FACS analysis of GFP-LC3 degradation

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METHOD

Induction of autophagy in GFP-LC3 expressing cells

1. Prepare cells stably expressing GFP-LC3.NOTE GFP-p62 or GFP-NBR1 can be used instead of GFP-LC3 (REF). In addition, degradation of these reporters can be more sensitively detected after blocking their synthesis using the Tet-regulated system (Larsen et al. 2010).

2. Seed the cells in 35 mm dishes in DMEM supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine, and maintain the cells at 37°C with 5% CO_2, and maintain the cells at 37°C with 5% CO_2.

3. Wash the cells twice with PBS and culture them in the regular medium or starvation medium (amino acid-free DMEM (Wako 048-33575) or EBSS) for 6-12 h at 37°C.

NOTE To ensure that reduction of the GFP fluorescence is due to autophagic degradation, it is recommended to include samples treated with autophagy inhibitors such as wortmannin and bafilomycin A_1.

Sampling

(steps 4-7 are options for adherent cells)

4. Wash the cells with PBS and add 250 µl of 0.05 % trypsin.

5. Incubate cells at 37°C for 5 min.

NOTE The incubation time should be identical among samples, because prolonged trypsinization may cause loss of fluorescence.

6. Place the culture dishes on ice.

7. Add 500 µl of ice cold PBS and transfer the cells into a 1.5-ml tube.

8. Cetrifuge the cells (2,300 x *g*, 2 min, 4°C) and remove the supernatant with an aspirator

9. Reuspend the cells in 400 µl of ice cold PBS.

NOTE The GFP fluorescence is stable for at least 2 h if the samples are kept on ice.

Data acquisition and analysis

10. Format a bivariate histogram correlating forward scatter (FS) and side scatter (SS) using linear scales and draw a region to exclude cell debris (low FS and SS).

11. Run cell samples. (Acquire at least 10000 events of each sample.)

12. Plot the cell number and the distribution of GFP intensity in a histogram format using Kaluza software

MATERIALS

REAGENTS

- Cells (e.g. MEF, HeLa, HEK293T) stably expressing GFP-LC3
- Dulbecco's modified eagle's medium - high glucose (Sigma, D6546)
- Fetal bovine serum (Equitech-Bio, Inc), heat inactivated (56 ˚C, 30 min)
- Penicillin-Streptomycin (5,000 U/mL) (Life technologies, 15070-063)
- L-glutamine (200 mM) (Life technologies, 25030-081)
- 10× DPBS (Life technologies, 14200-075, use after dilution)
- Hyclone™ Earle's balanced salt solution (EBSS; GE Healthcare Hyclone, SH3002902)

amino acid-free DMEM (Wako 048-33575)

EQUIPMENT

- 35 mm dish (Falcon, 353001)
- CO_2 incubator (Panasonic, MCO-175-PJ)
- EC800 cell analyzer (Sony) equipped with 488 nm laser and 525/50-nm bandpass (BP) filter
- Kaluza Flow Analysis Software (Beckman Coulter)

TROUBLESHOOTING TIPS

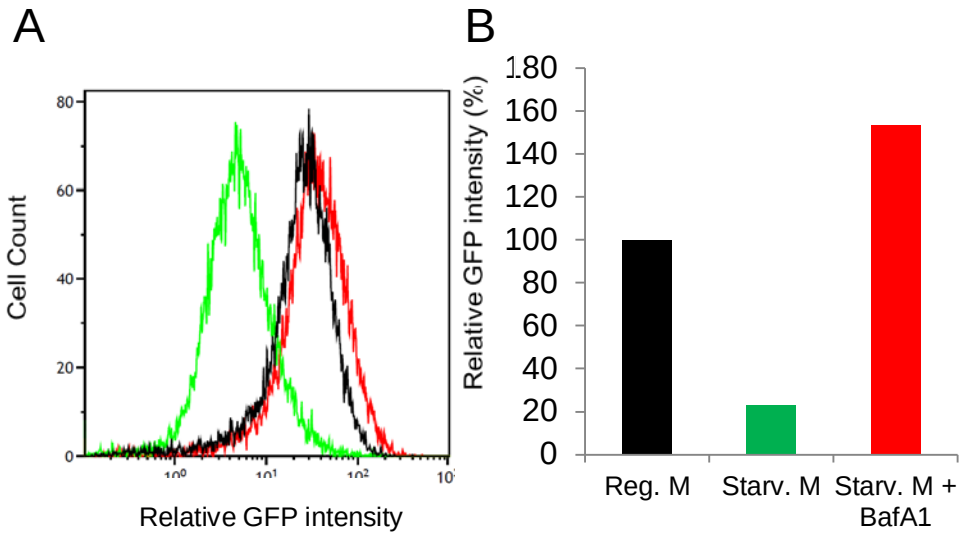
1. Starvation-induced reduction of GFP-LC3 may not be clearly detected if the GFP-LC3 expression level is too high. It is recommended to use clones with low expression levels of GFP-LC3.

2. The GFP fluorescence may be lost due to plasma membrane damage during sample preparation, particularly trypsinization of cells. Treating cells

cells are put on ice immediately after trypsinization.

REFERENCE

Larsen et al. Autophagy. 2010, 6:784-93.



(A) Mouse embryonic fibroblasts stably expressing GFP–LC3 were cultured in regular medium (DMEM supplemented with 10 % FBS) (black), starvation medium (amino acid free DMEM) (green) or starvation medium in the presence of 100 nM BafA1(red) for 12 h. Total cellular GFP–LC3 signals were analyzed by flow cytometry. (B) The geometric mean of fluorescence intensity was determined. Values indicate percentage of the mean of cells cultured in regular medium.