

## **Live imaging analysis of GFP-ATG proteins**

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### **METHODS**

#### **Induction of autophagy**

1. Prepare cells stably expressing GFP-ATG proteins for live imaging. Transient expression is not recommended because excess ATG proteins often impede autophagosome biogenesis (Fujita, et al., 2008, MBC; Hara, et al., 2008, JCB) or produce aggregates (Kuma et al. 2007, Autophagy).
2. Two days before observation, seed cells on glass bottom dish in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS and 50 U ml<sup>-1</sup> penicillin-streptomycin and 2 mM L-glutamine, and maintain the cells at 37°C with 5% CO<sub>2</sub>.
3. Just before starting observation, wash the cells twice with PBS and culture them in amino-acid free DMEM.

#### **Live cell imaging**

1. More than one hour before starting observation, turn on the power of all equipment for microscopy; a lamp, PC, microscope, camera, stage-top CO<sub>2</sub> incubator, and so on.

**NOTE:** Temperature-equilibration around the microscope is important to stabilize the focus position.

2. Optimize image-acquisition parameters, interval time and observation time; depending on purpose. For tracking whole life of an isolation membrane or an autophagosome, 10~30 sec time-interval and 20~60 min observation time are recommended.

**NOTE:** To reduce both photodamage to cells and photobleaching of the GFP signals during time-lapse imaging, light exposure should be minimized. Usually, ND filters are required to reduce the excitation power of the lamp.

**EXAMPLE:** Image-acquisition parameters for observation of GFP-ATG5 puncta formation

Exposure time, 300 ms

Interval time, 10 s

Observation time; 30 min

ND filter, 5%

## **Image analysis**

1. Make movies of time-lapse images using proper image-processing software (MetaMorph, ImageJ, etc.)
2. Using the software, analyze signal intensity; the structure of autophagosomes; the number of autophagosomes; trajectories, and so on.

## **MATERIALS**

### **REAGENTS**

Dulbecco's modified eagle's medium - high glucose (Sigma, D6546)  
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)  
Dulbecco's modified eagle's medium - high glucose, without Amino Acid (048-33575, Wako)

### **EQUIPMENTS**

Glass bottom dish (IWAKI, 11-004-008)  
Inverted microscope (Olympus, IX81-ZDC)  
60x PlanAPO (NA 1.24) oil-immersion objective lens (Olympus),  
Stage top incubator (TOKAI HIT, INUB-ONI-F2)  
Cooled CCD camera (Photometrics, CoolSNAP HQ2)  
Mechanical shutter (Unibritz, VS25S2ZM1)  
Shutter controller (Uniblitz, VMM-D3)  
Filter wheel (Ludl Electronic Products, 96A357) (optional for multicolor live imaging)  
Filter wheel controller (Ludl Electronic Products, MAC6000) (optional for multicolor live imaging)  
Image-processing software (Molecular Device, MetaMorph)  
CO<sub>2</sub> 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.

## **TROUBLESHOOTING TIPS**

1. Many punctate structures irrespective of autophagy induction.

Several ATGs can easily aggregate when they are overexpressed, which are often difficult to be distinguished from true autophagic structures. To avoid this artifact, it is highly recommended to use stable transformants.

2. Serious photodamage or photobleaching

Excitation may be too strong. Use ND filters or reduce the laser power. If not enough, reduce the exposure time or observation time, or extend time interval.

3. Difficult to distinguish specific GFP-ATG signals from autofluorescence

Check the fluorescence signals using another filter set, for example, for a red fluorescence. If the same punctate signals are still detected, there are likely autofluorescence. It is also important to examine untransfected control cells.

## **REFERENCES**

Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell*. 2008;19(5):2092-100

Hara T, Takamura A, Kishi C, Iemura S, Natsume T, Guan JL, Mizushima N., FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol*. 2008;181(3):497-510

Kuma A, Matsui M, Mizushima N. LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization. *Autophagy*. 2007;3(4):323-8

Koyama-Honda I, Itakura E, Fujiwara TK, Mizushima N. Temporal analysis of recruitment of mammalian ATG proteins to the autophagosome formation site., *Autophagy*. 2013;9(10):1491-9.

**FIGURE** Accumulation and disappearance of ATG proteins.

(Excerpt from Koyama-Honda et al., 2013, *Autophagy*.)

A, MEFs stably coexpressing SECFP. LC3 (green) and Venus. ZFYVE1 (red) were cultured in starvation medium. Scale bar, 20  $\mu$ m.

B, Time-lapse imaging was performed at 10-s intervals with a cooled CCD camera using 3  $\times$  3 binning, 500 ms exposure for CFP and 300 ms for Venus,

and  $20 \times 20$  pixel ( $6.45 \times 6.45 \mu\text{m}$ ) areas were extracted. Dashed-line boxes indicate the video frames at the maximum (peak) intensities. Scale bar,  $5 \mu\text{m}$ .

C, The time course of the fluorescence intensity of the punctate signals shown in the images (B) was plotted in the graphs (percent maximum intensity).

