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LC3 and Autophagy

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Summary

Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein with a molecular mass of ~17kDa that is distributed ubiquitously in mammalian tissues and cultured cells. During autophagy, autophagosomes engulf cytoplasmic components, including cytosolic proteins and organelles. Concomitantly, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are degraded by lysosomal hydrolases. At the same time, LC3-II in autolysosomal lumen is degraded. Thus, lysosomal turnover of the autophagosomal marker LC3-II reflects starvation-induced autophagic activity, and detecting LC3 by immunoblotting or immunofluorescence has become a reliable method for monitoring autophagy and autophagy-related processes, including autophagic cell death. Here we describe basic protocols to assay for endogenous LC3-II by immunoblotting, immunoprecipitation, and immunofluorescence.

Key Words: LC3; lipidation; ubiquitylation-like reaction; autophagosome; autolysosome; autophagy; *ATG* conjugation system.

1. Introduction

Autophagy is the major protein degradation system responsible for the turnover of bulky cellular constituents ***(1)***. As a pivotal cellular housekeeping system, autophagy contributes to maintain intercellular homeostasis ***(2)***. In addition, autophagy has been found to play significant roles in antigen presentation, bacterial and viral infection, and cell death ***(3–6)***. Dysfunction of autophagy has been proposed as an underlying mechanism for neurodegenerative diseases, muscle diseases, cancer, and hepatic inflammation.

From: *Methods in Molecular Biology, vol. 445: Autophagosome and Phagosome*
Edited by: V. Deretic © Humana Press, Totowa, NJ

During autophagy, unique double-membraned autophagosomes are formed to engulf intracellular components, including organelles such as mitochondria. Autophagosomes fuse with lysosomes to form autolysosomes, and sequestered intra-autophagosomal components are degraded by lysosomal hydrolases.

LC3 was originally identified as one of three light chains (LC1, LC2, and LC3) associated with purified MAP1A and MAP1B ***(7)***. Until its autophagy-specific role as a mammalian Atg8 homologue became established, LC3 had been long thought to be involved in the regulation of assembly and disassembly of microtubules ***(8,9)***. During the formation of autophagosomal membranes, cytosolic LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) ***(10,11)*** through two consecutive ubiquitylation-like reactions catalyzed by the E1-like enzyme Atg7 and the E2-like enzyme Atg3 ***(12–14)*** to LC3-II ***(8)***. During the fusion of autophagosomes with lysosomes, intra-autophagosomal LC3-II is also degraded by lysosomal proteases **(Fig. 1)**. Therefore, as a marker of autophagosomal membranes, changes in cellular LC3-II level are connected to the dynamic turnover of LC3-II via the lysosome, i.e., autophagic activity ***(9)***. Monitoring LC3-

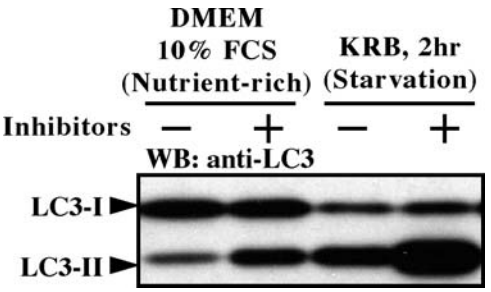

Fig. 1. Lysosomal turnover of endogenous LC3-II during autophagy. For nutrient-rich conditions, HEK293 cells were cultured in DMEM medium containing 10% FCS. Where indicated, cells were treated with the protease inhibitors, E64d (10 μg/mL) and pepstatin A (10 μg/mL) (Inhibitors +) for 2 h, or, as a negative control (Inhibitors -), with the solvent dimethylsulfoxide. For starvation conditions, cells were incubated for 4 h in Krebs Ringer bicarbonate buffer (KRB) in the presence (+) or absence (-) of protease inhibitors. The cells were lysed, total proteins (10 μg per lane) were separated by SDS-PAGE, and endogenous LC3 in the lysates was recognized by immunoblotting with an anti-LC3 antibody (WB: anti-LC3). LC3-I, soluble form of LC3; LC3-II, membrane-bound form of LC3. During the formation of autophagosomes, LC3 is lipidated, and LC3-II is localized on autophagosomes and autolysosomes. While intra-autophagosomal LC3-II is degraded by lysosomal hydrolases, a transient cellular amount of LC3-II does not simply reflect starvation-induced autophagic activity. Since endogenous LC3-II is considerably degraded by lysosomal hydrolases after formation of autolysosomes, lysosomal turnover of LC3-II should be investigated to estimate an autophagic activity using E64d and pepstatin A.

I and LC3-II by Western blotting, immunoprecipitation, and immunofluorescence is essential for investigating the mechanism of mammalian autophagy.

In assaying endogenous LC3-II by immunoblotting, it is important to consider the hydrophobicity of LC3-II. Since LC3-II is a protein–PE conjugate, only part of it can be extracted in the presence of 1% Triton X100 ***(11)***. Insufficient extraction, however, will lead to misleading results **(Fig. 2)**. Similar considerations apply to immunofluorescent analyses of endogenous LC3. In this chapter, we describe a protocol for effective extraction of LC3-II. In the latter section of this chapter, we mention several treatment precautions.

2. Materials

2.1. Cell Culture and Lysis

1. Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal calf serum (Invitrogen).
2. Krebs Ringer bicarbonate buffer (KRB) for nutrient starvation: 118.5 m*M* NaCl, 4.74 m*M* KCl, 1.18 m*M* KH_2PO_4, 23.4 m*M* $NaHCO_3$, 6 m*M* glucose, 2.5 m*M*

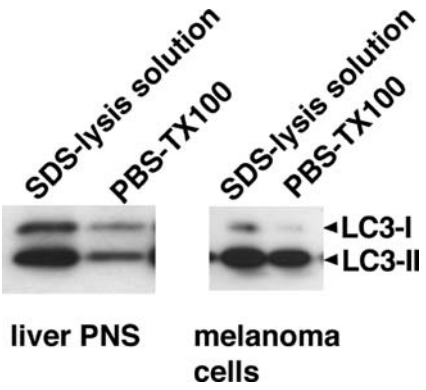

Fig. 2. Immunoprecipitation of LC3-I and LC3-II. Mouse liver postnuclear supernatant (2 mg protein in 100 µL) or total lysate of cultured B16 melanoma cells (0.5 mg in 100 µL) was mixed with an equal volume (100 µL) of SDS-lysis solution and subjected to immunoprecipitation as described in the text. The resulting immunoprecipitate was treated with modified Laemmli buffer, separated by SDS-PAGE, and analyzed by immunoblotting. For comparison, the same postnuclear supernatant or the lysate was mixed with an equal volume of PBS containing 1% TX-100. The mixtures were cleared by centrifugation at 15,000*g* for 10 min. LC3 was immunoprecipitated from the resultant supernatant and the precipitated antigens were analyzed by immonoblotting. SDS-lysis solution; LC3-I and LC3-II immunoprecipitated from the samples treated with SDS-lysis solution, PBS-TX-100; LC3-I and LC3-II immunoprecipitated from the samples treated with PBS containing 1% TX-100.

$CaCl_2$, 1.18 m*M* $MgSO_4$, and 6 mg/L phenol red ***(15)*** (*see* **Note 1**), adjusted to pH 7.6 by titration with 1 *N* NaOH.

3. E64d (Peptide Institute, Inc., Osaka, Japan) ***(16,17)*** and pepstatin A (Peptide Institute, Inc.) ***(16,18)*** dissolved at 10 mg/mL in dimethyl sulfoxide (special grade for biochemical analysis) (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). These 1000-fold concentrated stock solutions are stored at -20ºC, at which temperature they are stable for at least 3 months (*see* **Note 2**).
4. Phosphate-buffered saline (PBS): 20 m*M* sodium phosphate, pH 7.2, and 150 m*M* NaCl.
5. Complete™ protease inhibitor cocktail (Roche Diagnostics).
6. An ultrasonic generator with a micro-probe (model USP-300, Shimadzu, Tokyo, Japan).
7. Modified Laemmli buffer: 0.1 *M* Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) glycerol, 6% (v/v) *β*-mercaptoethanol, and 0.03% (w/v) bromophenol blue.
8. Teflon cell scrapers (Sumitomo Bakelite, Tokyo, Japan).
9. BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL).
10. TX100-lysis solution: 2% polyethylene glycol mono-*p*-isooctylphenyl ether (Triton X100, Nacalai Tesque, Inc., Kyoto, Japan) in PBS with the complete protease inhibitor cocktail (Roche Diagnostics) (for option 2b).

2.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Stock solutions for preparing 12.5% SDS-gels: 1.5 *M* Tris-HCl, pH 8.8 (at 25ºC), 0.5 *M* Tris-HCl, pH 6.8 (at 25ºC), 30% acrylamide solution (29:1 (w/w) acrylamide: methylene-bisacrylamide) and *N,N,N´,N´*-tetramethyl-ethylenediamide (TEMED, Wako).
2. Ammonium persulfate (APS): 10% (w/v) in water, prepared immediately before use.
3. Water-saturated isobutanol: Equal volumes of water and isobutanol are shaken in a glass bottle and allowed to separate; the top layer is stored at room temperature.
4. Running buffer: 25 m*M* Tris, 192 m*M* glycine, and 0.1% (w/v) SDS. Do not adjust pH. Store at room temperature.
5. Prestained molecular weight markers, broad range (6–175 kDa) (New England Biolabs, Beverly, MA): The solution contains 0.01% (w/v) phenol red and 0.01% (w/v) bromophenol blue.
6. Apparatuses for SDS-gel electrophoresis: NA-1030 (Nihon-Eido, Tokyo, Japan) for a mini-wide gel (1.0 mm thick, 200 mm wide, 105 mm high, suitable for 16–20 samples per gel).

2.3. Western Blotting for LC3-I and LC3-II

1. Bjerrum and Schafer-Nielsen‘s transfer buffer: 48 m*M* Tris, 39 m*M* glycine, 20% methanol (analytical grade), pH 9.0–9.4, depending on reagent purity. Do not adjust pH; if lower than pH 9.0, prepare again.

2. Polyvinylidene difluoride (PVDF) membrane (Durapore membrane GV, pore size 0.22 μm; Millipore, Bedford, MA) and chromatography paper (cat. no. 590; Advantec Japan, Tokyo, Japan).
3. Transblot SD Semi-Dry Transfer Cell (BioRad, Hercules, CA) for transferring proteins from polyacrylamide gels to PVDF membranes.
4. Tris-buffered saline containing polyixyehlene sorbitan monolaurate/Tween 20 (TTBS): 20 m*M* Tris-HCl, pH 7.5 at 25ºC, 150 m*M* NaCl, and 0.05% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20).
5. Blocking buffer: 5% (w/v) nonfat dry milk (Snow Brand Milk Products Co. Ltd., Tokyo, Japan) in TTBS.
6. Primary antibody solution: 1 μg/mL anti-LC3 IgG, 1% (w/v) fraction V bovine serum albumin (BSA), 20 m*M* Tris-HCl, pH 7.5 at 25ºC, 0.1% NaN_3 and 150 m*M* NaCl. When stored at 4ºC, it can be reused at least 10 times within a year.
7. Secondary antibody solution: 0.005% (v/v) goat anti-rabbit IgG antibody (minimum cross-reactivity with other species) conjugated to horse radish peroxidase and 1.5% (v/v) normal goat serum (Invitrogen) in TTBS.
8. Enhanced chemiluminescent reagents: SuperSignal West Dura Extended Duration Substrate or SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL).

2.4. Immunoprecipitation of Endogenous LC3

1. SDS-lysis solution: 2% (w/v) SDS in PBS with Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN).
2. TX100-solution: 1% polyethylene glycol mono-*p*-isooctylphenyl ether (Triton X100, Nacalai tesque) in PBS with Complete™ protease inhibitor cocktail (Roche Diagnostics).
3. TX100-wash solution: 2% polyethylene glycol mono-*p*-isooctylphenyl ether (Triton X100, Nacalai tesque) in PBS with Complete protease inhibitor cocktail (Roche Diagnostics).
4. Protein A-agarose (Santa Cruz Biotechnology, Santa Cruz, CA).
5. Modified Laemmli buffer: 0.1 *M* Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 6% (v/v) *β*-mercaptoethanol, and 0.03% (w/v) bromophenol blue.
6. A 5-mL syringe and 25G × 1" needle (Terumo, Tokyo, Japan).

2.5. Immunofluorescence Analysis of Endogenous LC3

1. Cell-fix solution: 4% paraformaldehyde in PBS, pH 7.2, prepared just before use and warmed to 37ºC.
2. Digitonin solution: 50 μg/mL digitonin (Wako) in PBS, pH 7.2.
3. Quenching solution: 50 m*M* NH_4Cl in PBS.
4. IF-blocking solution: 2% (w/v) BSA, 5% (v/v) normal goat serum (Invitrogen), 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
5. IF primary antibody solution: 5 μg/ml of affinity purified anti-LC3 IgG in IF-blocking solution.

6. IF secondary antibody solution: 0.05% (v/v) goat anti-rabbit IgG conjugated to Alexa488, 594 or Q-dot 605 (Invitrogen) in IF-blocking solution.
7. Tris-buffered saline (TBS): 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
8. Mounting medium: SlowFade light (Invitrogen).
9. Fluorescence microscope (e.g., a Zeiss Axioplan2 fluorescence microscope; Carl Zeiss, Thornwood, NY) and an ORCA-ER CCD camera and Aqua C-imaging system (Hamamatsu Photonics, Tokyo, Japan).

2.6. Antibodies

Polyclonal rabbit antibodies and mouse monoclonal antibodies to LC3 were raised to synthetic peptides corresponding to amino-terminal sequences ***(8)*** and to LC3 fused with GST expressed in *Escherichia coli* ***(19)*** (*see* **Note 3**).

3. Methods

It is important to consider the hydrophobicity of LC3-II when preparing cell lysates and during immunoprecipitation. Since LC3-II consists of LC3 conjugated to PE, autophagosomal and autolysosomal LC3-II is only partially solubilized in PBS containing 1% Triton X100, 1% Nonidet P40, or 1% Tween 20. To completely solubilize LC3-II from cells and tissue homogenates, we have boiled samples in PBS containing 1% SDS or used PBS containing 2% Triton X100. Under less stringent conditions, LC3-II is insufficiently extracted (**Fig. 2**).

Rather than immunostaining of endogenous LC3-II, a recombinant fluorescent protein, GFP-LC3, overexpressed in cells has often been employed to visualize autophagosomes. Even after autolysosomal degradation, however, a fraction of the free GFP cleaved from GFP-LC3 remains resistant to digestion in the lumen of autolysosomes. Therefore, when using GFP-LC3 as a reporter, care must be taken to avoid measuring the pseudo-fluorescence of free GFP. To avoid this potential artifact, immunofluorescence analysis of endogenous LC3-II is preferred. Due to the hydrophobicity of LC3-II, use of methanol and ethanol to permeabilize cells should be avoided. Since structures of autophagosomes and autolysosomes are sensitive to some detergents, we normally use digitonin to permeabilize cells.

3.1. Cell Culture and Preparation of Cell Lysates for Recognizing LC3-I and LC3-II

1. To investigate autophagic response under starvation conditions, prewarmed KRB buffer is prepared. When lysosomal degradation of LC3-II is suspected, one thousandth of the stock solutions of E64d and pepstatin A are added to the medium to yield final concentrations of 10 μg/mL. As a control, the solvent DMSO is

added to the medium. The cells, in a 60-mm dish, are incubated at 37ºC for 3–6 h to accumulate LC3-II; in general, LC3-II accumulates in cultured cells 3 h after adding the inhibitors. As a control of nutrient-rich conditions, HEK293 cells are incubated in DMEM containing 10% FCS.

2. The cells are washed twice in PBS, harvested with Teflon cell scrapers, resuspended in ice-cold 200 μL of PBS with Complete™ protease inhibitor cocktail (Roche Diagnostics), and lysed with an ultrasonic generator for 10 s (0.5-s pulse with 50% duty) on ice.
   [Option 2b for preparation of cell-lysate without sonication: TX100-lysis solution (final concentration: 2% Triton X-100) is added to washed cells and incubated with mild shaking.]
3. The protein concentration of the lysate is measured using a BCA protein assay kit.
4. Modified Laemmli buffer is added to the samples, and the samples are boiled for 5 min. (For recognizing endogenous LC3, 10–20 μg per lane should be loaded onto SDS-polyacrylamide gels.)
5. The samples are chilled on ice and centrifuged for 10 min at 15,000*g*.

3.2. SDS-PAGE for Recognizing Endogenous LC3-I and LC3-II

1. Glass plates are prepared for SDS-PAGE (for mini gels, spacer thickness is 1 mm). The inner plates should be cleaned with 70% ethanol.
2. A running gel solution (3.2 mL of distilled water, 4.2 mL of 30% acrylamide solution, 2.5 mL of 1.5 *M* Tris-HCl, pH 8.8 (at 25ºC), 0.1 mL of 10% SDS, 33 μL of 10% APS, and 5 μL of TEMED) is prepared and poured into the space between the glass plates.
3. The solution is gently overlaid with 150 μL of water-saturated isobutanol, and the gel is allowed to polymerize for 30–60 min at room temperature.
4. A stacking gel solution (2.89 mL of distilled water, 0.79 mL of 30% acrylamide solution, 1.25 mL of 1.5 *M* Tris-HCl, pH 6.8 [at 25ºC], 0.05 mL of 10% SDS, 17 μL of 10% APS, and 5 μL of TEMED) is prepared.
5. The isobutanol is removed from the polymerized running gel by washing the top with deionized water. Residual water is gently removed with a paper towel. The stacking gel solution is poured into the space between the glass plates, and a comb is inserted.
6. After the stacking gel polymerizes, the comb is removed, and the wells are washed.
7. The gel is mounted into the SDS-PAGE electrophoresis chamber, and running buffer is added.
8. Samples (10–20 μg per lane) are loaded onto the gel. A 5-μL aliquot of prestained molecular weight markers is employed as a standard for molecular weights.
9. The gel is electrophoresed at 1–1.5 mA current constant per cm of gel width, until the pink color of the phenol red in the markers reaches the bottom of the gel. (Good separation between LC3-I and LC3-II occurs when a fraction of the phenol red runs through the gel.)

3.3. Western Blotting for LC3-I and LC3-II

1. A PVDF membrane is soaked in methanol for 3 min, transferred to Bjerrum and Schafer-Nielsen's transfer buffer, and incubated for 5 min.
2. Four sheets of chromatography paper are soaked in Bjerrum and Schafer-Nielsen's transfer buffer.
3. After removing the glass plates and stacking gel, the running gel is rinsed in Bjerrum and Schafer-Nielsen's transfer buffer. Two sheets of chromatography paper are placed on the anode plate and overlaid with a PVDF membrane, the running gel, and two additional sheets of chromatography paper.
4. The stack is set onto a Transblot SD Semi-Dry Transfer Cell.
5. The stack is electrophoresed at 16 V for one hour.
6. The gel and papers are discarded, and the PVDF membrane is allowed to dry on a paper towel.
7. Blocking: The PVDF membrane is incubated in Blocking buffer at room temperature for 30 min.
8. The membrane is washed three times in TTBS at room temperature for 2 min each.
9. Primary antibody reaction: The PVDF membrane is incubated in primary antibody solution for 60 min at room temperature.
10. The membrane is washed at least five times, for 5 min each, in TTBS at room temperature.
11. Secondary antibody reaction: The PVDF membrane is incubated in secondary antibody solution for 60 min at room temperature.
12. The membrane is washed at least three times, for 10 min each, in TTBS at room temperature.
13. The reaction of horse radish peroxidase with chemiluminescent reagent is started on the membrane, and the signal is detected on x-ray film or CCD camera **(Fig. 1)**.

3.4. Immunoprecipitation of Endogenous LC3

1. Cells on a 60-mm dish are washed twice in PBS, harvested with Teflon cell scrapers, and resuspended in 100 μL of PBS. (When immunoprecipitating endogenous LC3 from rat (or mouse) tissues, postnuclear supernatant should be prepared.)
2. An equal volume of SDS-lysis solution is added to the cell suspension or postnuclear supernatant. After mixing gently by repeated inverting, the samples are boiled at 100ºC for 5 min and chilled on ice for 3 min.
3. Seven volumes of TX100-solution are added to each sample and mixed thoroughly. Each sample is centrifuged at 10,000*g* for 15 min at 4ºC, and the pellets are discarded.
4. One mL of TX100-solution is added to 50 μL of protein A–Agarose beads. The suspensions are centrifuged at 500*g* for 5 min at 4ºC to collect the beads.
5. Each cell suspension supernatant obtained at **step 3** is added to 50 μL of equilibrated protein A–Agarose beads.

6. The suspensions are incubated at 4ºC for one hour with rotating to remove proteins that bind nonspecifically to the beads. The suspensions are centrifuged at 500*g* for 5 min at 4ºC, and the supernatants are collected.
7. Protein A–Agarose beads are equilibrated as described in **step 4**.
8. The precleared supernatants are added to the equilibrated protein A–Agarose beads, along with 1 μg of affinity-purified anti-LC3 IgG or, as a negative control, 1 μg of normal rabbit IgG.
9. The mixtures are incubated at 4ºC for 16 h with rotation.
10. The mixtures are centrifuged at 500*g* for 5 min at 4ºC, and the supernatants are discarded using a 5-mL syringe with 25G × 1" needle (0.5 mm × 25 mm) (Terumo, Tokyo, Japan).
11. The beads are washed at least five times by vortexing in TX100-wash solution.
12. The beads are collected by centrifugation at 500*g* for 5 min.

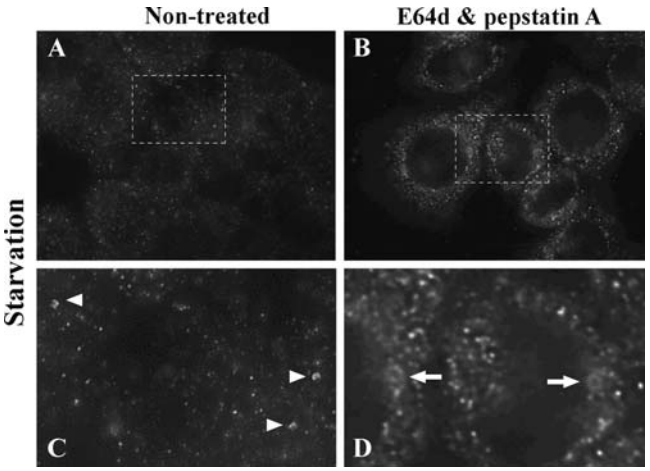

Fig. 3. Accumulation of endogenous LC3-positive puncta under starvation conditions in the presence of E64d and pepstatin A in HeLa cells. HeLa cells were transferred to KRB medium for 4 h (starvation). "E64d & pepstatin A" (**B** and **D**) indicates that cells were incubated for 4 h with these protease inhibitors, whereas "nontreated" (**A** and **C**) indicates addition of a solvent instead of these inhibitors. The cells were fixed and permeabilized with digitonin, and endogenous LC3 was recognized using rabbit anti-human LC3 antibody and goat anti-rabbit IgG conjugated to Q-dot 605. Intracellular fluorescence of endogenous LC3 in HeLa cells was observed. A series of Z-scanned fluorescent images was deconvoluted by a program for two-dimensional blind deconvolution with Aqua C-imaging software (Hamamatsu Photonics). Images **C** and **D** are about fourfold magnifications of the images shown in boxes with dotted lines in **A** and **B**, respectively. An arrowhead indicates a cup-shaped preautophagosome, and an arrow indicates an autophagosome/autolysosome.

13. Fifty µl of modified Laemmli buffer is added to the beads, boiled for 5 min, and chilled on ice for one minute.
14. The immunoprecipitates are analyzed as described in **Subheadings 3.2.** and **3.3.** **(Fig. 2)**.

3.5. Immunofluorescence Analysis of Endogenous LC3

1. After washing in TBS, cells are incubated in cell-fix solution at 37ºC for 5 min.
2. The cells are rinsed twice in TBS.
3. Cells are permeabilized in digitonin solution at 37ºC for 5 min. The solution is discarded by aspirating.
4. Excess digitonin is quenched by incubation in quenching solution at 37ºC for 5 min. The solution is discarded by aspirating.
5. The cells are rinsed twice in TBS.
6. Blocking: Cells are incubated in IF-blocking solution at 37ºC for 30 min.
7. The cells are rinsed three times in TBS.
8. Primary antibody reaction: Cells are incubated in IF-primary antibody solution at 37ºC for 60 min.
9. The cells are washed three times in TBS for 5 min.
10. Secondary antibody solution: Cells are incubated in IF secondary antibody solution at 37ºC for 60 min.
11. The cells are washed five times in TBS for 5 min.
12. The cells are mounted on glass slides with mounting medium.
13. Fluorescent images are obtained using a fluorescence microscope **(Fig. 3)**.

4. Notes

1. Earle‘s balanced salt solution (EBSS) can be substituted for KRB for starvation conditions. If using Hanks’ balanced salt solution (HBSS) instead of KRB as a starvation medium, more care should be taken regarding buffering and incubation. HBSS should be buffered by adding 10 m*M* hydroxyethyl piperazine ethane sulfonate (HEPES). When cells are incubated in HBSS, it may be better to incubate them at 37ºC without control of CO_2.
2. Storage of pepstatin A for over 3 months should be avoided.
3. Polyclonal and monoclonal anti-LC3 antibodies are now available from the Medical & Biological Laboratories, Co. Ltd (http://www.mbl.co.jp/) and Cell Signaling Technology (http://www.cellsignal.com/).

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