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Analyzing autophagy in zebrafish

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Abstract

The transparency, external development and simple drug administration of zebrafish embryos makes them a useful model for studying autophagy during embryonic development in vivo. Cloning of zebrafish *lc3* and generation of a transgenic GFP-Lc3 fish line provide excellent tools to monitor autophagy in this organism.[1] This protocol discusses several convenient autophagy assays in zebrafish, including immunoblotting of Lc3 lipidation, microscopy imaging of GFP-Lc3 and lysosomal staining.

Keywords

lysosome; protein degradation; protein targeting; stress; vacuole

1. Lc3 Immunoblotting

Upon autophagy induction, cytosolic microtubule-associated protein 1 light chain 3 (LC3, the mammalian homolog of Atg8) is conjugated to PE (phosphatidylethanolamine) and incorporated into autophagosomal membranes.[2] The two forms of LC3, i.e., cytosolic (LC3-I) versus membrane-associated (LC3-II), migrate at different rates during SDS-PAGE. In a similar manner, zebrafish Lc3, which is highly conserved and similar to mammalian LC3, is converted to the membrane-conjugated form during autophagy. Thus, western blot of Lc3-II conversion can be used as an indication of autophagy induction in zebrafish.

1.1 Materials

1.1.1 PBS (phosphate buffered saline): 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3.

1.1.2 PMSF (phenylmethylsulphonyl fluoride): make 100 mM (100x) stock solution in isopropanol and store at −20°C in aliquots.

1.1.3 Tricaine (ethyl 3-aminobenzoate, an anesthetic): make 30x tricaine stock by dissolving 200 mg tricaine powder (Sigma, A5040) in 1 ml of 1 M Tris buffer (pH 9) and adjusting pH to 7 with NaOH. Add double distilled (dd) H_2O to a total volume of 50 ml. Store the stock at −20°C.[3]

1.1.4 1x SDS-PAGE sample buffer: 2% SDS, 8.7% glycerol, 80 mM Tris-HCl pH 6.8, bromophenol blue powder, and freshly added 2.5% β-mercaptoethanol.

1.1.5 Dumont #5 tweezers (World Precision Instruments, 500342).

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1.1.6 Glass pipettes (Fisher Sci., 13-678-30) with drawn-out tips. The tips are drawn out after heating the pipette in a flame, and pulling them with a forceps. Break the drawn-out pipette at the appropriate position to generate a desired opening with a similar diameter as the yolk.

1.1.7 Kontes disposable pellet pestles (Fisher Sci., K749521-1500).

1.2. Methods.[3]

1.2.1 Zebrafish embryos are raised in a 28.5°C incubator. Transfer ~20 embryos into a culture dish with fish water. Under a dissection microscope, remove chorions of the embryos using #5 tweezers. Hold the embryo with one tweezer and use a second one to remove the chorion.

1.2.2 Transfer dechorionated embryos into ice-cold PBS containing freshly added 1 mM PMSF and 1x tricaine for sedation. Remove yolk by passing the embryo several times through a glass pipette with the tip drawn out to a similar size as the yolk.

1.2.3 Pipette embryos in a 1.7 ml microcentrifuge tube and rinse twice with fresh cold PBS containing PMSF. Note that it is not necessary to centrifuge during the washing procedure because the embryos rapidly settle to the bottom of the tube.

1.2.4 Centrifuge at 3,000 rpm for 5 s and remove as much liquid as possible. Do not centrifuge at a high speed as this may damage the embryos.

1.2.5 Add 50 μl SDS-PAGE sample buffer for 1 d embryos (100 μl for 3 d embryos), and homogenize with pestles for 10 s. Sonicate for approximately 1 to 2 min until the lysate is not viscous.

1.2.6 Immediately boil at 95°C for 5 min.

1.2.7 Spin in a microcentrifuge at the top speed for 1~2 min and transfer the supernatant fraction into a new tube; discard the pellet fraction. Embryo lysates can be stored until needed at −20°C or −80°C, or can be processed immediately.

1.2.8 Load 15 μl lysate on 15% SDS-PAGE gels and probe by western blot with anti-LC3 (Novus Biologicals, NB100-2331) or anti-tubulin (Sigma, T6793) antibodies.

1.2.9 Membrane-associated Lc3 (Lc3-II) migrates faster than the cytosolic form (Lc3-I), at 14 kD and 16 kD, respectively. Tubulin can be used as a loading control.

2. GFP-Lc3 Microscopy

Recruitment of Lc3 to autophagosomes can also be analyzed by microscopy. Zebrafish embryos are transparent and can be directly observed live through confocal fluorescence microscopy. In transgenic embryos expressing GFP-tagged Lc3 under normal conditions the GFP signal is largely cytosolic, whereas after autophagy induction, GFP-Lc3 displays punctate localization. Therefore counting GFP-Lc3 puncta per fixed area represents a near-quantitative measure of autophagic activity.

2.1. Materials

2.1.1 GFP-Lc3 transgenic zebrafish.[1]

2.1.2 PTU (1-phenyl-2-thiourea; Sigma, P7629): make 10x stock solution by dissolving 30 mg PTU in 100 ml ddH_2O [0.03% (w/v)]. Avoid light exposure by wrapping with aluminum foil. Store at room temperature.[3] This solution is stable for at least four months.

2.1.3 Tricaine (see 1.1.3).

2.1.4 Stock solutions

Autophagy-inducing drugs (store at −20°C):

Rapamycin: 1 mg/ml in DMSO.

Calpeptin (Biomol, Pl101): 2.5 mg/ml in DMSO.

2′5′-dideoxyadenosine (2′5′-ddA; Biomol, CN110-0005): 2.5 mg/ml in DMSO.

Clonidine (Sigma, C7897): 1.5 mM (500x) stock in DMSO.

Verapamil (Sigma, V4629): 1.5 mM (500x) stock in DMSO.

Lysosomal protease inhibitors (store at −20°C):

Pepstatin A (Sigma, P5318): 1 mg/ml in DMSO.

E-64d (Sigma, E8640): 2 mg/ml in DMSO.

3-methyladenine (3-MA; Sigma, M9281): 0.33 M stock in ddH_2O (33x). The stock solidifies at room temperature. Immediately before use, heat to 70°C for 3 min to dissolve.

2.1.5 24-well plates for drug treatment (Fisher Sci., 08-772-1H).

2.1.6 Plastic disposable droppers.

2.2. Methods

2.2.1 To prevent pigmentation, transfer embryos into fish water containing 1x PTU prior to 24 h post-fertilization and thereafter.

2.2.2 With lysosomal protease inhibitors, chemicals can be used to induce or inhibit autophagy. Add chemicals to the embryo water at a working concentration indicated in parentheses and incubate at 28.5°C:[1,4] rapamycin (1 μM), calpeptin (50 μM), 2′5′-ddA (100 μM), verapamil (3 μM or higher), clonidine (3 μM or higher), pepstatin A (10 μg/ml), E64d (5 μg/ml), and 3-MA (10 mM).

2.2.3 After chemical treatment for 24 h and 48 h, mount embryos live in fish water containing 1x tricaine in dishes. Capture images using a confocal fluorescence microscope under a 40x water immersion lens and count the number of GFP-Lc3 puncta per unit area.

2.2.4 Autophagy can be induced efficiently in zebrafish embryos by 24 h-calpeptin or rapamycin treatment. If longer treatment time is needed, change the water every 24 h and add fresh chemicals. Embryos treated with only DMSO can be used as a negative control, in which the volume of DMSO added should be the same as that of chemicals applied to the testing group.

3. LysoTracker Staining[1]

Autophagosomes are transported to, and degraded after fusion with, lysosomes. During the maturation process, autophagosomes fuse with endo/lysosomal compartments and gradually become acidic. Thus, increased acidification of autophagosomes, triggered by autophagy-inducing conditions, can be assessed by cell-permeable acidotropic dyes, such as LysoTracker Red.[5] Fluorescent signals from acidic lysosomes and autolysosomes, and their colocalization with GFP-Lc3 puncta, can be used to investigate upregulation of autophagy.

LysoTracker dyes are used in studies of retinal degeneration in embryos of blind cavefish,[6] and synaptic vesicle acidification in purified retinal bipolar cells from goldfish,[7] at a concentration of 50 μM for 45–60 min and 100 nM for 10 min, respectively. However, LysoTracker dyes are only reported in one publication in the zebrafish system.[1] In this protocol, we optimized the concentration and duration of LysoTracker Red usage for zebrafish embryos where the dye can be directly applied to the water.

3.1. Materials

3.1.1 LysoTracker Red DND-99 (Invitrogen, L7528): 1 mM (100x) stock in DMSO. Store at −20°C.

3.1.2 PTU (see 2.1.2).

3.1.3 Tricaine (see 1.1.3).

3.1.4 48-well plates (Fisher Sci., 08-772-1C).

3.1.5 Plastic disposable droppers.

3.3. Methods

3.2.1 To prevent pigmentation, transfer embryos into fish water containing 1x PTU prior to 24 h post-fertilization and retain in PTU thereafter.

3.2.2 Embryos are transferred into 48-well plates. Add LysoTracker Red to a final concentration of 10 μM.

3.2.3 Mix the embryo medium by gently pipetting up and down using plastic droppers.

3.2.4 Incubate the 48-well plates in the 28.5°C incubator 1 h for 4 d embryos (and 45 min for 1–3 d embryos).

3.2.5 Remove the water and rinse the embryos with approximately 1 ml fresh fish water three times immediately before fluorescence microscopy imaging. Centrifugation is not needed during the rinse procedure.

3.2.6 Lysosomes show punctate or patch staining using the RFP filter set, and the number of LysoTracker-positive signals per unit area should be counted. The negative control should be embryos treated with 1% DMSO only, to determine the specificity of LysoTracker Red signals.

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