Fluorescence Microscopy Analysis of the Pre-Autophagosomal Structure (PAS)

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METHOD

Cultivation of yeast cells

1. Yeast cells expressing GFP/mCherry-tagged Atg proteins are constructed.

NOTE GFP/mCherry-tagged Atg proteins should be expressed by the *ATG* own promoters. We recommend genomic expression of the GFP/mCherry-tagged Atg proteins, because the expression levels are nearly equal in each cell, which allows us to observe the PAS accurately.

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. If needed, 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 (using a micro slide glass)

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, because the PAS is less mobile in this time period.

NOTE If the protein of interest is highly mobile, images should be acquired with 16–32 msec exposure.

Fluorescent microscopy (using a glass bottom dish)

1. Spot 20 µl of 0.1% concanavalin A (ConA) onto a glass bottom dish and incubate for 3 min at room temperature.
2. Remove ConA and wash twice with distilled water by pipetting.
3. Spot 20 µl of the cell culture (OD_{600}=1.2–1.8) onto the ConA-coated glass bottom dish and incubate for 3 min at room temperature.
4. Remove the cell culture and wash twice with medium by pipetting.
5. Fill the dish with the medium.
6. Set the glass bottom dish onto an inverted fluorescence microscope with immersion oil.
7. Observe fluorescence using GFP and mCherry channels simultaneously.
8. Images are acquired by AQUACOSMOS software (Hamamatsu Photonics) or MetaMorph software (Molecular Devices).

NOTE Images should be acquired with 500–1,000 msec exposure, because the PAS is less mobile in this time period.

NOTE If the protein of interest is highly mobile, images should be acquired with 16–32 msec exposure.

MATERIALS

REAGENTS

• Immersion oil (Olympus, TYPE-F IMMOIL-F30CC)

• Rapamycin (funakoshi, LC Laboratories, R-5000)

• Concanavalin A (Wako, 037-08771)

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)
• Glass base dish (IWAKI, 35 mm, No.1-S, 3971-035)
• Laser microscope (Olympus, IX81)

Inverted microscope (Olympus, IX81) is equipped with a 150× TIRF objective (Olympus, UAPON 150×OTIRF, 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".

0.1% (w/v) concanavalin A

Dissolve 1 mg of concanavalin A in 1 ml of 25 mM Tris-HCl pH 7.0 and 1 mM $MnCl_2$. 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.

TROUBLESHOOTING TIPS

1. No PAS signals can be detected.

   When GFP-Atg8 is used as a probe, the PAS signals will be efficiently detected as bright dots in each cell. However, other Atg-GFP proteins will exhibit only minor dots, and the signals may be hardly detected. If so, images should be acquired with a long exposure at 2,000–3,000 msec. Alternatively, the PAS may be disassembled during sample preparation using a micro cover glass (probably because of a stress by sandwich with glass slides). In particular, the PAS is easily disassembled in *atg11*Δ cells. If you examine the PAS formation in these cells, you should use a glass bottom dish.