Lysophagy analysis

Ryo Iwamoto, Junya Hasegawa, Tamotsu Yoshimori

METHOD

Induction of Lysophagy

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 cells) on sterilize coverslips in 24-well plate in DMEM.
3. Incubate HeLa cells with 1000 µM LLOMe dissolved in DMEM for 15 min.

NOTE Prepare the LLOMe solution just prior to use.

NOTE Responsiveness to LLOMe and recovery after washout varied among cell types. The concentration of LLOMe (100-1000 µM) and incubation time (15-60 min) should be examined depending on cell lines.

4. Wash the cells twice with DMEM and culture them at 37°C.

NOTE Since some cell lines (e.g. MEF cells) are easily detached after LLOMe treatment, the cells should be washed gently.

Immunostaining

5. Wash the cells with PBS, and fix them in 4% PFA/PBS for 15 min at room temperature. Wash the cells with PBS twice.
6. Permeabilize the cells with 50 µg/ml digitonin in 0.1% gelatin in PBS for 10 min.
7. Wash the cells with PBS twice and incubate them with 0.1% gelatin in PBS for 30 min at room temperature.
8. Incubate the samples with 30 µl of rat monoclonal anti-galectin-3 antibody diluted 1:1000 in 0.1% gelatin in PBS for 1hr at room temperature. Wash the samples three times with PBS.
9. Incubate the samples with secondary antibody (e.g. Alexa Fluor® 488 Goat Anti-Rat IgG (H+L) Antibody) diluted 1:1000 and DAPI in 0.1% gelatin in PBS for 40 min at room temperature. Wash the samples three times with PBS.
10. 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.

11. Observe the galectin-3 staining by microscopy.
12. Count the number of galectin-3 dots per cell. Analyze the images by G-count or Cell Profiler.

Method (observing GFP-galectin-3 dots)

Endogenous galectin-3 dots staining may show less signal-noise ratio, resulting in an inaccurate dot counting. To overcome this obstacle, exogenous expression of GFP-galectin-3 is useful. Using cells stably expressing GFP-galectin-3, a more precise dot counting might be achieved.

MATERIALS

REAGENTS

• LLOMe (Leu-Leu methyl ester hydrobromide) (Sigma, L7393)
• Rat monoclonal anti-galectin3 antibody (Santacruz, sc-23938)
• Alexa Fluor® 488 Goat Anti-Rat IgG (H+L) Antibody (Life technologies, A-11006)
• Fetal bovine serum (FBS) (Gibco), heat inactivated (56 ˚C, 45 min)
• Dulbecco's modified eagle's medium (DMEM) (Sigma, D6429)
• Penicillin-Streptomycin (Sigma, P4333)
• Sodium chloride (Wako, 191-01665)
• Potassium chloride (Wako, 163-03545)
• Disodium hydrogenphosphate (Wako, 197-02865)
• Potassium dihydrogenphosphate (Wako, 169-04245)
• ProLong Gold (Life technologies, P36930)
• 4% Paraformaldehyde (Nacalai, 09154-85)
• Gelatin (Wako, 043-21371)
• Digitonin (Wako, 043-21371)
• Dimethyl sulfoxide (Wako, 045-24511)
• Manicure

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)
• CO_2 incubator (Thermo, HERACELL 150i)

REAGENT PREPARATION

333 μM LLOMe solution

A suggested stock solution is 333μM LLOMe dissolved in ethanol and solution should be firmly sealed and stored at -20°C. Dissolve 1.13 g of LLOMe in 10 ml Ethanol. Agitate the solution and make crystals completely dissolved. Since frequent use the solution in the same tubes would lead to inactivation of LLOMe solution, dividing into small aliquots is highly recommended.

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.

0.1% Gelatin-PBS

Add 0.2 g of gelatin in 200 ml of PBS. Subsequently this solution should be autoclaved (121°C, 30min) to make gelatin completely dissolved. This reagent should be stored at 4°C.

50 μg/ml digitonin in gelatin-PBS

For 50 mg/ml stock solution, dissolve 500 mg of digitonin in 10 ml of dimethylsulfoxide. This reagent can be stored at –20°C. For 1× solutions, dilute 50 mg/ml stock solutions 1:1000 with gelatin-PBS. This solution should be prepared just before use.

TROUBLESHOOTING TIPS

1. Cell shrinkage after LLOMe treatment

Some cell lines show less tolerance to LLOMe treatment. In this case, cell shrinkage during LLOMe treatment might be recovered several hours after

washout.

2. Cell detachment after LLOMe treatment
   Some type of cells are easily detached after LLOMe treatment, In this case, decreasing the concentration of LLOMe solution might work. Or it might be worth trying collagen coat in another way. Coat coverslips with 0.1 mg/ml collagen (Nitta gelatin, Cell matrix Type I-C) for at least 10 min and subsequently discard the solution and make the coverslip and the dish bottom completely dried in a clean bench. After washing the dish with PBS in prior to the use, seed the cells on that.

3. No lysosomal rupture triggered by LLOMe treatment
   LLOMe solution might be inactivated. It would be better to prepare fresh LLOMe solution.

4. No galectin-3 dots recovery can be seen.
   Too much concentration of LLOMe solution or too long incubation of LLOMe treatment might be the cause. A condition of LLOMe treatment should be optimized.