mRFP-GFP tandem fluorescent-tagged LC3 (tfLC3)

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METHOD

1. Coat coverslips with 0.1 mg/ml collagen (Nitta gelatin, Cell matrix Type I-C) for at least 10 min and subsequently wash with PBS once.
2. Seed cells (e.g. HeLa-Kyoto cells) stably expressing mRFP-GFP tandem fluorescent-tagged LC3 (tfLC3) on sterilize coverslips in 24-well plate in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Incubate the cells at 37°C in CO_2 incubator for at least 24 h.

NOTE The cells should be seeded to become 60%–80% confluent at the time of the experiment.

NOTE Refer to the paper (Autophagy, 3. 452-460, 2007) for more detail on tfLC3.

3. For starvation treatment, discard the medium and wash the cells with PBS prewarmed at 37°C once, then culture the cells with Earle's Balanced Salt Solutions (EBSS) prewarmed at 37°C for 1-4 h depending on the cell types.

NOTE We usually perform EBSS treatment for 2 h in MEF cells, for 4 h in HeLa-Kyoto cells.

4. Wash the cells with PBS once, and fix them in 4% PFA/PBS for 20 min at room temperature. Wash the cells with PBS twice.

NOTE Since cells attachment onto the bottom becomes much weaker after starvation treatment, all steps should be gently done.

5. Wash the coverslip with distilled water once and remove extra water. Mount the samples on slide glass with 5 µl prolong gold, a mounting reagent. Seal them with manicure.
6. Image the tfLC3 by microscopy
7. Count the number of GFP/RFP LC3 puncta per cell and/or measure intensity of them per cell. These measurements can be quantified by computer softwares such as G-count and CellProfiler.

MATERIALS

• ptfLC3 plasmid (addgene plasmid, ID 21074)

• Fetal bovine serum (FBS) (Gibco), heat inactivated (56 ˚C, 45 min)
• Dulbecco's modified eagle's medium (DMEM) (Sigma, D6429)
• Earle's Balanced Salt Solutions (EBSS) (Sigma, E2888)
• 4% Paraformaldehyde (Nacalai, 09154-85)
• ProLong Gold (Life technologies, P36930)
• Manicure
• Sodium chloride (Wako, 191-01665)
• Potassium chloride (Wako, 163-03545)
• Disodium hydrogenphosphate (Wako, 197-02865)
• Potassium dihydrogenphosphate (Wako, 169-04245)

EQUIPMENT

• Micro cover glass (Matsunami, 12 mm, ◯, 0.12 - 0.17 mm), sterilized by autoclave (121 ˚C, 30 min)
• Confocal laser microscope (Olympus, FV1000 IX81), equipped with a 60× PlanApoN oil immersion lens (Olympus, 1.42 NA), and FV12-HSD.
• CO_2 incubator (Thermo, HERACELL 150i)

Reagent Preparation

PBS

For 10 × PBS stock solutions, dissolve 400 g of sodium chloride, 10 g of potassium chloride, 72 g of disodium hydrogenphosphate, 12 g of potassium dihydrogenphosphate in 5 L of distilled water. Dilute 10 × PBS 1:10 with distilled water. This reagent can be stored at room temperature.

Trouble shooting

1. No response to starvation treatment

Make sure that PBS wash can completely remove culture medium away, or EBSS treatment time should be optimized.

2. Abnormal green-dots accumulation even in nutrient-rich condition

We use HeLa-Kyoto cells stably expressing tfLC3 for this assay, and have experienced above problem before. We recommend that high frequency of

passage (within 24 h after cell splitting) should be avoided. When Cell Matrix is incompatible with cells, such a problem may happen. In a case of HeLa-Kyoto cells, we use collagen (Nitta gelatin, Cell matrix Type I-C) for coating glass.

3. Colocalization between mRFP and GFP is not visualized clearly

Confocal microscopy is the best choice to visualize the colocalization between mRFP and GFP. Since tfLC3 expression level is relatively low, some settings (e.g., lens, high sensitive detector) to catch more signals may be needed for better visualization.

4. Only mRFP dots already accumulate, that could influence measurements you want.

As long as you use the cell lines stably expressing tfLC3, the constant accumulation of mRFP is inevitable. To avoid this, transient expression approaches such as plasmid transfection, Tet-on/off system and Adenoviral expression system are widely used.

5. The tfLC3 assay for high throughput screening

tfLC3 can be used for monitoring autophagic flux by counting the number of GFP/RFP LC3 puncta per cell, or measuring total intensity of them per cell; the latter can be considered as useful way for high throughput screening because measuring total intensity is easy, fast and reproducible way, and is less likely to be hardly affected by various factors (e.g. variation in the number of LC3 dots between cells, quality of dot-segmentation, confluency of cells).