Fluorescence Microscopy Analysis of GFP-Atg8 Translocation into the Vacuole and Observation of Autophagic Bodies

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METHOD

Cultivation of yeast cells and FM4-64 staining

1. Prepare yeast cells expressing GFP-Atg8.

NOTE For expression of GFP-Atg8 by the *ATG8* own promoter, we usually use a plasmid pRS316 GFP–Atg8 (Suzuki et al. (2001) EMBO J). Alternatively, we use yeast cells genomically expressing GFP–Atg8 (integrated in the *ATG8* locus or *LEU2* locus).

2. Inoculate 0.5-2.5 µl of preculture into 5 ml of liquid medium (SD/CA or SD/DO medium) in a test tube.

NOTE We recommend SD/CA or SD/DO medium, because YPD medium exhibits much autofluorescence.

3. Cultivate overnight at 30°C by using a rotator RT-50 at 50 rpm.

NOTE A doubling time of yeast cells is 90–120 min.

4. Measure OD_{600} of the yeast culture by diluting 100 µl of the yeast culture into 900 µl of water.

NOTE A yeast culture containing 1×10^7 cells/ml gives the OD_{600} of ~1.0.

5. Add 16 µM FM4-64 (add 1/2,000 volume of 3.2 mM FM4-64) into the culture.

6. Cultivate for 15 min, wash two times with a fresh medium, and then chased for 30 min at 30°C using a rotator.

NOTE Cells should be stained before induction of autophagy because FM4-64 is hardly internalized during autophagy.

7. Autophagy is induced by starvation or rapamycin treatment (final 0.2 µg/ml rapamycin; add 1/5,000 volume of 1 mg/ml rapamycin into the cell culture).

NOTE To induce autophagy efficiently, the OD_{600} should be over 1.0.

Fluorescence microscopy of GFP-Atg8 translocation into the vacuole

1. Transfer 1 ml of the cell culture (OD_{600}=1.2–1.8) to an eppendorf tube and centrifuge at 3,000 × *g* for 30 sec at room temperature.

2. Remove the supernatant (30–50 µl of the supernatant is left in the tube).

3. Resuspend the cells into the rest of the supernatant.
4. Spot 2 µl of the suspension onto a micro slide glass (24×32 mm) and cover with a micro cover glass (18×18 mm).
NOTE Squeeze out air bubbles.
5. Set the preparation onto a fluorescence microscope with immersion oil.
6. Observe fluorescence using GFP and mCherry channels simultaneously.
7. Images are acquired by AQUACOSMOS software (Hamamatsu Photonics) or MetaMorph software (Molecular Devices).
NOTE Images should be acquired with 500–1,000 msec exposure.
NOTE If the protein of interest is highly mobile, images should be acquired with 16–32 msec exposure.

Fluorescence microscopy of autophagic bodies containing GFP-Atg8
NOTE If you use *pep4*Δ cells or you treated cells with 1 mM PMSF (add 1/1,000 volume of 1 M PMSF into the cell culture), you can observe autophagic bodies containing GFP–Atg8 in the vacuolar lumen.
NOTE Images should be acquired with 16–32 msec exposure, because autophagic bodies are highly mobile in the vacuolar lumen.

MATERIALS

REAGENTS

- FM4-64 (Life technologies, T-3166)
- Immersion oil (Olympus, TYPE-F IMMOIL-F30CC)
- Rapamycin (funakoshi, LC Laboratories, R-5000)
- PMSF (phenylmethylsulfonyl fluoride) (SIGMA, P7626)

EQUIPMENT

- Rotator RT-50 (TAITEC)
- Centrifuge MX-100 (TOMY)
- Micro slide glass (Matsunami, 24×32 mm, 0.16-0.19 mm, No.1-S)
- Micro cover glass (Matsunami, 18×18 mm)
- Laser microscope (Olympus, IX81)

Inverted microscope (Olympus, IX81) is equipped with a 150× TIRF objective

(Olympus, UAPON 150XOTIRF, 1.45 NA) and an electron-multiplying CCD camera (Hamamatsu Photonics, ImagEM C9100-13). For excitation of GFP and mCherry, a 488-nm blue laser (50 mW, Coherent) and a 561-nm yellow laser (50 mW, Coherent) are used, respectively. For simultaneous observation of GFP and mCherry, both lasers are combined and guided to the specimen with a Di01-R488/561-25 dichroic mirror (Semrock) without an excitation filter. The fluorescence is filtered with an Em01-R488/568-25 bandpass filter (Semrock) and separated into two channels using a U-SIP splitter (Olympus) equipped with a DM565HQ dichroic mirror (Olympus). The fluorescence is further filtered with an FF02-525/50-25 bandpass filter (Semrock) for the GFP channel and an FF01-624/40-25 bandpass filter (Semrock) for the mCherry channel.

REAGENT PREPARATION

SD/CA medium

- 0.17% (w/v) yeast nitrogen base w/o amino acids and ammonium sulfate
- 0.5% (w/v) ammonium sulfate
- 0.5% (w/v) casamino acids
- 2% (w/v) glucose

See also the protocol "Cultivation of Yeast Cells and Induction of Autophagy".

SD/DO medium

- 0.17% (w/v) yeast nitrogen base w/o amino acids and ammonium sulfate
- 0.5% (w/v) ammonium sulfate
- 1× dropout mix
- 2% (w/v) glucose

See also the protocol "Cultivation of Yeast Cells and Induction of Autophagy".

3.2 mM FM4-64 in DMSO

Dissolve 1 mg of FM4-64 in 514 µl of DMSO. Divide into several aliquots of 20–50 µl. This reagent can be stored at –30°C.

1 mg/ml rapamycin

Dissolve 10 mg of rapamycin in 10 ml of 90% ethanol and 10% Tween 20. This

reagent can be stored at –30°C.

1 M PMSF in DMSO

Dissolve 174.19 mg of PMSF in 1 ml of DMSO. Divide into several aliquots of 20–50 µl. This reagent can be stored at –30°C.

TROUBLESHOOTING TIPS

1. No induction of autophagy.

   Make sure that the culture medium is completely washed away. As needed, the culture medium is removed by aspiration.

2. No autophagic bodies can be detected.

   Autophagic bodies may be degraded because of insufficient treatment with PMSF. Add 1/500 volume of 1 M PMSF into the cell culture before starvation or rapamycin treatment. Alternatively, you should use protease-deficient cells such as *pep4*Δ cells and *pep4*Δ *prb1*Δ cells.