LC3 immunostaining

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METHOD

Induction of autophagy

1. Seed cells on sterile coverslips in 24-well plates in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS and 50 U ml^{-1} penicillin-streptomycin and 2 mM L-glutamine, and maintain the cells at 37°C with 5% CO_2.
2. Wash the cells twice with PBS and culture them in the regular medium or EBSS for 1 h at 37°C.

Immunostaining

3. Wash the cells with PBS, and fix them in 4% (w/vol) paraformaldehyde/PBS for 10 min at room temperature.

NOTE Fixed cells can be stored at 4°C for at least 1 week.

4. Wash the cells three times with PBS and permeabilze them with 50 µg/ml digitonin/PBS for 5 min at room temperature.

NOTE Do not exceed the 5 min incubation, as longer treatments might influence visualization of LC3 signals.

5. Wash the cells three times with PBS and incubate them with 3% (w/vol) BSA/PBS for 30 min at room temperature.
6. Pick up the cover glasses and place them on a humidified chamber (A simple chamber can be made from a 10 cm dish with water-soaked filter papers covered with parafilm). Spot 50 µl of LC3 antibody solution (1:200) in 3% BSA/PBS onto each cover glass. Incubate for 1 h at room temperature.

NOTE No primary antibody sample should be prepared as a negative control.

7. Pick up the cover glasses and place in 24-well plates. Wash the cells five times with PBS and incubate them with 200 µl of secondary antibody solution (1:2000) in 3% BSA/PBS for 1 h in the dark at room temperature.
8. Wash the cells five times with PBS.
9. Rinse the slides briefly in distilled water, remove the majority of liquid and mount them on slide glass in a small droplet (~3 µl) of SlowFade® Gold antifade mountant. Remove extra mountant and seal with manicure.

10. Dry the slides for 1 h at room temperature and store them at 4 ˚C.

NOTE Mounted samples can be stored at 4°C for at least 1 week.

11. Image the slides with the confocal laser microscope, using the appropriate excitation and emission filters.

MATERIALS

REAGENTS

• Mouse embryonic fibroblasts (MEF)
• Bovine serum albumin fraction V (FBS; Roche, 10735094001)
• SlowFade® Gold antifade mountant (Life technologies, S36936)
• Dulbecco's modified eagle's medium - high glucose (Sigma, D6546)
• Sodium chloride (Wako, 197-01667)
• Disodium hydrogenphosphate 12-water (Wako, 196-02835)
• Potassium chloride (Wako, 163-03545)
• Potassium dihydrogen phosphate (Wako, 169-04245)
• Trypsin-EDTA (0.05%), phenol red (Life technologies, 25300-062)
• 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)
• Paraformaldehyde (Wako, 162-16065)
• Sodium hydroxide (Wako, 197-02125)
• Digitonin (Wako, 043-21376)
• Dimethyl sulfoxide (Wako, 045-24511)
• Anti-LC3 (Clone: LC3-1703) monoclonal antibody (Cosmo bio, CTB-LC3-2-IC)
• Alexa Fluor® 488 goat anti-mouse IgG (H+L), highly cross-adsorbed (Life technologies, A-11029)
• Manicure
• Hyclone™ Earle's balanced salt solution (EBSS; GE Healthcare Hyclone, SH3002902)

EQUIPMENT

• Micro cover glass (Matsunami, 12 mm, ◯, 0.12 - 0.17 mm), sterilized by autoclave (121 ˚C, 30 min)
• Micro slide glass (Matsunami, S2441)
• Multiwell™ 24 well (Falcon, 353047)
• Liquid aspirator setup (ULVAC, DAP-15)
• Confocal laser microscope (Olympus, FV1000D IX81), equipped with a 60× PlanApoN oil immersion lens (Olympus, 1.42 NA)
• CO_2 incubator (Panasonic, MCO-175-PJ)

REAGENT PREPARATION

PBS for cell culture

Dilute 10× DPBS 1:10 with distilled water and autoclave (121 ˚C, 30 min). This reagent can be stored at room temperature.

PBS for immunostaining

For 25× PBS stock solutions, dissolve 2000 g of sodium chloride, 725 g of disodium hydrogenphosphate 12-water, 50 g of potassium chloride and 50 g of potassium dihydrogen phosphate in 10 L of distilled water. Dilute 25× DPBS 1:25 with distilled water. This reagent can be stored at room temperature.

10N NaOH

Dissolve 8 g of sodium hydroxide in 20 ml of distilled water. This reagent can be stored at room temperature.

4% (w/vol) paraformaldehyde/PBS

For 5× stock solutions, dissolve 200 g of paraformaldehyde in 600 ml of distilled water and add 4 ml of 10N NaOH. The solution need to be carefully heated (use a stirring hot-plate in a fume hood) to dissolve. Adjust the volume to 1000 ml with distilled water. This reagent can be stored at –30°C. Aliquot to avoid repeated freeze and thaw. For 1× working solutions, thaw 5× stock solutions by heating at 65°C and dilute 1:5 with PBS. This reagent can be stored at 4°C for at least a week.

50 µg/ml (w/vol) digitonin/PBS

For 50 mg/ml stock solutions, dissolve 500 mg of digitonin in 10 ml of dimethyl sulfoxide. This reagent can be stored at –30°C. Aliquot to avoid repeated freeze and thaw. For 1× working solutions, dilute 50 mg/ml stock solutions 1:1000 with PBS. Freshly prepare this reagent before use.

3% BSA/PBS

Dissolve 0.3 g of bovine serum albumin in 10 ml of PBS. Freshly prepare this reagent before use.

TROUBLESHOOTING TIPS

1. No induction of autophagy.

   PBS wash might not be properly performed. Make sure that culture medium are completely washed away. If floating cell lines are used, the mTOR inhibitor Torin1 treatment is better instead of serum/amino acid starvation.

2. No LC3 signals can be detected.

   Autophagosome structures might be disrupted in a permeabilization step. Do not exceed 5 min incubation of digitonin, as longer treatments might influence visualization of LC3 signals. Note that TritonX-100 treatment will influence LC3 immunostaining, though low concentration of TritonX-100 treatment might be O.K.

3. Non-uniform LC3 staining.

   Membrane permeabilization might not be sufficient. Replace digitonin batch a new one.

4. No increase of LC3 puncta by an autophagy induction.

   The number of LC3 puncta at steady state is reflected not only by autophagosome formation, but also by autophagosome degradation. Therefore, in some cell line, an increase of LC3 puncta is not clear even after an autophagy induction. If cells are treated with the lysosomal inhibitor bafilomycin A_1, an increase of LC3 puncta should be more clearly observed.

5. It is hard to take a beautiful image of LC3 puncta.

   Confocal optical sections might be too thin to take enough LC3 signals. If so, pinhole size should be increased. Note that, however, the signal to noise ratio would increase according to increasing pinhole size. Instead of the confocal laser microscope, a conventional light microscope is also useful.