EDTA solution	Sigma-Aldrich	Cat# T4174
L-Glutamine solution	Sigma-Aldrich	Cat# G7513
Penicillin-Streptomycin	Sigma-Aldrich	Cat# P4333
ImmunoStar LD	FUJIFILM Wako	Cat# 296-69901
Collagen; Cell matrix Type I-C (3 mg/mL)	Nitta Gelatin	Cat# 631-00771
NP-40	Nacalai Tesque	Cat# 25223-04
Protease Inhibitor Cocktail	Roche	Cat# 11873580001
phosphatase inhibitors	Roche	Cat# 4906837001
M-PER mammalian protein extraction reagent	Thermo Fisher Scientific	Cat# 78501
Experimental models: Cell lines		
Human: HeLa Kyoto	Prof. Shuh Narumiya (Kyoto Univ.)	N/A
Human: HeLa Kyoto TFEB-mNeonGreen	Nakamura et al., 2020	N/A
Software and algorithms		
ImageJ/Fiji	National Institutes of Health (NIH)	N/A
CellProfiler	Broad Institute	https://cellprofiler.org/
Image Lab	Bio-Rad Laboratories	N/A
GraphPad Prism	GraphPad Software	https://www.graphpad.com/
Other		
Polybead Carboxylate Microspheres 3.00 μm	PolySciences, Inc.	Cat# 09850-5
Glass slide	Matsunami Glass Ind.	Cat# FF-001
Micro cover glass (12 mm, No.1-S)	Matsunami Glass Ind.	Cat# C501813
FLUOVIEW FV3000RS Laser Confocal Microscope operated by FV31S-SW (version 2.3.1.163)	Olympus	N/A
IX83 widefield fluorescence microscope operated by MetaMorph (Molecular Devices)	Olympus	N/A
ChemiDoc Touch imaging system	Bio-Rad Laboratories	17001401JA

MATERIALS AND EQUIPMENT

Solutions for cell culture and inducing lysosomal damage

Culture medium		
Reagent	Final concentration	Amount
Dulbecco's modified Eagle's medium (DMEM) -high glucose	n/a	500 mL
Fetal Bovine Serum (FBS)	10%	50 mL
Penicillin-Streptomycin	1%	5 mL
L-glutamine	1%	5 mL
Total	**n/a**	**560 mL**
Store at 4°C for up to a month.		

10× Phosphate Buffered Saline (PBS)		
Reagent	Final concentration	Amount
NaCl	1.37 M	400 g
KCl	27 mM	10 g
Na_2HPO_4	100 mM	72 g
KH_2PO_4	18 mM	12 g
ddH_2O	n/a	up to 5 L
Total	**n/a**	**5 L**
Adjust pH to 7.4 using 2N HCl or 2N NaOH. In this protocol, all PBS solutions are used as 1×. Store at 20°C–25°C for up to 3 months.		

Note: 1× PBS for cell culture should be autoclaved.

Note: 1× PBS is used at 20°C–25°C unless otherwise stated.

LLOMe solution		
Reagent	Final concentration	Amount
LLOMe	333 mM	500 mg
EtOH	n/a	4.426 mL
Total	**n/a**	**4.426 mL**
Add 4.426 mL of EtOH to the bottle of LLOMe. Voltex the bottle well and dissolve LLOMe completely. Aliquot LLOMe solution to 1.5 mL tubes. Store at −20°C for up to a year.		

⚠ CRITICAL: Seal the cap of tubes by paraffin film to avoid evaporation of EtOH.

Solutions for immunofluorescence

Blocking buffer		
Reagent	Final concentration	Amount
Gelatin	0.2% (w/v)	1 mg
PBS	n/a	500 mL
Total	**n/a**	**500 mL**
Autoclaved. Store at 20°C–25°C for up to a year.		

Digitonin solution		
Reagent	Final concentration	Amount
Digitonin	50 mg/mL	50 mg
DMSO	n/a	1 mL
Total	**n/a**	**1 mL**
Store at −20°C for up to a year.		

Note: Dilute 1:1000 with PBS before use.

Solutions for immunoblotting

5× SDS sample buffer		
Reagent	Final concentration	Amount
0.5 M Tris-HCl (pH6.8)	0.28 M	28 mL
Glycerol	30 %	15 g
Sodium Dodecyl Sulfate	10 %	5 g
DTT (Dithiothreitol)	0.5 M	3.86 g
BPB (Bromophenol blue)	n/a	6 mg
ddH_2O	n/a	up to 50 mL
Total	**n/a**	**50 mL**
Store at −20°C for up to a year.		

10× Tris Buffered Saline with Tween 20 (TBST)		
Reagent	Final concentration	Amount
NaCl	138 mM	400 g
KCl	2.7 mM	10 g
Tris-base	50 mM	121 g
Tween 20	0.1 %	50 mL
ddH_2O	n/a	up to 5 L
Total	**n/a**	**5 L**
Adjust pH to 7.4 by HCl. Store at 20°C–25°C for up to 3 months.		

STEP-BY-STEP METHOD DETAILS

Induction of lysosomal damage

Timing: 2 days

This step describes induction of lysosomal damage by two methods, pharmacological treatment and beads transfection. L-Leucyl-L-Leucine methyl ester (LLOMe) is one of the lysosomotropic agent which induces membrane permeabilization of lysosomes (Uchimoto et al., 1999). Polystyrene beads coated with Effectene, a transfection reagent, are known to damage endolysosomes after being endocytosed into cells (Fujita et al., 2013).

1. Seed cells onto plates, incubate the cells with 5% CO_2 for 18–24 h at 37°C.

2. Treat the cells with lysosomal damage inducing reagents. Troubleshooting 1
   a. LLOMe treatment
      i. Prepare LLOMe containing culture medium (final concentration is 1 mM).

⚠ CRITICAL: Make sure that LLOMe dissolved in EtOH completely before use, it sometimes crystallizes.

ii. Wash the cells with PBS once.

Note: Do not use ice cold PBS during cell culture.

iii. Add LLOMe containing medium to the cells, incubate for 1 h at 37°C.
iv. Remove the medium from the cells, wash the cells gently with PBS once.
v. Add the fresh medium to the cells, incubate for 3 h at 37°C.

b. Bead transfection
i. Mix the 2 μL beads and 1 μL Effectene (Qiagen Cat# 301425) transfection reagent in 100 μL Buffer PC.
ii. Incubate transfection reagent-coated beads for 20 min at 20°C–25°C.
iii. Add the 1 mL growth medium to the bead mixture.
iv. Remove the medium from the cells, add to resulting bead mixture to the cells.
v. Incubate the cells for 3 h at 37°C in a CO_2 incubator.
vi. Wash the cells with PBS twice to remove attached beads to the extracellular.

⚠ CRITICAL: Cell cultures containing reagent-coated beads must remain to stand still in order for the beads that fall on the cells to be taken up into the cells (step 2-b-v).

Assessment of the lysosomal damage using microscopy

Timing: 2–3 days

This step describes verification of lysosomal damage by observing Galectin3 (Gal3) puncta. Gal3, one of the beta-galactoside binding lectins, binds to glycans in the lumen of endocytic compartments including lysosomes. Gal3 can be useful for a marker of damaged lysosomes, since it can bind to glycans only when endolysosomal membrane is ruptured (Maejima et al., 2013). Before proceeding to the monitor TFEB activity, we recommend checking whether lysosomes are actually damaged in the experimental condition by this assay. Once confirming the lysosomal damage, step3 to 20 can be omitted accordingly (see also a flowchart in Figure 1).

3. Place sterile coverslips (12 mm in diameter) into a sterile cell culture plate (for 6-well plate, 4 to 5 coverslips/well).
4. Coat coverslips with collagen; Cellmatrix (Nitta Gelatin, 631-00771) diluted by 10 times with sterile water, incubate the plate at least 30 min at 37°C.
5. Wash coverslips with PBS twice.
6. Seed cells onto collagen coated plate, incubate the cells with 5% CO_2 for 18–24 h at 37°C.

⚠ CRITICAL: The cells should be seeded to become below 70% confluent at the next day (In the case of HeLa cells, 2.0×10^5 cells/well).

7. Treat the cells with lysosomal damage inducing reagents as described in step 2a or 2b.
8. Wash the cells with PBS once.
9. Fix the cells with ice cold 4% PFA, incubate the cells for 20 min at 20°C–25°C.
10. Wash the cells with PBS twice.

Pause point: After fixation, the cells can be stored in PBS at 4°C within 1 week.

11. Permeabilize the samples with 50 μg/mL Digitonin-PBS solution for 10 min at 20°C–25°C.
12. Wash the samples with PBS twice

13. Incubate with 0.1% gelatin-PBS for 30 min at 20°C–25°C .
14. Wash the samples with PBS twice.
15. Incubate with the primary antibodies (anti-Gal3 and anti-Lamp1 antibodies) diluted in 0.1% gelatin-PBS for 1h at 20°C–25°C .
16. Wash the samples with PBS twice.
17. Incubate with secondary antibodies diluted in 0.1% gelatin-PBS for 40 min, in the dark at 20°C–25°C .
18. Wash the samples with PBS and ddH_2O for three times (PBS twice → ddH_2O once).
19. Mount the samples with a drop of mounting media with DAPI onto glass slide. Remove excess media and seal the coverslips with nail polish.

Pause Point: After mounting, samples can be stored within one month in the dark at 4°C.

20. Observe the samples by confocal microscopy. Troubleshooting 2

⚠ CRITICAL: After wash, drain excess PBS or ddH_2O from coverslips with paper towel to avoid carryover to following steps.

⚠ CRITICAL: Keep samples from drying out, which cause enhancement of background signal.

Monitor and analysis of TFEB nuclear translocation using microscopy

Timing: 3 days

This step describes observation of TFEB nuclear translocation by microscopy and following quantification of nuclei/cytoplasm ratio using CellProfiler software. This is the free open-source software for analysis of imaging data.

Note: Because the expression level of endogenous TFEB is low, it is recommended to use the cells in which fluorescent protein tagged TFEB is stably expressed for observation of TFEB nuclear translocation by microscopy. In the protocol described below, HeLa cells expressing TFEB-mNeonGreen are used.

Note: It is better to adjust the expression of exogenous TFEB at the same level within the cells by FACS.

21. Place sterile coverslips (12 mm in diameter) into a sterile cell culture plate (for 6-well plate, 4 to 5 coverslips/well).
22. Coat coverslips with Cellmatrix diluted by 10 times with sterile water, incubate the plate at least 30 min at 37°C.
23. Wash coverslips with PBS twice.
24. Seed cells onto collagen coated plate, incubate the cells with 5% CO_2 for 18–24 h at 37°C.

⚠ CRITICAL: The cells should be seeded to become below 70% confluent at the next day (In the case of HeLa cells, 2.0×10^5 cells/well). It is difficult to distinguish each cell in the images of high confluency by CellProfiler.

25. Induce lysosomal damage by LLOMe or beads transfection as described in step 2a or 2b.
26. Wash the cells with PBS once.
27. Fix the cells with ice cold 4% PFA, incubate the cells for 20 min at 20°C–25°C .
28. Wash the samples with PBS and ddH_2O for three times (PBS twice → ddH_2O once).
29. Mount the samples with a drop of mounting media with DAPI onto glass slide. Remove excess media and seal the coverslips with nail polish.

30. Observe the cell using widefield fluorescence microscopy, take images of the cells (images of DAPI and TFEB).

Note: More than 100 cells/sample are required for the following analysis.

31. Quantify the nucleus/cytoplasm ratio of TFEB using CellProfiler software. Troubleshooting 3
    a. Identify the nuclei from DAPI images by following modules.
        i. Smooth: Smoothing DAPI images.
        ii. IdentifyPrimaryObjects: Identify the regions of nuclei from smoothed DAPI images, name these primary objects <Nuclei>.
    b. Identify the cells from TFEB images based on <Nuclei> by following modules.
        i. Smooth: Smoothing TFEB images.
        ii. IdentifySecondaryObjects: Identify the regions of cells from smoothed TFEB images, name these secondary objects <Cells>. Also, name the primary objects which have matched secondary objects <filtered Nuclei>.
    c. Identify the cytoplasm from TFEB images by following module.
        i. IdentifyTertiaryObjects: Identify the regions of cytoplasm by subtracting the nuclei objects <filtered Nuclei> from the cell objects <Cells>, name these tertiary objects <Cytoplasm>.
    d. Measure intensity of TFEB in nuclei and cytoplasm by following module.
        i. MeasureObjectIntensity: Measure intensity of <filtered Nuclei> and <Cytoplasm>.
    e. Calculate the ratio of intensity between nuclei and cytoplasm TFEB by following module.
        i. CalculateMath: Divide the mean intensity of <filtered Nuclei> by the mean intensity of <Cytoplasm>.

Monitor of nuclear translocation of TFEB by western blotting

Timing: 4 days

This step describes observation phosphorylate state or subcellular localization of TFEB by immunoblotting.

Under normal conditions, TFEB is phosphorylated at multiple sites, including the S211 site, by mTOR and other protein kinases and is present in the cytoplasm (Puertollano et al., 2018), and TFEB is detected as a single band in immunoblotting. Upon starvation or lysosomal damage, some sites of TFEB are dephosphorylated (including the site of S211) and translocated to the nucleus, and TFEB is detected as a smear band in immunoblotting.

Sample preparation

Timing: 2 days

32. Seed cells to a sterile cell culture plate, incubate the cells with 5% CO_2 for 18–24 h at 37°C.
33. Treat the cells with LLOMe or transfection reagent coated beads.
34. Wash the cells with ice-cold PBS twice.

Note: The cells grow until 70–80% confluence.

35. Prepare sample for immunoblotting.
    a. Preparation of total cell lysate
        i. Suspend the ice-cold cells in the 200 μL (for 1 well in 6-well dish) M-PER mammalian protein extraction reagent (Thermo) containing protease and phosphatase inhibitors for total cell lysate. Incubate cell lysates for 10 min on ice.
        ii. Centrifugate the cell lysates at 15,000 × g for 10 min and collect the supernatant.

iii. Add the 5× SDS sample buffer and boiled for 5 min at 95°C.
iv. The samples are used for standard method of SDS-PAGE and western blotting.

b. Fractionation of cytosolic fraction and nuclear fraction Troubleshooting 4
i. Suspend the ice-cold cells in the 300 μL (for 1 well in 6-well dish) PBS containing 0.1% NP-40, protease and phosphatase inhibitors as lysis buffer. Incubate cell lysates for 10 min on ice.
ii. Centrifugate the cell lysates at 9,100 × g for 10 sec, and take 100 μL supernatant (cytosolic fraction).
iii. Remove supernatant and wash the pellet with the 300 μL PBS containing 0.1% NP-40.
iv. Centrifugate the cell lysates at 10,000 rpm for 10 sec and remove supernatant.
v. Add the 200 μL PBS containing 0.1% NP-40 (nuclear fraction) and mix 5× SDS sample buffer and boil for 5 min at 95°C.
vi. The fractionation samples are used for standard method of SDS-PAGE and western blotting.

SDS-PAGE of the sample of total cell lysate and fractionation

Timing: 2 days

36. Load equal amounts (about 20–30 μg of total protein) of above samples and molecular weight marker into the wells of the SDS-PAGE gel.

⚠ CRITICAL: The samples load into 8% polyacrylamide gels to separate phospho-TFEB protein.

37. The loaded gels run for 1–1.5 h at 20 mA per a mini gel.
38. Transfer the protein from the gel to the polyvinylidene fluoride membrane.
39. Incubate the membranes with blocking solution (TBST containing 1% skim milk) for 30 min at 20°C–25°C.
40. Incubate the membranes with primary antibodies diluted in blocking solution for 16–24 h at 4°C.

Note: In the immunoblotting using fractionation sample, we recommend detecting loading controls. In this protocol, we detect Lamin B (control for nuclear fraction) and GAPDH (control for cytoplasmic fraction).

41. Wash the membranes with TBST (5 min ×3).
42. Incubate the membranes with 5,000× dilutions of HRP-conjugated secondary antibodies in blocking solution for 1h at 20°C–25°C.
43. Wash the membranes with TBST (5 min ×3).
44. Detect the immunoreactive bands using luminescent regents on a ChemiDoc Touch imaging system (Bio-Rad). Troubleshooting 5

EXPECTED OUTCOMES

After LLOMe treatment, some lysosomes (Lamp1 positive puncta) are positive for Galectin-3, which represents membrane damage (Figure 2). These treatments trigger TFEB translocation from cytoplasm to nucleus (Figures 3A and 3B). Also, in the cells where Galectin3 positive endolysosomal membrane rupture occurred, TFEB translocates to nucleus (Figure 3C).

This immunoblotting protocol for total cell lysate allows detection of TFEB protein and its phosphorylation status in lysosomal damage condition. As shown in our previous report (Nakamura et al., 2020), by detecting amount of phosphorylated TFEB at serine 211, the states of TFEB

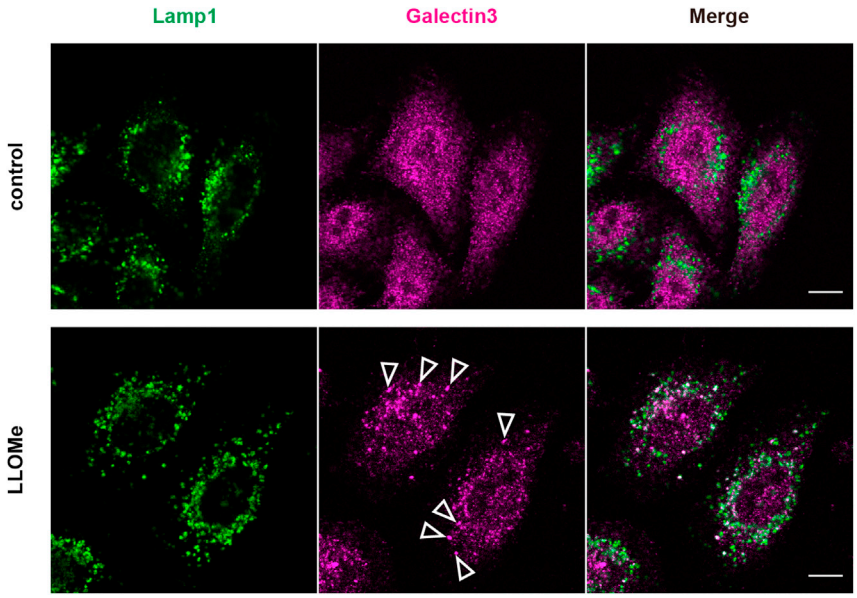

Figure 2. Galectin3 localizes on lysosomes during the lysosomal damage response

Immunofluorescence images of LAMP1 (green) and Galectin3 (magenta) localization in control and LLOMe-treated HeLa cells. Galectin3 puncta after LLOMe treatment indicate damaged lysosomes (arrowheads). Scale bars, 10 μm.

nuclear translocation can be indirectly probed. In this protocol, signal of phospho-TFEB (Ser211) markedly decrease by LLOMe treatment, and most of TFEB bands appear as a smear state and shift down compared to control cells (Figure 4A). Furthermore, the nucleus/cytoplasm fractionation strategy allows us to analyze nuclear localization of TFEB directly. In control conditions, most of TFEB signals is detected at cytosolic fraction compared to nuclear fraction. By contrast, the band size of TFEB is shifted down in response to LLOMe treatment and mainly detected in nuclear fraction (Figure 4B).

QUANTIFICATION AND STATISTICAL ANALYSIS

The quantification result of the nucleus/cytoplasm ratio of TFEB by CellProfiler is saved as .xls file. The .xls result file contains information of each identified object and the nucleus/cytoplasm ratio of TFEB calculated in CalculateMath were shown in the Math column. After calculating the average of the ratio in each condition, make graphs and determine statistical significance using GraphPad Prism.

LIMITATIONS

These protocols are designed for monitoring activation and translocation of TFEB during lysosomal damage condition in HeLa cells. Because LLOMe is highly toxic for the cells, it may induce cell death in other cell lines. Therefore, if you want to utilize these protocols in other cell lines, we recommend optimizing lysosomal damage inducing condition, especially concentration and treatment time of LLOMe. Of note, we succeeded to observe nuclear translocation of TFEB-mNeonGreen after LLOMe treatment in MEF cells (1 mM for 1 h, incubate 3 h after washout) and PTECs (proximal tubular epithelial cells) (1 mM for 1 h, incubate 1 h after washout) (Nakamura et al., 2020). Since the activation and translocation of TFEB during starvation can be observed in other cell lines including HEK293T and APRE19 cells (Napolitano et al., 2018), the current protocol should be used in various cell types with some modification.

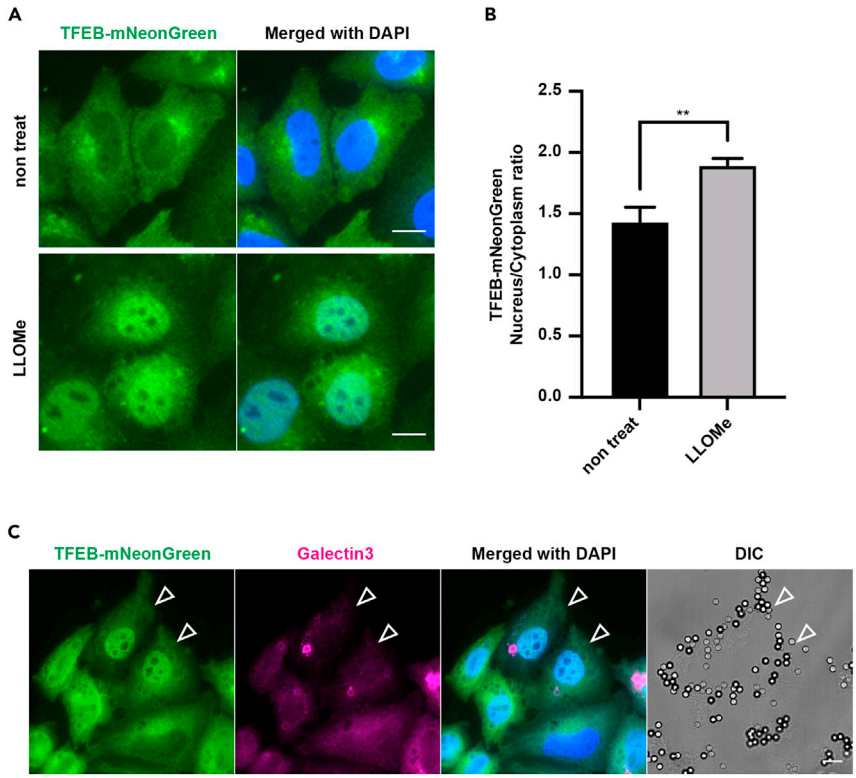

Figure 3. Monitor and analysis of TFEB nuclear translocation using microscopy

(A) Representative immunofluorescence images of exogenous TFEB-mNeonGreen in HeLa cells stained with DAPI. Control cells (top) and LLOMe treated (1 mM for 1 h, incubated 3 h after wash off) cells (bottom) are shown. Scale bars, 10 μm.

(B) The nucleus/cytoplasm ratio of TFEB-mNeonGreen in (A). Cells harboring a clear nuclear (more than 100 cells) were quantified per each condition. Values in the graph are means ± SD (n = 3). **P = 0.0037 (unpaired two-tailed t test).

(C) Representative immunofluorescence images showing TFEB-mNeonGreen (green) and Galectin3 (magenta) counterstained by DAPI in beads transfected cells. Arrowheads show that transfected beads were internalized in cells and caused endolysosomal membrane rapture. Scale bars, 10 μm.

TROUBLESHOOTING

Problem 1

Cells die after LLOMe treatment (step 2).

Potential solution

Lower the concentration of LLOMe. We confirm that Galectin3 puncta were formed after 0.2 mM LLOMe treatment for 1 h. Alternatively, shorten the time of LLOMe treatment to 30 min.

Problem 2

Low signal-to-noise ratio of Galectin3 (step 20).

Potential solution

It would be helpful for enhancement of signal-to-noise ratio to modify the concentration of first and second antibodies. As alternative method, we used GFP-Galectin3 stably expressing HeLa cells to monitor damaged lysosomes. In that cell, strong signal of GFP-Galectin3 positive puncta could be seen after LLOMe treatment (Maejima et al., 2013).

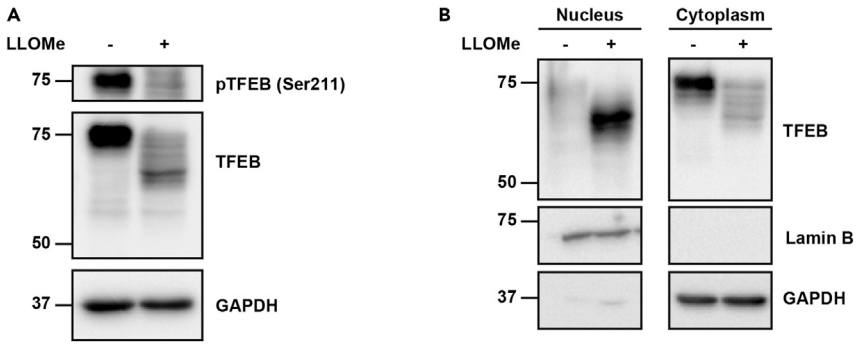

Figure 4. Monitor of nuclear translocation of TFEB by western blotting

(A) Representative immunoblots showing the levels of TFEB and phospho-TFEB (Ser211) in control and LLOMe treatment cells.

(B) The nuclear and cytosol fractions from control and LLOMe treatment cells were subjected to immunoblotting to detect TFEB, Lamin B1 (nucleus) and GAPDH (cytoplasm). Representative images are shown.

Problem 3

Cells or nuclei are not recognized correctly in identification steps of CellProfiler (step 31).

Potential solution

It is very important to prepare images suitable for analysis. Especially, following points are crucial for proper analysis.

The signal-to-noise ratio of the image should be high. If the ratio is low, cells or nuclei are not recognized, or multiple cells or nuclei in proximity to each other are recognized as one object.

The confluency of the cells should be below 70%. In the image of high confluency, it is difficult to distinguish each cell.

It is also effective to adjust the parameters in the identification steps (step 31-a-ii and 31-b-ii). Before running the modified pipeline, make sure that it processes images as you needed by test mode.

Problem 4

Contamination of cytosolic fraction into nuclear fraction (step 35-b).

Potential solution

Make sure that the supernatant is completely removed before adding SDS sample buffer into the pellet. In addition, it is effective for reducing contamination to repeat the washing steps (step 45-b-iii), though it is accompanied by loss of the pellet.

Problem 5

Weak signal of phospho-TFEB (step 44).

Potential solution

Use highly sensitive luminescent regents. It is difficult to detect the band of phospho-TFEB with normal luminescent reagents.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Shuhei Nakamura (shuhei.nakamura@fbs.osaka-u.ac.jp).

Materials availability

All unique reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

Investigation, M.O. and S.N.; Writing – Original Draft, T.S. and M.O.; Writing – Review & Editing, T.Y. and S.N.; Supervision, S.N.

DECLARATION OF INTERESTS

T.Y. is founder for AutoPhagyGO.

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