Published in final edited form as:

Curr Protoc Toxicol. ; 69: 20.12.1–20.12.26. doi:10.1002/cptx.11.

Methods for the Detection of Autophagy in Mammalian Cells

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Abstract

Macroautophagy (hereafter referred to as autophagy) is a degradation pathway that delivers cytoplasmic materials to lysosomes via double-membraned vesicles designated autophagosomes. Cytoplasmic constituents are sequestered into autophagosomes, which subsequently fuse with lysosomes, where the cargo is degraded. Autophagy is a crucial mechanism involved in many aspects of cell function, including cellular metabolism and energy balance; and alterations in autophagy have been linked to various human pathological processes. Thus, methods that accurately measure autophagic activity are necessary. In this unit, we introduce several approaches to analyze autophagy in mammalian cells, including immunoblotting analysis of LC3 and p62, detection of autophagosome formation by fluorescence microscopy, and monitoring autophagosome maturation by tandem mRFP-GFP fluorescence microscopy. Overall, we recommend a combined use of multiple methods to accurately assess the autophagic activity in any given biological setting.

Keywords

autophagy; autophagic flux; LC3; p62; fluorescence microscopy; immunoblotting

INTRODUCTION

Macroautophagy (referred to as autophagy hereafter) is an intracellular bulk degradation pathway in which cytoplasmic components, such as protein aggregates and damaged organelles are degraded and recycled for maintaining normal cellular homeostasis (Yoshimori, 2004). The process of autophagy is initiated by the formation of phagophore (or isolation membrane), which is likely derived from the lipid bilayer of the endoplasmic reticulum, Golgi apparatus, mitochondria or plasma membrane (Mari et al., 2011). The phagophore elongates and subsequently engulfs a portion of cytoplasm, thereby sequestering the cargo in a double membrane structure known as autophagosome (Mijaljica et al., 2012).

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Conflicts of Interest

The authors declare they have no conflict of interest.

The outer membrane of autophagosome fuses with the lysosomal membrane to form an autolysosome, leading to the degradation of autophagosomal inner membrane along with the sequestered materials by lysosomal acid proteases. The degradation products are then transported back into the cytoplasm to be recycled.

A set of autophagy-related (ATG) proteins organize into functional complexes and mediate the autophagosome formation which involves nucleation and elongation of isolation membrane (Arroyo et al., 2014). The only known mammalian ATG protein that is specifically associated with autophagosome is microtubule-associated protein light chain 3 (LC3) (Mizushima et al., 2010). Upon the autophagy induction, the cytosolic LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) to form lipidated LC3 (LC3-II). LC3-II binds to the expanding isolation membrane and remains bound to complete autophagosome. Therefore, LC3-II is widely used as a marker for autophagosome (Mizushima et al., 2010).

One critical point is that autophagy is a multi-step process which includes not just the formation of autophagosomes, but most importantly, flux through the entire system, including the degradation upon fusion with lysosomes (Klionsky et al., 2016). The term “autophagic flux” is used to denote the complete process of autophagy including autophagosome biogenesis, maturation, fusion with lysosomes, and breakdown of autophagic substrates inside the lysosome. Assays that monitor autophagic flux are crucial for the assessment of the dynamic autophagy process, since such assays help to distinguish between the accumulation of autophagosomes due to induced autophagic activity versus accumulation due to inefficient lysosomal clearance.

Currently there is no single “gold standard” for determining autophagic activity that is applicable in every experimental context. This unit describes some basic protocols that are currently used to detect macroautophagy in cultured mammalian cells. We present four different methods that are widely used to analyze autophagy. Three methods are aimed to monitor autophagic flux, including LC3 turnover by immunoblotting (basic protocol 1), degradation of autophagic substrate p62 (alternative protocol 1) and tandem fluorescent-tagged LC3 assay (alternative protocol 2). In addition, the fluorescence microscopy of LC3-positive vesicles (basic protocol 2) is used to assess the static levels of autophagosomes. We also discuss the critical points for the successful application of these methods. In most cases, it is highly recommended to combine multiple approaches to detect an autophagic response induced by a drug or treatment.

NOTE: Use deionized, distilled water in all recipes and protocol steps.

NOTE: Different cell lines and primary cells may respond differently to the drugs and/or treatments mentioned in the following protocols. Optimum concentration and incubation time must be determined empirically for each cell culture investigated.

BASIC PROTOCOL 1. MONITORING LC3 TURNOVER BY IMMUNOBLOTTING TO ASSESS AUTOPHAGIC FLUX

To date, the most experimentally straightforward method to monitor autophagic activity is the detection of LC3 protein processing. LC3 is the only known protein that is specifically associated with all types of autophagic membranes, including phagophore, autophagosome and autolysosome (a hybrid organelle formed by fusion of the autophagosome and lysosome) (Kabeya et al., 2000). Therefore, the amount of LC3-II correlates well with the number of autophagosomes, which provides a good index of autophagy induction.

LC3 is initially synthesized in an unprocessed form, proLC3, which is cleaved at its C terminal by ATG4 to form cytoplasmic LC3-I. LC3-I is then conjugated to PE to become a membrane bound form, LC3-II; this step involves an ubiquitination-like reaction mediated by ATG7 (E1) and ATG3 (E2). The lipidated LC3-II binds to both inner and outer membrane of autophagosome, with the former being degraded after fusion with lysosomes; whereas LC3 on the outer membrane is deconjugated by ATG4 and returns to the cytosol (Jiang and Mizushima, 2015). Thus, LC3 conversion (LC3-I to LC3-II) and lysosomal degradation of LC3-II reflect the progression of autophagy, and immunoblotting can easily be used to monitor changes in LC3 amount.

One critical point is that autophagy is a highly dynamic, multiple-step process that can be modulated at several steps. The amount of autophagosomes detected at any specific time point is a function of the balance between the rate of their generation and the rate of degradation through fusion with lysosomes. Accordingly, increased LC3-II could reflect either increased autophagosome formation due to autophagy induction, or a blockage in the downstream steps in autophagy, such as inefficient fusion or decreased autophagosome degradation (Rubinsztein et al., 2009). Thus, the mere detection of levels of LC3-II at a specific time point is insufficient for an overall estimation of autophagic flux, which refers to the complete process of autophagy including the sequestration of cargo within the autophagosome, the delivery of cargo to lysosomes and the subsequent release of the breakdown products. Moreover, a number of studies have reported that not all LC3-II is present on autophagic membranes. Some population of LC3-II seems to be generated in an autophagy-independent manner, since knockdowns of critical upstream autophagy factors could reduce autophagic activity but fail to reduce LC3-II (Yamada and Singh, 2012). Thus, we detect LC3-II turnover by immunoblotting in the presence and absence of lysosomal degradation. Preventing lysosomal degradation of LC3-II can be achieved through the use of lysosomal protease inhibitors (e.g. leupeptin, pepstatin A and E64d), or drugs that alter lysosomal pH (e.g. NH₄Cl, bafilomycin A and chloroquine), or agents that inhibit autophagosome-lysosome fusion (e.g. bafilomycin A) (Mizushima et al., 2010). In the current method, we use the combination of leupeptin and NH₄Cl to block the lysosomal degradation. The differences in the amount of LC3-II between samples in the presence and absence of inhibitors reflect the amount of LC3-II transit through the autophagic pathway.

Materials

Reagents and Solutions

Cultured cell line or primary culture of interest

Drug or treatment to be tested

Growth medium

Phosphate-buffered saline (1xPBS, pH 7.4, Thermo Fisher Scientific)

Lysosome inhibitors: leupeptin stock solution (10 mM) and NH₄Cl stock solution (2 M) (see recipe)

Lysis Buffer (see recipe)

Protein quantitation assay kit (e.g. Pierce BCA protein assay kit)

Sample loading buffer 6x (see recipe)

Pre-stained molecular weight marker

12% Pre-cast gels (Bio-Rad)

Running buffer (see recipe)

Transfer buffer (see recipe)

Tris-buffered saline with Tween 20 (TBST, see recipe)

Blocking buffer: 5% (w/v) Bovine serum albumin (BSA) in TBST

Primary antibody to detect LC3 (rabbit polyclonal anti-LC3B antibody; Cell Signaling #2775)

Primary antibody to detect β -actin (mouse monoclonal anti- β -Actin antibody; Sigma #A1978)

Secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Scientific #31460) and HRP-conjugated goat anti-mouse IgG (#31432)

Enhanced chemiluminescent (ECL) substrate (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific #34080)

Restore Western Blot Stripping Buffer (Thermo-Fisher Scientific)

Equipment

6-well culture plates

Incubator (37°C with controlled humidity and 5% CO₂)

Cell scraper

Polyvinylidene fluoride (PVDF) protein blotting membrane

Electrophoresis chamber (Mini-PROTEAN Tetra Cell, Bio-Rad)

Protein transfer apparatus (Mini Trans-Blot Cell, Bio-Rad)

Power supply

Shaker

Film cassette

X-ray film

Automated film developer

Software for data analysis (e.g., ImageJ)

Cell culture and preparation of cell lysate

- 1 Plate the appropriate number of cells in 2 ml of medium per well in a six-well plate to obtain 80%–90% confluency by 48 hrs.

This assay can be performed in various cell lines as well as primary cultures. The cell number for initial plating depends on the cell type and must be determined by the investigator. Seed cells at an appropriate density to ensure that there will be enough cells to harvest, but that they will not reach complete confluency by the end of the experiment.

- 2 At 24 hrs after plating, remove culture medium from the well and replace with medium either with or without the drug or treatment of interest to the appropriate well and return to incubator for 24 hrs (or alternative period of time, as required by the particular experiment).

- 3 At 2 hrs before harvesting the cells, add 1:100 dilutions of the stock solutions of leupeptin (10 mM) and NH_4Cl (2 M) to the medium to yield final concentrations of 100 μM and 20 mM, respectively.

This step requires the inhibitors concentrations that are saturating for LC3-II accumulation, but do not provoke cell death. Investigators should also be careful not to use lysosome inhibitors for too long, as long-term autophagy blockade will start mediating non-specific effects (e.g. inhibiting clearance via the ubiquitin-proteasome system) (Rubinshtein et al., 2009). It is noteworthy that the saturating concentrations of lysosomal inhibitors can vary among different cell lines and need to be determined for each cell type (Chittaranjan et al., 2015). Besides leupeptin and NH_4Cl , alternative lysosome inhibitors such as lysosomal proton pump inhibitor bafilomycin A and lysosomal protease inhibitors E-64d, pepstatin A or chloroquine can also be used.

- 4 Discard the medium and place the plate on ice. Wash the cells twice with ice-cold PBS. Aspirate the PBS, then add 50–100 μl pre-cooled lysis buffer.

- 5 Collect adherent cells with a plastic cell scraper, and then transfer the cell lysate into a pre-cooled 1.5 ml Eppendorf tube. Sonicate 10–15 sec to complete cell lysis and shear DNA to reduce sample viscosity.
- 6 Centrifuge the lysate at 12,000 rpm for 10 min at 4°C. Transfer the supernatant to a new tube. Keep all samples on ice.
- 7 Measure protein concentration of each sample (e.g., using a commercially available BCA kit) and then adjust the volume of the samples so they have equal protein concentrations.
- 8 Mix preferred amount of protein with 6x loading buffer (final 1x) and boil at 100°C for 5 min on a heating block. Put the sample tubes on ice for 1–2 min, then spin briefly to bring all solution to the bottom of the tube. Samples can be stored frozen at –80°C for 4–6 months.

LC3-I is more labile than LC3-II and more sensitive to freezing-thawing and to degradation in RIPA buffer (Klionsky et al., 2012). We recommend preparing electrophoresis samples rapidly after cell lysis. Avoid freezing-thawing the sample more than once.

SDS-PAGE

- 9 Rinse the pre-cast gel with water and remove the tape on the bottom of the gel.
- 10 Assemble the gel cassette in the electrophoresis tank. Fill the tank with running buffer. Rinse the wells with running buffer to remove salt precipitates and gel debris.
- 11 Before loading individual wells, briefly vortex and spin the samples. Load equal amounts of protein (10–20 µg) into the wells of the SDS-PAGE gel, along with molecular weight marker. Run the gel at 100 V for approximately 2 hrs until the dye reaches 1/4 inch from the bottom of the gel.

As LC3-II migrates at ~14 kD, it is important to ensure that the blue dye does not run too close to the bottom of the gel.

Western blotting and Quantitation

- 12 Cut the PVDF membrane into approximately the same size of the running gel then soak it in methanol for 1–2 min. Incubate in ice cold transfer buffer for 5 min.

Previous studies reported that LC3-II binds more avidly to PVDF than nitrocellulose.

- 13 Disassemble the gel apparatus. Cut wells and bottom of gel off to ensure gel is flat, and equilibrate the gel in ice cold transfer buffer for 10 min.
- 14 Assemble the transfer sandwich and make sure no air bubbles are trapped in the sandwich. The blot should be on the cathode and the gel on the anode.

- 15** Place the cassette in the transfer tank. To control the temperature, place an ice block next to the transfer cassette. Fill the tank with transfer buffer. Surround the apparatus with ice. Transfer at the constant voltage of 90 V for 70 min.
- These steps describe wet transfer. Investigators should optimize the experimental conditions if semi-dry transfer is used. Experience from other research groups shows no significant difference in results when using a semi-dry blotter.
- 16** After transfer, disassemble the sandwich. Block the membrane (immunoblot) by incubating in 5% BSA blocking buffer with rocking for 1 hr at room temperature.
- 17** Dilute the primary antibody in 5% BSA blocking buffer (anti-LC3B at 1:1000). Discard blocking buffer and incubate the membrane in primary antibody solution overnight on a rocking platform at 4°C.
- The antibody against LC3B can be incubated for 1 hr at room temperature, but the best signal may be obtained by overnight incubation. Each primary antibody requires different dilutions.
- 18** Wash the membrane three times for at least 15 min each in TBST on a rocker at room temperature.
- 19** Dilute the secondary antibody in 5% BSA blocking buffer (HRP-conjugated anti-rabbit IgG at 1:5000) and incubate for 1 hr at room temperature with rocking.
- 20** Wash as step 7.
- 21** Mix enough amount of ECL solution A and B. Add enough ECL solution mixture to cover the surface of membrane. Incubate for 1 min.
- 22** Remove excess reagent and cover the membrane in transparent plastic wrap. Place the blots in a film cassette. Expose the blots to film with appropriate exposure time in dark room, and then develop the film in an automated developer. Appropriate imaging apparatus (e.g., Bio-Rad scanner with chemiluminescence) can also be used instead of conventional X-ray film.
- 23** To ensure equal loading of the samples onto the gel, dehybridize the bound antibodies by incubating the membrane in stripping buffer for 15 min at room temperature. Wash 3 times in TBST for 5 min each.
- 24** Repeat the steps 16–22 with the following antibodies:
- β-actin primary antibody: 1:50,000 in blocking buffer.
- Secondary antibody (goat anti-mouse): 1:20,000 in blocking buffer.
- LC3 is easily lost from membranes that are stripped and reprobed. For that reason, LC3 should be the first protein to be detected on a membrane.

- 25 Use ImageJ to determine the densitometry levels of LC3-II and β -actin (alternative comparable imaging software can be used).
- 26 Normalize the levels of LC3-II in each sample to those of β -actin.

ALTERNATIVE PROTOCOL 1. MONITORING AUTOPHAGIC DEGRADATION OF P62/SQSTM1 BY IMMUNOBLOTTING

In addition to LC3, levels of p62 (also known as SQSTM1/sequestosome 1) can also be used to monitor autophagic flux. p62 possesses a C-terminal ubiquitin-binding domain and a short LC3-interacting region sequence (Lippai and Low, 2014). It serves as an adaptor protein that links ubiquitinated proteins to the autophagic machinery and enables their clearance in the lysosome (Bjorkoy et al., 2009). p62 and p62-bound ubiquitinated proteins become incorporated into the completed autophagosome and are degraded in autolysosomes. Since p62 itself is removed mainly by autophagy, its amount is generally considered to inversely correlate with autophagic activity (Bjorkoy et al., 2009). Accumulation of p62 has been used as a marker for autophagy suppression, and similarly, a decreased p62 level indicates autophagic activation.

Like LC3, western blotting analysis of p62 is only a snapshot of a dynamic process. For that reason, measurement of p62 in the presence and absence of lysosomal blockade provides essential information about the amount and rate of autophagic substrates sequestered and degraded. The procedures used in this analysis are the same as those used in LC3 turnover assay.

Additional Materials

8% Pre-cast gels (Bio-Rad)

Primary antibody to detect p62 (rabbit polyclonal anti-p62 antibody; Enzo Life Science)

1. Use a precast-gel containing 8% acrylamide for separation of p62.
2. Transfer at the constant voltage of 90 V for 90 min.

All the other steps are the same as the procedures in LC3 turnover assay.

BASIC PROTOCOL 2. MONITORING THE NUMBER OF AUTOPHAGOSOMES BY VISUALIZING LC3 PUNCTA USING FLUORESCENCE MICROSCOPY

As described above, upon induction of autophagy, LC3 becomes associated to the autophagosomal membrane. This change in the subcellular distribution of LC3 can be observed through either indirect immunofluorescence or by examining the signal of fluorescent protein tagged to LC3 (e.g., green fluorescent protein (GFP)-LC3). Detecting endogenous LC3 by anti-LC3 antibodies in immunocytochemistry offers advantages in terms of reducing cellular manipulation and avoiding potential artifacts resulting from overexpression.

In some cell types, or under some experimental conditions, endogenous amount of LC3 is below the level of detection through immunofluorescence. Cells can also be transfected with a recombinant form of LC3 fused with GFP (or other fluorescent proteins). Regardless of the approach, LC3 appears as a diffuse signal throughout the cytoplasm under normal growth condition, representing soluble cytosolic LC3-I. When the cells are exposed to an autophagic stimulus, LC3 is recruited to autophagosome membranes, exhibiting a characteristic pattern of LC3-II puncta. It is important to note that the simple determination of numbers of autophagosomes by LC3 puncta is insufficient for an estimation of autophagic activity. We suggest using additional approaches (e.g., LC3 turnover by immunoblotting) along with fluorescence microscopy to detect autophagy in order to obtain convincing data.

Materials

Reagents and Solutions

Cells of interest: untransfected cells and/or cells stably expressing GFP-LC3

0.001% poly-L-lysine (sterile)

GFP-LC3 plasmid (Kabeya et al., 2000)

Transfection reagent: Lipofectamine 2000 (Invitrogen)

PBS (1x, pH 7.4)

Fixative: 4% paraformaldehyde in PBS (see recipe) or 100% methanol (store overnight at -20°C)

Blocking buffer (5% goat serum/0.25% Triton X-100 in PBS, prepare fresh and store up to one day at 4°C)

Antibody dilution buffer (1% goat serum/0.25% Triton X-100 in PBS, prepare fresh and store up to one day at 4°C)

Primary antibody to detect LC3 (rabbit polyclonal anti-LC3B antibody; Cell Signaling #2775)

Secondary antibody: goat anti-rabbit conjugated to Alexa Fluor 488 (Thermo Fisher Scientific #A11008)

Mounting Medium with DAPI (Vector Laboratories)

Equipment

13×13 -mm or 22×22 -mm glass coverslips

6-well culture plates or 35 mm Petri dishes

Aspirator

Forceps

Glass slides

Humidified chamber

To make a humidified chamber, place a large piece of parafilm at the bottom of a 150-mm Petri dish. Surround the parafilm with small pieces of moist paper towels. Make sure that the moist paper towels are not in direct contact with the parafilm.

Aluminum foil

Nail polish, clear

Microscope slides

Confocal or wide-field fluorescence microscope

Note: Do not allow the cells to dry out during any steps. Keep the coverslip in a 35-mm Petri dish, aspirate off buffer washes, and then add new buffer to the dish. Perform changes of buffer and coverslip transfer quickly.

Cell preparation for imaging

- 1** Sterilize glass coverslips by soaking them in 95% ethanol for at least 24 hrs. Carefully dry them over a flame for a few seconds. Place a coverslip in each well of a 6-well plate or 35-mm Petri dish.
- 2** *Optional (for the cells only grow well on coated coverslip):* Coat coverslips with sterile 0.001% poly-L-lysine for 1 hr in the cell incubator. Rinse coverslips with sterile water 3 times. Allow coverslips to dry completely in the cell culture hood.
- 3** Plate the cells of interest at a density that will yield 50%–70% confluency on the day of experiment. Grow the cells in normal culture medium under standard conditions overnight.
- 4** *Optional (for transiently expressed GFP-LC3):* Transfect cells the following day with 0.5–1 µg GFP-LC3 expression construct using Lipofactamine 2000 according to the manufacturer's protocol for as short a time as possible, preferably no more than 4 hrs.
- 5** The day after transfection or seeding, aspirate the growth medium. Switch the cells to the appropriate culture medium for application of the experimental treatment.
- 6** At the end of treatment, aspirate the culture medium from each well and gently wash the cells twice with PBS at room temperature.
- 7** Fix the cells for 15 min with 4% paraformaldehyde in PBS at room temperature.

Paraformaldehyde is toxic, use only in a fume hood. Alternatively, fix the cells with 100% methanol for 5 min at –20°C.
- 8** Wash the cells 3 times with PBS at room temperature.

Cells are ready for immunostaining of endogenous LC3 (steps 9–13) or direct microscopy of GFP-tagged LC3 (proceed to step 14).

Immunostaining of endogenous LC3

- 9** Incubate the cells with 5% goat serum in 0.25% Triton-X PBS for 1 hr at room temperature to block unspecific binding of the antibodies.

It is better to use the serum from the species in which the secondary antibodies are raised in. (e.g. if the secondary antibody is made in goat, use goat serum. Likewise if the secondary antibody is made in donkey, use donkey serum.)

- 10** Dilute the desired concentration of primary antibody in antibody dilution buffer. Incubate the cells in the primary antibody at 4°C overnight in the humidified chamber.

- i.** Remove the coverslip from the plate. Drain the excess buffer by holding the coverslip on its edge with forceps and carefully touching its edges with a piece of paper towel.
- ii.** Place 70 µl of diluted primary antibody on the parafilm of a humidified chamber.
- iii.** Invert the coverslip onto the drop of the diluted primary antibody. Be sure cell-side face down.

Always include a negative control using incubation buffer without primary antibody to identify non-specific staining of the secondary antibody. The optimal antibody concentration will vary depending on the antibody and need to be determined by the user.

- 11** Wash the cells 3 times in PBS, 10 min each wash.

Perform step 11–15 at room temperature.

- 12** Incubate the cells in diluted secondary antibody in a humidified chamber covered with aluminum foil for 1 hr at room temperature.

From the step forward samples should be protected from light to minimize photobleaching of the fluorophore.

- 13** Wash the cells 3 times in PBS for 10 min each.

- 14** Rinse the coverslips quickly in water and mount the coverslips on glass microscope slides.

- i.** Remove the coverslip from the plate with forceps. Dip it into water and drain the excess water with a piece of paper towel.
- ii.** Place a drop of mounting medium on a slide. Carefully lower the coverslip (cell-side down) onto the mounting medium to avoid any air bubbles.
- iii.** Allow the mounted sample to set for 1 hr in the dark.

- 15** Seal the coverslip edges with clear nail polish to prevent drying and moving under the microscope. Alexa Fluor dyes are particularly stable, so samples may be analyzed weeks after experiments if slides are properly stored at 4°C in the dark, although the best results will always be obtained with early analysis.

Image capture and data collection

- 16** Visualize the cells using epifluorescence microscopy or confocal laser scanning fluorescence microscopy. Count the number of LC3 puncta per cell and measure the size of the punctate structures using computerized software image analysis program (e.g. ImageJ).

The number of punctate structures can be counted visually (by experiment-blind personnel) or automatically determined using software by setting a threshold for puncta size and pixel intensity and then applying to images to extract the puncta signal from cytosolic background.

ALTERNATIVE PROTOCOL 2. USE OF TANDEM FLUORESCENT-TAGGED LC3 TO ASSESS AUTOPHAGIC FLUX

It is noteworthy that an increased LC3-positive puncta does not invariably correspond to increased cellular autophagic activity. Given that autophagosomes (as represented by LC3-positive puncta) constitute a mid-point in the dynamic autophagy pathway, accumulation of autophagosomes at a specific time point reflects either increased autophagosome formation or, alternatively, suppression of autophagosome turnover. The latter can occur, for example, due to impairment of fusion between autophagosomes and lysosomes, or compromised lysosomal activity. Therefore, a novel autophagic flux report system has been developed by using a tandem monomeric RFP-GFP-tagged LC3 (tfLC3) (Kimura et al., 2007). The GFP fluorescent signal is quenched by the low pH inside the lysosomes, whereas mRFP (and other red fluorescent proteins, such as mCherry) maintain their fluorescence in acidic compartments. Thus, colocalization of GFP and mRFP fluorescence (yellow puncta) indicates that the tandem protein localizes in a compartment that has not fused with a lysosome, such as the phagophore and the autophagosome. On the contrary, an mRFP signal without GFP (red puncta) indicates delivery of tfLC3 into lysosomes, that is, the formation of autolysosomes. The dual color analysis using tfLC3 enables a direct estimation of both autophagy induction and flux without using any potentially toxic inhibitors.

Additional Materials

Cells of interest: untransfected cells and/stable transformants expressing mRFP-GFP-LC3

mRFP-GFP-LC3 plasmid (Kimura et al., 2007)

The procedure is the same as basic protocol 2 steps 1–8 and 14–16.

REAGENTS AND SOLUTIONS

Leupeptin stock solution

Dissolve leupeptin in water to make a 10 mM stock solution. Sterilize the solution through syringe filter (pore size 0.2 μm). Dispense into 0.5 ml aliquots and store for up to 6 months at -20°C .

Lysis buffer (1 ml)

860 μl RIPA buffer (Sigma #R2078)

Store up to 2 years at 4°C

Add right before use to a final volume of 1 ml lysis buffer:

50 μl 40 mM β -glycerophosphate stock solution (dissolve in water) to a final concentration at 2 mM

50 μl 100 mM sodium pyrophosphate stock solution (dissolve in water) to a final concentration at 5 mM

20 μl 100 mM sodium orthovanadate stock solution (dissolve in water) to a final concentration at 2mM

10 μl 100 mM phenylmethylsulfonyl fluoride stock solution (dissolve in isopropanol) to a final concentration at 1 mM

10 μl protease inhibitors cocktail (Sigma #8340)

Store all the stock solution in aliquots at -20°C for up to 6 months.

Do not re-use sodium orthovanadate after defrosted.

NH_4Cl stock solution

Dissolve NH_4Cl in water to make a 2 M stock solution. Sterilize the solution through syringe filter (pore size 0.2 μm). Prepare fresh before use.

Paraformaldehyde, 4% (w/v)

To 4 g paraformaldehyde, add 10 ml $10 \times \text{PBS}$ and 70 ml water. Heat the mixture to 60°C in a fume hood, stirring continually. If paraformaldehyde does not dissolve, slowly raise the pH by adding 1 M NaOH dropwise until the solution clears. Once the paraformaldehyde is dissolved, the solution should be cooled and filtered. Adjust the pH to 7.4 with 1 M HCl (~1 mL), then add water to a final volume of 100 ml. Prepare the paraformaldehyde solution fresh before use. Store in aliquots at -20°C for 6 months. Avoid repeated freeze/thaw.

Phosphate buffered saline (PBS) 10x

1.37 M NaCl

27 mM KCl,

80 mM Na_2HPO_4

20 mM KH_2PO_4

Adjust to pH 7.4

Sterilize by autoclaving

Store up to 6 month at room temperature.

For 1x PBS: Dilute 100 ml of 10x buffer with 900 ml water. Prepare fresh.

Running buffer

25 mM Tris base

192 mM glycine

0.1% Sodium dodecyl sulfate (SDS)

Check the pH and adjust to pH 8.3 if necessary

Store up to 1 month at room temperature.

Sample loading buffer, 6x

375 mM Tris-Cl, pH 6.8

12% (w/v) SDS

60% (v/v) glycerol

0.6 M DTT

0.06% bromophenol blue

Dispense into 0.5 ml aliquots and store up to one year at -20°C .

Transfer buffer

25 mM Tris

192 mM glycine

20% methanol

Check the pH and adjust to pH 8.3 if necessary

Store up to 1 month at 4°C .

Tris-buffered saline with Tween 20 (TBST) buffer

20 mM Tris-Cl pH 7.3–7.5

150 mM NaCl

0.1% Tween 20

Store up to one week at room temperature.

COMMENTARY

Background Information

Over the past 15 years, studies have uncovered a vast array of homeostatic, developmental and other physiological functions of autophagy (Mizushima et al., 2010). Moreover, compromised autophagy has been highlighted as a key factor of pathological processes in many human diseases (Zhang et al., 2013). Thus, it is critical to precisely monitor autophagy and to study its function in diverse physiological systems. In this unit, we describe some of the widely used methodologies to assess autophagy in mammalian cells. Given the complexity of both the autophagy process itself and its regulation, there is presently no single marker or assay that can be used as a stand-alone assay to monitor autophagy. It is recommended to use a combination of several assays to accurately monitor the status of autophagy in any given experimental condition.

LC3 turnover—Immunoblotting assessment of the LC3-II protein processing is currently considered as a simple, quick, and straightforward method to investigate autophagy in cells. This method relies on endogenous LC3 conversion and thus avoids the need to transfect cells with a reporter-based system, making it suitable for monitoring flux in primary cells. In this assay, it is essential to use lysosomal inhibitors to “clamp” the LC3-II degradation because the static LC3-II level is a function of the amount of autophagosomes generated and the amount degraded. Lysosomal inhibition results in the accumulation of autophagosomes that would have progressed through the pathway during that period. Therefore, the difference in the amount of LC3-II in the presence and absence of saturating levels of inhibitors reflects the transit of LC3-II through the autophagic pathway.

While monitoring LC3-II turnover is considered as one of the principal assays in current use to measure autophagic flux, it does not formally assess flux of substrates through the autophagic pathway (the rate of autophagy-dependent substrate degradation); but rather it assess the amount of LC3-II delivered to lysosome in a certain time period. Since the assay arrests the autophagic flux at the point of lysosomal proteolysis, it precludes the evaluation of lysosomal functions. In addition, although the LC3-II level correlates well with the number of autophagosomes, a previous study has reported that the cells can still perform autophagy-mediated protein degradation in the absence of LC3-II (Gimenez-Xavier et al., 2008). Moreover, it has been observed that an autophagic-lysosomal flux of LC3-II can take place in the absence of an accompanying flux of cytosolic bulk cargo (Klionsky et al., 2016). Therefore, we recommend combining this protocol with complementary methods that examine the turnover of autophagy substrates and/or visualize autophagic structure before conclude whether autophagy is affected by a particular drug or treatment.

p62 degradation—In addition to LC3-II, turnover of a second endogenous autophagic substrate p62 may also be used to examine the autophagic flux in a manner similar to LC3-II. After all, the rate at which autophagic substrates are delivered to lysosomes and degraded is the most direct reflection of autophagic activity. p62 targets the ubiquitinated cargos to the autophagic machinery by binding directly to LC3 and is efficiently degraded in autolysosomes. Thus, the effect of a particular compound on autophagic flux can be inferred

by comparing the differences in p62 levels in the presence and absence of lysosomal inhibition. Although monitoring cellular p62 level is a valuable complement to LC3-II turnover assay, this experiment has some potential pitfalls. First, p62 can also deliver ubiquitinated cargos to the proteasome, so its level may increase when proteasome is inhibited (Zhang et al., 2013). Second, the protein level of p62 can be regulated at the transcriptional level by many factors (e.g. oxidative stress), which further complicates the interpretation of the results (Bjorkoy et al., 2009). Finally, p62 contains domains that interact with several signaling pathways and it may have additional functions in autophagy regulation (Zhang et al., 2013). In conclusion, caution should be taken when evaluating the rate of autophagy based on the p62 degradation alone. However, in combination with other methods such as LC3-II turnover, analysis of p62 may provide a valuable addition to the toolbox used to study autophagy in mammalian cells.

Fluorescence microscopy—The classical method used to detect autophagosome is electron microscopy. However, this method requires expensive equipment and skilled technical expertise, and is also time consuming. Therefore, its use as the primary method to detect autophagy declines upon the development of light microscopic approaches using LC3-II as autophagosome marker. LC3-I is uniformly distributed in the cell when autophagy levels are low, whereas, upon induction of autophagy, lipidated LC3-II relocates to the autophagosome, which can be visualized and quantified by counting LC3 puncta with fluorescence microscopy. Monitoring LC3-positive puncta relies on either immunofluorescence using an anti-LC3 antibody or the signal of fluorescent protein tagged to LC3. Advantages of fluorescence microscopy include a fast turnaround time, low cost, ease of performance and widespread familiarity (Martinet et al., 2013). The main limitation of method is that while appearance of LC3-positive puncta reflects a snapshot of the number of autophagy-related structures in a cell, it does not necessarily imply that the autophagosomes will reach the final degradative step. As mentioned earlier, fluorescence microscopy *per se* is insufficient in determining whether increased LC3 puncta is due to induction of autophagy or inhibition of autophagosome clearance. Therefore, it is recommended to use assays that monitor autophagic flux along with fluorescence microscopy.

Tandem mRFP-GFP fluorescence microscopy—The dual color analysis using tfLC3 is based in the nature of the fluorescent signal of GFP, which is highly susceptible to acidic conditions of the lysosome lumen, whereas mRFP fluorescence is relatively stable. The key advantage of this method is that it enables simultaneous monitoring of both autophagy induction and flux in naive conditions without any potentially toxic inhibitor. It is noteworthy that this assay does not directly measure the endpoints of lysosomal degradation; rather, the use of tfLC3 depends on the acidification and fusion capacity of the lysosome (Mizushima et al., 2010). Therefore, this approach may not be used in conjunction with agents that neutralize lysosomal pH. Moreover, some fixation protocols may alter lysosomal acidification, leading to restoration of GFP signal (Klionsky et al., 2012). Thus, samples must be properly processed to avoid losing the acidic environment of the autolysosome. In some lysosomal storage diseases, lysosomes are unable to degrade sequestered cargos despite normal fusion activity. In these cases, it is recommended to perform an autophagic

flux assay in parallel with quantification of tandem fluorescent markers to confirm completion of carrier flux (Klionsky et al., 2016).

Critical Parameters and Troubleshooting

Determination of LC3 turnover—When using lysosomal inhibitors, it is of fundamental importance to establish proper conditions of inhibitor concentration and incubation time that ensure the complete inhibition of the lysosomal degradation. Non-saturating doses or insufficient treating times may result in additive effects on LC3-II protein levels when testing a compound that blocks autophagosome-lysosome fusion, which may lead to incorrect interpretation of an increase in autophagosome synthesis (Rubinsztein et al., 2009). The saturating concentration of the lysosomal inhibitor assessed on a certain cell culture cannot be generalized and used on other cell types (Chittaranjan et al., 2015). In theory, dose-curve and time-course standardization for the use of lysosomal inhibitors should be established for the initial optimization of the conditions to detect LC3-II accumulation in each cell culture. In addition, one should avoid incubating the cells with inhibitors for too long, as long-term of autophagy blockade will start to mediate non-specific effects and provoke cell death (Rubinsztein et al., 2009).

In some experimental settings, it can be challenging to reliably detect autophagic flux by this LC3 turnover assay. For example, in cases of high basal autophagy, the control samples may show very high levels of LC3-II in the presence of lysosomal inhibitors; while the tested treatment upregulates the autophagy, the additional changes in LC3 turnover may be subtle and hard to detect (Chittaranjan et al., 2015). In those cases, we suggest using additional methods that examine the degradation of autophagy substrates (e.g., p62, see alternative protocol 1) to offer more useful information.

It is also noteworthy that the saturating lysosomal inhibitors usually induce a very high level of LC3-II accumulation. Therefore, one may not be able to get the bands of samples in the absence of inhibitors without over-exposing the bands of samples treated with inhibitors on the same blot (Rubinsztein et al., 2009). This issue can be addressed by using different exposure time for lanes in the presence and absence of inhibitors and normalizing against a common loading control.

Determination of p62 degradation—It is of importance to note that an alternation in the p62 levels may not be immediately evident upon the treatment of certain autophagy modulator (Klionsky et al., 2012). For example, there is no obvious difference in p62 protein level after 30 min of starvation, whereas a significant increase of LC3 can be detected by the same time (Mizushima and Yoshimori, 2007). The reason is that while LC3-II synthesis is rapid, the clearance of p62 targeted-substrates may take a longer time. Therefore, we suggest assessing p62 levels at later time points in order to determine the maximal effect on substrate clearance.

Puncta quantification with fluorescence microscopy—It is important to analyze LC3 puncta only from high-resolution, high-quality (low autofluorescence) images. Decreased resolution of puncta will result in identification of overlapping or lustered puncta as a single large particle. Because most cells display minimal amounts of LC3 puncta even

under basal conditions, it is inappropriate to express results as percent cells positive for LC3 puncta (theoretically it should be 100% in most cell types) (Mizushima et al., 2010). Therefore, it is important to establish a cutoff value for the number of puncta per cell to define active and inactive autophagy. The results should be expressed as the “percentage of cells with more than a certain number of puncta.” It is preferable to determine the number of puncta on a per cell basis by quantifying the number of puncta in each of a selected number of cells and calculating the average puncta count per cell.

Avoid false-positive in fluorescence microscopy—Although the number of punctate LC3 or GFP-LC3 structures per cell is usually an accurate measure of autophagosome number, caution must be exercised due to the limitations of fluorescence microscopy. Transfected GFP-LC3 and even endogenous LC3 can be incorporated into intracellular protein aggregates in an autophagy-independent manner (Kuma et al., 2007). Many cellular stresses can induce the formation of aggregates, including transfection reagents. It is usually difficult to distinguish aggregate-related LC3 dots from true autophagosomes by fluorescence microscopy. As a result, the number of autophagosomes detected by quantifying fluorescent dots tends to be overestimated. To avoid these problems, it is recommended to use stable GFP-LC3 transformants because one can select clones expressing appropriate levels of LC3 that precludes artificial LC3 aggregation (Yamada and Singh, 2012). In experiments require transient GFP-LC3 transfection, it is important to include proper negative controls to ensure that the signal is derived from the autophagosome. Transfection of untagged GFP can be used as a control for background levels of puncta. Moreover, we recommend using a C-terminal glycine mutant LC3 (GFP-LC3^{G120A}) which is defective for LC3 lipidation as a negative control (Yamada and Singh, 2012). Since the mutant LC3 is incapable of conjugation with PE, it will allow one to distinguish between true increases in LC3-positive autophagosomes as opposed to the lack of it in these mutants.

Starvation is a potent inducer of autophagy, and when cells are incubated for prolonged period of time, depletion of nutrients of the medium creates starvation-like conditions. Autophagy may increase without any additional stress when cell culture reaches 100% confluency. Therefore, the cell culture should be always maintained as subconfluent.

Both lipofectamin-mediated transfection and viral infection may activate stress pathways in cells, which could stimulate autophagy. One may need to shorten the period of lipofectamin exposure as much as possible. Also, changing the medium and waiting 24–48 hours after the transfection help to reduce the background level of LC3 puncta that is due to the transfection reagent (Klionsky et al., 2012). Here again, proper controls need to be included to rule out the LC3 puncta increase results from transfection protocol.

Anticipated Results

LC3 turnover—A typical result of the LC3-based autophagic flux is shown in Figure 1. It is of note that although the actual molecular weight of LC3-II (PE-conjugated) is larger than that of LC3-I, LC3-II migrates faster than LC3-I on SDS-PAGE due to its extreme hydrophobicity. Consequently, immunoblotting of LC3 gives two bands: LC3-I (upper band) and LC3-II (lower band). Appropriate standardization controls are necessary when

evaluating LC3 by western blotting. In general, we do not suggest using LC3-II/LC3-I ratio as an indicator of autophagic activity since the LC3-II may be more sensitive to detection by certain anti-LC3 antibodies in immunoblotting analysis. Thus, measuring the levels of LC3-II to appropriate housekeeping proteins (e.g., β -actin) is likely to be a more reliable method.

The relevant parameter in this assay is the difference in the amount of LC3-II in the presence and absence of lysosomal inhibitors, which is used to represent the amount of LC3-II that is delivered to lysosomes for degradation (i.e., autophagic flux). Thus, we calculate the net LC3 flux by subtracting densitometric values of samples in the absence of lysosomal inhibitors from values of corresponding inhibitors-treated samples. Higher values indicate increased LC3 flux. For example, in Figure 1, the difference in LC3-II levels between lane 1 and lane 2 indicates the amount of LC3-II that is delivered to lysosomes under normal growth condition; whereas the difference between lane 3 and lane 4 indicates the amount of LC3-II that is delivered to lysosomes under starvation condition. Since the difference in LC3-II levels in the presence and absence of inhibitors is larger under starvation condition, this result suggests that starvation increases autophagic flux.

If a particular drug increases LC3-II levels on its own but does not further increase LC3-II in the presence of lysosomal inhibitors, then there is likely a block in LC3-II degradation (Figure 2, scenario 1). Conversely, a drug may induce a further increase in LC3-II in the presence of inhibitors but cause no obvious change in the absence of inhibitors, suggesting increased autophagosome formation and concomitant enhanced delivery of cargo to lysosomes (Figure 2, scenario 2). More frequently, a drug may increase LC3-II levels in the presence and absence of inhibitors (Figure 2, scenario 3 and 4). In scenario 3, the net LC3 flux is increased in drug-treated groups. This situation may occur if there is increased autophagosome synthesis concurrently with increased delivery of autophagic substrates to lysosomes. If the net LC3 flux is decreased (scenario 4), then the result suggests increased autophagosome synthesis occurring simultaneously with reduced degradation.

p62 degradation—Figure 3 shows the expected p62 immunoblotting results in the different theoretical scenarios. The differences in the amount of p62 between samples in the presence and absence of lysosomal inhibitors represent the amount of p62 that is recruited into autophagosome and delivered to lysosomes for degradation. The densitometric value of (lane 2 - lane 1) reflects the amount of p62 degraded through autophagy in untreated control cells; while (lane 4 - lane 3) value reflects the amount of p62 degraded in the cells pre-exposed to a particular compound. In scenario 1, (lane 2 - lane 1) < (lane 4 - lane 3) suggests that the compound promotes the delivery of p62 into lysosomes, which in turn indicates an increase in autophagic activity. Conversely, (lane 2 - lane 1) > (lane 4 - lane 3) in scenario 2 suggests that the compound inhibits the clearance of p62 through autophagy, which in turn indicates a decrease in autophagic activity.

Fluorescence microscopy—In both indirect immunofluorescence and GFP-LC3 transfected cells, autophagosomes are primarily visualized as fluorescent dots or sometimes ring-shaped structures. In the control cells, LC3 signal should mainly distribute diffusely in the cytoplasm and nucleus. Since most cells display basal levels of constitutive autophagy, a few punctuate dots may be detected in the control cells. A strong autophagic stimulus (e.g.,

starvation) is expected to induce a large number of LC3 puncta in the cells, as shown in Figure 4, which indicates an increase in the absolute number of autophagosomes. The presence of LC3 puncta can be less evident in response to other stimuli. Furthermore, the increases in LC3 dots need to be interpreted with caution, since accumulation of autophagosomes is a function of the balance between the rate of their generation and the rate of degradation. An increase in LC3 puncta may reflect autophagy induction and/or suppression of autophagosome clearance. It has been reported that larger LC3 punctate structures are detected when the autophagosome-lysosome fusion is blocked, which is possibly due to autophagosome-autophagosome fusion (Kuma et al., 2007). However, it needs to be pointed out that although one may detect changes in the size of LC3 puncta, it is not possible to correlate the puncta size with autophagy activity by fluorescence microscopy alone.

Tandem mRFP-GFP fluorescence microscopy—To determine the autophagic flux, the relative abundance of red versus yellow puncta is compared. An induction of autophagy can be tracked by increases in both red and yellow puncta. An inhibition of autophagy due to a defect in autophagosome formation is shown by a decrease in yellow and red puncta. If autophagy is impaired at autophagosome maturation into autolysosome, one may observe an increase in yellow puncta without a concomitant increase in red puncta.

Time Considerations

Cells can be exposed to the tested drug or treatment for different time periods, usually from 30 min to 48 hrs. The immunoblotting assay can be completed in two days. The gels can be run and transferred on day 1, and the blots are incubated overnight with primary antibody; so the membranes are ready to be exposed to film on day 2. Alternatively, one can also finish the immunoblotting assay in one day by using 1 hr primary antibody incubation at room temperature. Blots may need to be stripped and reprobed with a primary antibody of loading control, in which case the whole process takes 3 days in total. Samples collection including cell lysis and protein quantitation may take several hours depending on the number of samples.

The timeline of immunostaining is similar to the one of immunoblot, with an overnight incubation of primary antibody. If GFP-LC3 is used, the transient transfection usually takes 1–2 days before the cells are ready for treatments. The fixation and slide making can be done in 2 hours. Analyzing cells by epifluorescence or confocal microscope can be time consuming, which usually take more than 1 day depending on the number of samples and the way of quantification.

Acknowledgments

This work was supported in part by a grant from the National Institutes of Health (R01 ES07331, R01 ES10563 and R01 ES020852) and an Albert Einstein College of Medicine startup fund.

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SIGNIFICANCE STATEMENT

Autophagy plays an important homeostatic role in cells by producing substrates for both energy metabolism and vital protein synthesis, as well as by removing damaged organelles and misfolded proteins. Autophagy has been implicated in multiple physiological processes, such as embryogenesis and regulation of immunity. Conversely, dysregulation of autophagy may contribute to certain human diseases, including cancer, neurodegeneration and inflammatory disorders. Given the strong significance of autophagy in physiological and pathological processes, there is a growing need for methods that accurately identify and quantify autophagy in a given biological context. This unit is intended to introduce several basic methods that are reliable and convenient for measuring autophagy in mammalian cells.

Figure 1.

Representative immunoblots for LC3 turnover assay with β -actin as a protein loading control. Mouse primary astrocytes were cultured in regular serum-supplemented Dulbecco's modified Eagle's medium (DMEM) (10% horse serum) or incubated for 2 hrs in starvation conditions (serum-free DMEM) in the presence(+)/absence(-) of lysosomal inhibitors, leupeptin (100 μ M) and NH_4Cl (20 mM) for 2 hrs. Positions of LC3-I and -II are indicated.

Figure 2.

Schematic representation of the LC3 flux assay in different theoretical scenarios. Cartoons depict immunoblots and densitometry for LC3-II showing four different possible outcomes of autophagy modulation by a drug. Calculations for determination of net LC3 flux are at the right of each blot. Densitometric values of samples are subtracted from corresponding inhibitor-treated value, and the results represent the amount of LC3-II delivered to lysosomes. Higher values correspond to increased autophagic flux. Levels of loading control are assumed to be constant.

Figure 3. Schematic representation of the p62 degradation assay in different theoretical scenarios. Cartoons depict immunoblots and densitometry for p62 showing two different possible outcomes of autophagy modulation by a drug. Calculations for the amount of p62 degraded inside autophagosomes are at the right of each blot. Higher values correspond to increased autophagic flux. Levels of loading control are assumed to be constant.

Figure 4.

Representative indirect immunofluorescence images for endogenous LC3 puncta formation upon induction of autophagy. Mouse primary astrocytes were cultured in regular serum-supplemented DMEM (10% horse serum) or incubated for 4 hrs in starvation conditions (serum-free DMEM) and then subjected to immunofluorescence analysis using anti-LC3 antibody. Distinct LC3 puncta were observed in response to serum removal. Nuclei are in blue (DAPI). Scale bar, 10 μ m.