

Overview: autophagy methods in mammals

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1. Induction of autophagy by nutrient starvation

Withdrawal of amino acids or both amino acids and serum is the strongest method to induce autophagy. This can be mimicked by suppression of mTORC1 using catalytic inhibitors such as Torin 1. Note that autophagy induction by glucose starvation or rapamycin treatment is much weaker.

2. Monitoring autophagic activity in cultured cells

It is very important to notice that "accumulation of autophagosomes" does not always indicate "increased autophagic flux (or autophagic degradation)". As the number of autophagosomes is determined by both the rate of autophagosome formation and the rate of their conversion into autolysosomes, "accumulation of autophagosomes" can indicate either "an increase in autophagosome formation" or "a blockage of autophagosome degradation". In general, the following assays are mainly for prediction of the number of autophagosome.

- *Number of LC3 puncta (endogenous or GFP-LC3)*
- *Steady state level of LC3-II (or its ratio to an internal control)*
- *Electron microscopy*

On the other hand, the following methods can monitor the autophagic flux, if appropriately conducted. Each method has its advantages and disadvantages.

- *LC3 flux assay (LC3 turnover assay)*

As endogenous protein can be used, no transfection is required. However, it is essential to prepare two samples one with and the other without lysosomal inhibitor(s) (bafilomycin A1 is recommended). The dynamic range appears to be relatively narrow. This assay is very sensitive and, therefore, the signals become easily saturated, which may underestimate or miss a true difference.

- *p62 degradation assay*

As endogenous protein can be used, no transfection is required. The most problematic point of this assay is that p62 can be also regulated at the transcriptional level. For example, oxidative stress and starvation can induce p62 mRNA. This assay can be used as far as its mRNA level is constant. Thus, it is essential to monitor its mRNA level simultaneously.

- *Detection of mRFP-GFP tandem fluorescent-tagged LC3*

This is a microscopy-based method and requires careful quantification, which is ideally achieved by an automatic system.

- *Flow cytometry-based monitoring of GFP-LC3 degradation*

Although this is a less sensitive method compared to the above methods, the dynamic range of this assay is wide. Moreover this is a highly objective and quantitative method. This method can be recommended as a first choice.

- *Long-lived protein degradation assay*

Although this is one of the classical methods, this is still the most direct method. This is less sensitive compared to other methods and not strictly specific to autophagy. Using radioactive amino acids can be another limitation.

3. Monitoring autophagic activity in whole organisms

Although these "autophagic flux" assays work in cultured cells, it is still challenging to measure autophagic flux in whole organisms like mice. The lysosome activity differs among tissues and cell types, which makes it difficult to efficiently inhibit lysosomal degradation when the LC3 turnover assay is performed. The use of p62 is also problematic because its mRNA level is highly fluctuated in vivo. Several trials have been reported:

Heart: Intraperitoneal administration of chloroquine (10-50 mg/kg) 2-4-h prior to sampling (Hariharan et al. *Circ Res*, 107:1470, 2010, Gurney et al. *Methods Mol Biol*, 1219:187, 2015).

Liver: Intraperitoneal administration of leupeptin (18-40 mg/kg) 1-4-h prior to sampling (Haspel et al. *Autophagy* 7:1, 2011, Kageyama et al. *J Biol Chem*, 289:24944, 2014).

Muscle: Intraperitoneal administration of colchicine (0.4 mg/kg) 6-h or 2-days prior to sampling (Ju et al. *Autophagy* 6:929, 2010, Quy et al. *J Biol Chem*

288:1125, 2013).

4. Frequently Asked Questions

Q1. What is the best methods to monitor autophagy?

A1. Flux assays are essential. However, there is no golden standard method. As each flux assay method has its own limitation, it is important to use more than one methods. Nonetheless, as stated above, the flow cytometry-based method can be recommended as an initial option because of its wide dynamic range and objectivity.

Q2. Can the mRNA levels of ATG genes indicate autophagic activity?

A2. It is true that some of the ATG genes are transcriptionally upregulated during autophagy. However, there is no clear evidence that these changes always correlate with the autophagic activity. At least, transcriptional regulation of ATG genes is not required for short-term starvation-induced autophagy. Thus, it is not recommended to use the ATG mRNA levels as autophagy indicators.

Q3. Does an increase in LC3 staining in tissue histology indicate active autophagy?

A3. No, in general. As LC3 is degraded in the lysosome, LC3 is one of the typical autophagy substrates. An increase in LC3 staining may rather indicate autophagy suppression. However, the expression level of LC3 is also regulated by transcription. One cannot predict the autophagic activity just by the staining intensity of LC3.

Q4. What is the best gene to knockout or knockdown to suppress autophagy?

A4. This is a difficult choice. As many ATG proteins have been reported to have non-autophagy functions, it is recommended to knockout/down more than two ATGs. A recommendation would be one from upstream ATGs (e.g. FIP200, ATG9A) and one from downstream ATGs (e.g. ATG5, ATG7). ULKs, ATG2s, WIPIs, ATG4s, and LC3s are not recommended because there are multiple homologs.

Q5. What is the best marker for mitophagy?

A5. At least in Parkin-mediated mitophagy, many outer mitochondrial membrane proteins as well as even intermembrane proteins are degraded by the ubiquitin-proteasome system. To precisely monitor autophagic degradation of mitochondria, it is highly recommended to use proteins on the inner mitochondrial membrane or matrix, or mitochondrial DNA as markers.

Q6. *What is the best autophagy inhibitor?*

A6. There is no specific inhibitor. Thus, it is generally essential to use cells depleted with appropriate ATGs. For preliminary experiments, wortmannin (to inhibit starvation-induced autophagosome formation) and chloroquine or bafilomycin A₁ (to inhibit degradation inside autolysosomes) can be used. 3-methyladenine (3-MA) can also be used but it is effective only at a higher concentration (5-10 mM).

Further information:

Klionsky et al. Guidelines for the use and interpretation of assays for monitoring autophagy. ***Autophagy*** 8:445-544 (2012)

Mizushima et al. Methods in mammalian autophagy research. ***Cell*** 140; 313-326 (2010)

Mizushima & Yoshimori. How to interpret LC3 immunoblotting. ***Autophagy*** 3:542-545 (2007)