

Detection of LC3-Associated Phagocytosis (LAP)

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Phagocytes, notably macrophages, are critical sentinels of their environment, patrolling for and eradicating unwanted components. The ability of cells to process extracellular cargo in an appropriate manner is important for both clearance of the cargo and eventual return to homeostasis. Although the evolutionarily conserved pathway of autophagy involves the degradation and recycling of unnecessary or dysfunctional cellular components during starvation, we now appreciate that the reach of autophagy extends beyond nutrient deprivation, notably including cellular quality control (e.g., mitophagy) and host defense against internalized pathogens (i.e., xenophagy). Despite being seemingly disparate, autophagic functions are unified as conserved mechanisms for containment and immunosuppression, suggesting an original immune function for autophagy. A recently described pathway called LC3-associated phagocytosis (LAP) marries the ancient concepts of phagocytosis and autophagy, revealing new ways in which the autophagy machinery, in a molecularly distinct pathway, contributes to the inflammatory response. In this article, protocols to detect LAP by electron microscopy, immunofluorescence, flow cytometry, and phagosome purification are described, allowing the user to detect multiple characteristics of LAP in both qualitative and quantitative manners. Published 2020. U.S. Government.

Basic Protocol 1: Detection of LAP by electron microscopy

Basic Protocol 2: Detection of LAP by confocal microscopy of LC3-GFP-expressing cells

Alternate Protocol 1: Detection of LAP by confocal microscopy using immunofluorescence

Basic Protocol 3: Detection of LAP using flow cytometry of LC3-GFP-expressing cells

Alternate Protocol 2: Detection of LAP using antibody staining and flow cytometry

Basic Protocol 4: Detection of LAP by western blot of purified LAPosomes

Keywords: autophagy • confocal microscopy • electron microscopy • flow cytometry • LC3-associated phagocytosis (LAP) • phagosome purification

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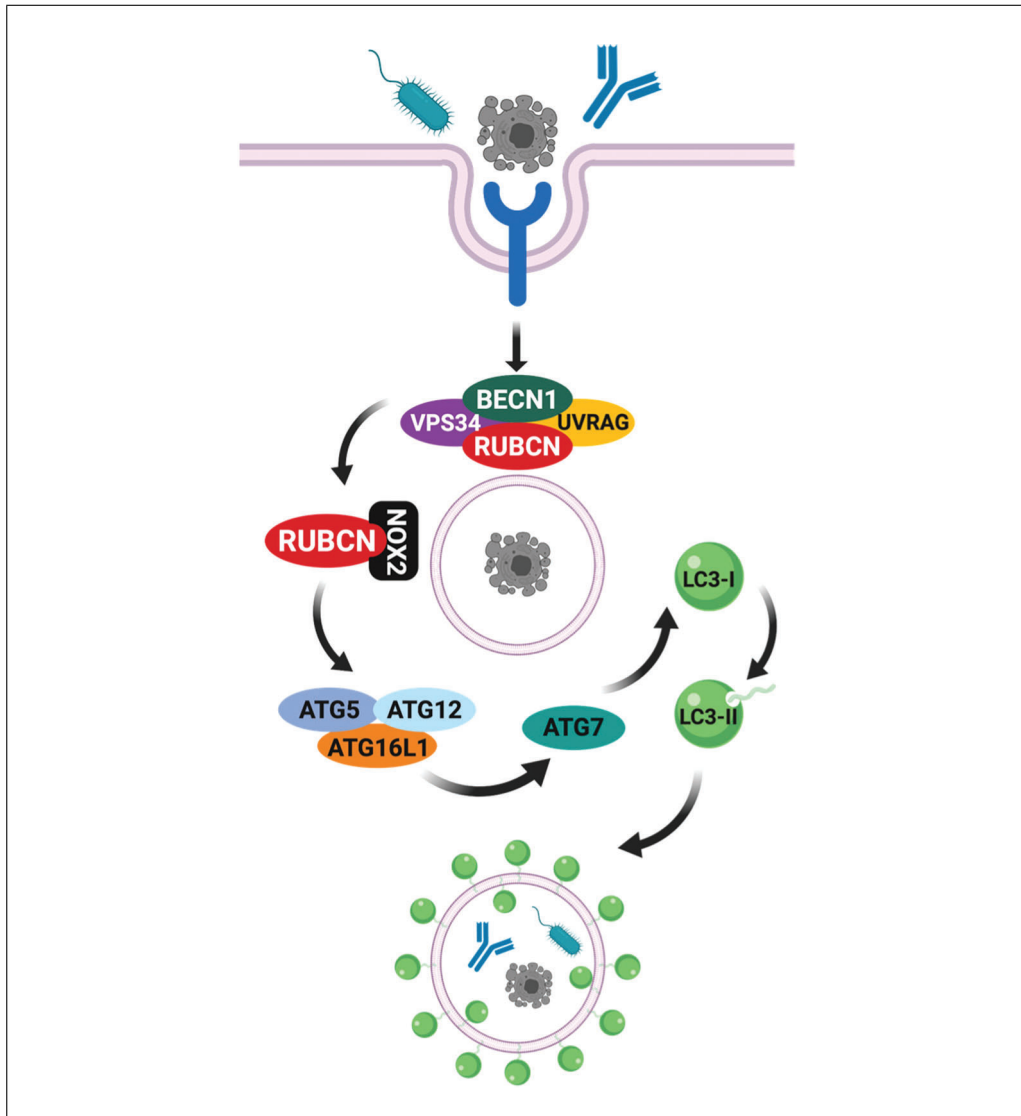


Figure 1 Molecular mechanisms of the LC3-associated phagocytosis (LAP) pathway. Engulfment of pathogens, dying cells, or antibody-coated particles via TLR, PtdSer, and FcR, respectively, triggers recruitment of the RUBCN-containing Class III PI3K complex to the cargo-containing phagosome. RUBCN also binds and stabilizes the NOX2 complex. RUBCN activity is required for recruitment of the downstream ubiquitin-like conjugation systems to generate LC3-II and localize it to the cargo-containing phagosome to form the LAPosome.

INTRODUCTION

LC3-associated phagocytosis (LAP) is a novel form of non-canonical autophagy, wherein receptor engagement during phagocytosis triggers the recruitment of some, but not all, members of the autophagy machinery to the single-membraned cargo-containing phagosome, or LAPosome (Sanjuan et al., 2007) (Fig. 1). When it was originally discovered by Sanjuan and colleagues in 2007 (Sanjuan, Milasta, & Green, 2009), LAP was initially detected and distinguished from canonical autophagy using electron microscopy (EM) and fluorescence techniques. In addition to these techniques (Basic Protocols 1 and 2 and Alternate Protocol 1), this article describes other key methods for measuring LAP, including flow cytometry (Martinez et al., 2015) (Basic Protocol 3 and Alternate Protocol 2) and phagosome purification (Henault et al., 2012; Martinez et al., 2015) (Basic Protocol 4). Although multiple stimuli have been described to engage LAP, these methods primarily use zymosan (Basic Protocols 1 to 3) or LAP ligand-coated latex beads (Basic Protocol 4) as examples to demonstrate the principle of the procedures.

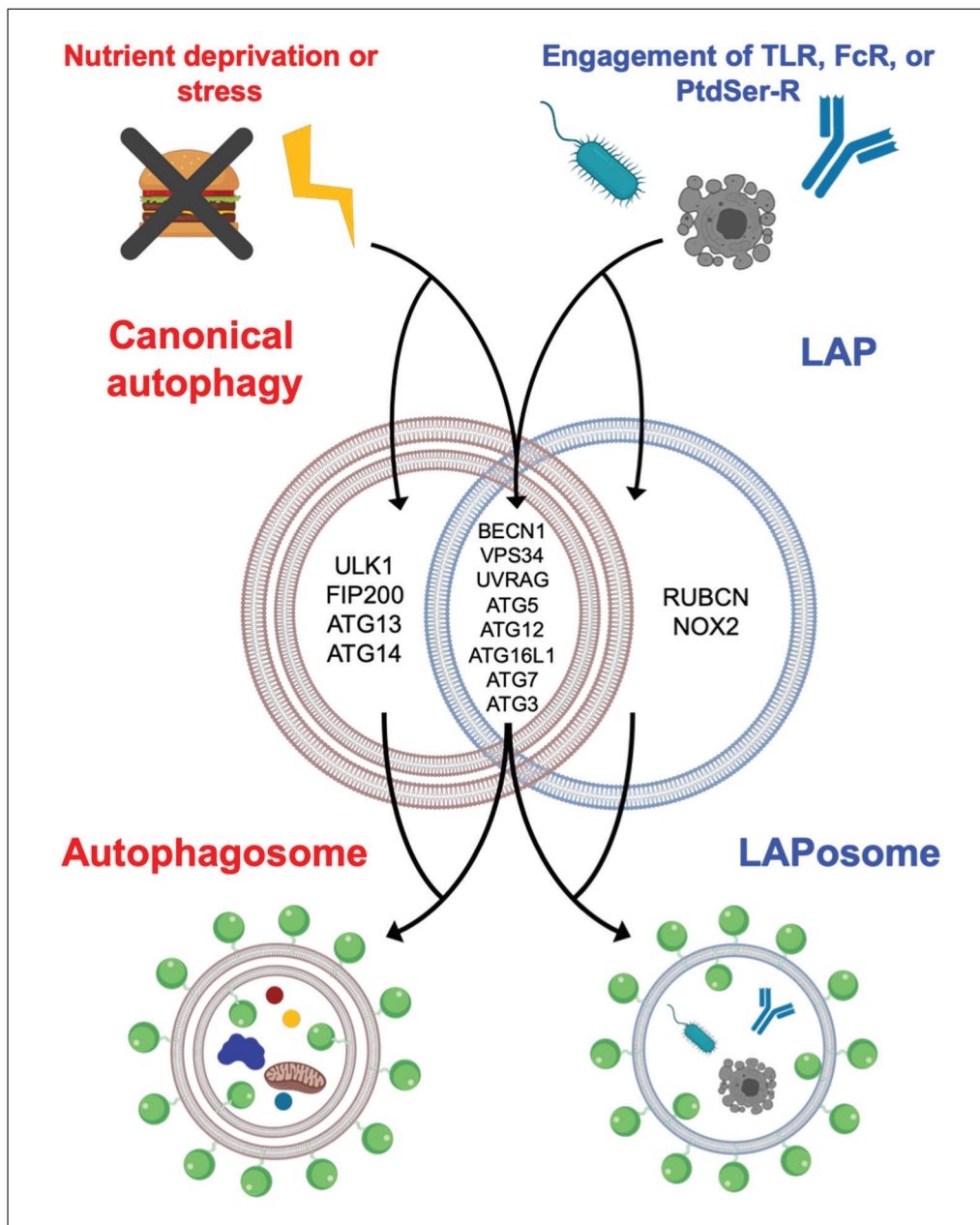


Figure 2 Molecular overlap of canonical autophagy and LAP. The genes required for canonical autophagy are shown on the left; for LAP, on the right; and for both, in the center.

These methods, however, can be adapted to be useful for the detection of LAP with other stimuli.

STRATEGIC PLANNING

Both canonical autophagy and LAP result in the lipidation of LC3-I to form LC3-II (Martinez et al., 2015). Therefore, it is necessary to use healthy, viable cells for these assays to avoid induction of autophagic LC3-II, which could hinder the detection of LAP-dependent LC3-II. It is also important to remember that LAP is a dynamic process. It is advisable to perform a time course using one of the below techniques to determine the timing of LC3-II association with the phagosome using your stimuli of interest in your cells of interest.

There is much molecular overlap between LAP and canonical autophagy (Fig. 2). Thus, it is important to characterize any methods in wild-type cells, autophagy-deficient but

LAP-sufficient cells (such as ULK1-, FIP200-, ATG13-, or ATG14-deficient cells), and LAP-deficient but autophagy-sufficient cells (such as RUBCN-deficient cells).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a 37°C, 5% CO₂ incubator unless otherwise specified.

**BASIC
PROTOCOL 1**

DETECTION OF LAP BY ELECTRON MICROSCOPY

As described by Sanjuan et al. (2009), EM was used to detect one of the first discernible differences between canonical autophagy and LAP. Whereas the autophagosome comprises a well-described double membrane, the LAPosome is a single-membraned structure that contains engulfed cargo. However, detection of engulfed cargo within a single-membraned phagosome is only enough to distinguish LAP from autophagy, as EM, in the absence of immunogold staining (discussed in Melo, Morgan, Monahan-Earley, Dvorak, & Weller, 2014), will not allow one to detect the presence of phagosome-associated LC3-II. Further assays, such as those described in Basic Protocols 2 to 4, are needed to definitively define a process as LAP.

Materials

Cell line or primary cells of interest in cell culture medium
Zymosan A [Zymosan A (*Saccharomyces cerevisiae*) BioParticles™, unlabeled, Invitrogen, cat. no. Z2849] or equivalent LAP stimulus
Phosphate-buffered saline (PBS), 4°C
2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.35; Tousimis, cat. no. 1051)
0.1 M sodium cacodylate buffer (Sigma, cat. no. 70114, or equivalent)
3.5% (w/v) molten water agar
1% (v/v) tannic acid (CAS no. 1401-55-4) in 0.1 M sodium cacodylate buffer
1% (w/v) osmium tetroxide (CAS no. 20816-12-0) in 0.1 M sodium cacodylate buffer
30%, 50%, 70%, 90%, and 100% (v/v) ethanol in PBS
100% propylene oxide (CAS no. 75-56-9)
1:1, 1:2, and 1:3 Poly/Bed 812 resin (Polysciences, cat. no. 08791-500, or equivalent)/acetone (CAS no. 67-64-1)
Poly/Bed 812 resin (Polysciences, cat. no. 08791-500, or equivalent)
3% (w/v) aqueous uranyl acetate (see recipe)
Distilled water
2.66% (w/v) leadcitrate (see recipe)

6-well plates (Corning™, cat. no. 3516, or equivalent)
Cell lifter (Corning™, cat. no. 3008, or equivalent)
Microcentrifuge tubes
Microcentrifuge
50-ml conical tubes or beakers
Fine-tipped tweezers
Capped containers (e.g., conical tubes)
Platform shaker
Wide-mouth plastic transfer pipet
Embedding capsules or molds
~60°C oven
JEOL 1200 EXII transmission electron microscope with digitalized Gatan camera (ES500W) or equivalent

1. Plate cell line or primary cells of interest in cell culture medium at the desired concentration in 6-well plates (≤ 3 ml/well) and incubate for 4 hr to overnight at 37°C and 5% CO₂ to allow cells to adhere.
2. Add zymosan A or an equivalent LAP stimulus at desired particle-to-cell ratio.
For zymosan A, a ratio of 8:1 is recommended. Other LAP stimuli may require different particle-to-cell ratios.
3. Carefully aspirate cell culture medium and harvest cells with gentle scraping with a cell lifter in ~ 1 ml cold PBS per well. Pellet cells in microcentrifuge tubes with centrifugation for 5 min at $500 \times g$.
4. Aspirate PBS and pre-fix cells in 1 ml of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.35) for 1 hr at room temperature.
5. Pellet cells with centrifugation for 5 min at $500 \times g$. Aspirate glutaraldehyde solution and wash two times with 1 ml of 0.1 M sodium cacodylate buffer.
6. For ease of staining, embed cell pellet in 2 ml of 3.5% molten water agar, let cool, and then cut agar into 1-mm cubes.
7. Incubate cells in agar with excess volume of 1% tannic acid in 0.1 M sodium cacodylate buffer for 1 hr at room temperature.
8. Remove cells in agar from the tannic acid using fine-tipped tweezers, transfer to a 50-ml conical tube or beaker, and submerge cells in agar in excess volume of 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hr at room temperature.
9. Remove cells in agar from the osmium tetroxide solution and wash cells in agar with excess volume of 0.1 M sodium cacodylate buffer three times.
10. Perform dehydration in excess volume of a graded ethanol series in PBS in 50-ml conical tubes or beakers:
 - a. Submerge cells in agar in 30% ethanol in PBS for 15 min.
 - b. Transfer cells in agar to 50% ethanol in PBS and submerge for 15 min.
 - c. Transfer cells in agar to 70% ethanol in PBS and submerge for 15 min.
 - d. Transfer cells in agar to 90% ethanol in PBS and submerge for 15 min.
 - e. Transfer cells in agar to 100% ethanol and submerge for 15 min.
 - f. Transfer cells in agar to fresh 100% ethanol and submerge for 15 min.
11. Transfer cells in agar to excess volume of 100% propylene oxide in a 50-ml conical tube or beaker and submerge for 15 min. Repeat for a total of two times.
12. Transfer cells in agar to a solution of PolyBed 812 resin/acetone in a capped container at a 1:3 ratio. Incubate for 1 hr on a platform shaker.
13. Transfer cells in agar to a solution of PolyBed 812 resin/acetone in a capped container at a 1:2 ratio. Incubate for 1 hr on a platform shaker.
14. Transfer cells in agar to a solution of PolyBed 812 resin/acetone in a capped container at a 1:1 ratio. Incubate for 1 hr on a platform shaker.
15. Transfer cells in agar to a solution of 100% PolyBed 812 resin in a capped container. Incubate for 1 hr on a platform shaker.
16. Transfer cells in agar to a new solution of 100% PolyBed 812 resin in a capped container. Incubate overnight on a platform shaker.
17. Using a wide-mouth plastic transfer pipet, place a drop of the resin mixture in the bottom of each embedding capsule or mold. Then, carefully place sample in the capsule or mold. Use pipet to fill the capsule or mold with resin.

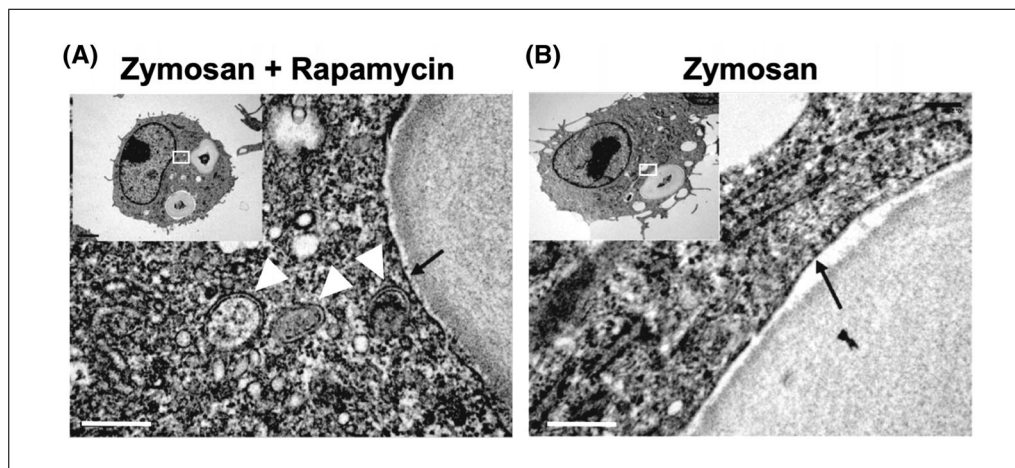


Figure 3 Structural comparison of rapamycin-induced autophagosomes (**A**, white arrowheads) and zymosan-induced LAPosomes (**A** and **B**, black arrow) by electron microscopy. Reprinted with permission from Springer Nature (Sanjuan et al., 2007).

18. Incubate samples in an oven at $\sim 60^{\circ}\text{C}$ for ≥ 24 hr but ≤ 3 days.
19. After polymerization, incubate for 15 min with excess volume of 3% aqueous uranyl acetate solution.
20. Rinse with excess volume of distilled water.
21. Incubate for 5 min with excess volume of 2.66% lead citrate solution.
22. Rinse with excess volume of distilled water.
23. Capture images with a JEOL 1200 EXII transmission electron microscope with a digitalized Gatan camera (ES500W) or equivalent.

LAP is characterized by the engulfed cargo being contained within a single-membraned structure (LAPosome, Fig. 3A), whereas starvation or stress-induced autophagy results in the formation of double-membraned autophagosomes (Fig. 3B).

BASIC PROTOCOL 2

DETECTION OF LAP BY CONFOCAL MICROSCOPY OF LC3-GFP-EXPRESSING CELLS

Immunofluorescence imaging of LC3 localization with cargo-containing phagosomes has proven to be a reliable and robust technique to detect LAP. Localization of LC3 can occur as early as 15 min post-phagocytosis, and fusion of the LAPosome with the lysosome, which degrades LC3-II, can occur as early as 45 min post-phagocytosis. It is important to establish stimulus- and cell-type-specific timing of LAP early in a project's timeline. Other proteins may also be visualized using this protocol (via expression of fluorescently tagged components) or Alternate Protocol 1 (via immunofluorescent staining).

Materials

50 $\mu\text{g/ml}$ poly-D-lysine (see recipe; make fresh) or equivalent extracellular matrix solution

PBS

Cell line or primary cells expressing LC3-GFP in cell culture medium

Zymosan A–Alexa Fluor 594 [Zymosan A (*Saccharomyces cerevisiae*)

BioParticlesTM, Alexa FluorTM-594 conjugate, Invitrogen, cat. no. Z23374] or equivalent LAP stimulus

Chamber slides (NuncTM Lab-TekTM II Chambered Coverglass, Thermo Fisher, cat. no. 155360, 155379, 155382, or 155409, or 1.5 borosilicate glass equivalent)

Table 1 Recommended Volumes and Cell Densities for Chamber Slides

Nunc™ Lab-Tek™ II Chambered Coverglass cat. no.	Number of wells	Cell culture volume (ml)	Recommended cell concentration ($\times 10^4$ /ml)	Wash or reagent volume (ml)
155360	1	2.0-4.5	20	~2.0
155379	2	1.0-2.0	10	~1.0
155382	4	0.5-1.0	5	~0.5
155409	8	0.2-0.5	2.5	~0.25

Environmental control chamber (optional)

Laser-scanning confocal microscope (LSCM) equipped with confocal system,
argon-ion laser at 488 nm, and DPSS lasers at 561 or 594 nm

Oil-immersion 40 \times 1.3 N.A. objective with phase-contrast optics

NOTE: If performing live-cell imaging in an environmental control chamber (see step 4), prepare the chamber's temperature ($\sim 37^\circ\text{C}$) and carbon dioxide (5% CO_2) ≥ 1 hr prior to imaging.

1. Coat bottom of chamber slides with 50 $\mu\text{g/ml}$ poly-D-lysine or equivalent extracellular matrix solution for 5 min. Remove poly-D-lysine solution and return to container for reuse (store at 4°C). Wash chamber slides once with PBS.

Perform coating in a biosafety cabinet.

2. Plate cell line or primary cells expressing LC3-GFP in cell culture medium at the desired concentration in the chamber slides and incubate at 37°C and 5% CO_2 for >4 hr to allow cells to adhere.

Primary cells isolated from LC3-GFP^{+/-} animals are suitable for experimentation.

See Table 1 for the recommended volumes and cell densities for chamber slides.

3. Add zymosan A–Alexa Fluor 594 or an equivalent LAP stimulus at desired particle-to-cell ratio.

For zymosan A, a ratio of 8:1 is recommended. Other LAP stimuli may require different particle-to-cell ratios.

4. For live-cell imaging, place chamber slides in an environmental control chamber, with conditions maintained at $\sim 37^\circ\text{C}$ and 5% CO_2 .

For acquisition of images at static intervals (see step 5), an environmental control chamber is not required.

5. To capture static images, use an LSCM equipped with a confocal system, an argon-ion laser at 488 nm (LC3), and DPSS lasers at 561 or 594 nm (zymosan A).

6. Acquire images at desired intervals using an oil-immersion 40 \times 1.3 N.A. objective with phase-contrast optics following the manufacturer's instructions.

DETECTION OF LAP BY CONFOCAL MICROSCOPY USING IMMUNOFLUORESCENCE

Although expression of fluorescently tagged LC3 (Basic Protocol 2) allows for live-cell imaging of its localization with cargo-containing LAPosomes, visualization of LAPosomes can also be achieved via immunofluorescent staining. Moreover, this technique can be adopted for visualization of other proteins localizing with the LAPosome.

**ALTERNATE
PROTOCOL 1**

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Additional Materials (also see Basic Protocol 2)

Cell line or primary cells of interest in cell culture medium
Image-iTTM Fixative Solution (Invitrogen, cat. no. FB002) or equivalent
1 µg/ml DAPI (CAS no. 28718-90-3) in PBS or other nucleic acid dye (optional)
0.15% (v/v) Triton X-100 (CAS no. 9002-93-1) in PBS
2% (w/v) bovine serum albumin (BSA; CAS no. 9048-46-8) in PBS (store at 4°C)
Anti-LC3B antibody (Abcam, cat. no. ab51520)
Goat anti-rabbit antibody conjugated to Alexa Fluor 488 [Goat Anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Invitrogen, cat. no. A11008, or equivalent]

1. Coat bottom of chamber slides with 50 µg/ml poly-D-lysine or equivalent extracellular matrix solution for 5 min. Remove poly-D-lysine solution and return to container for reuse (store at 4°C). Wash chamber slides once with PBS.

Perform coating in a biosafety cabinet.

2. Plate cell line or primary cells of interest in cell culture medium at the desired concentration in the chamber slides and incubate for 4 hr to overnight at 37°C and 5% CO₂ to allow cells to adhere.

See Table 1 for the recommended volumes and cell densities for chamber slides.

3. Add zymosan A–Alexa Fluor 594 or an equivalent LAP stimulus at desired particle-to-cell ratio. Incubate at 37°C and 5% CO₂.

For zymosan A, a ratio of 8:1 is recommended. Other LAP stimuli may require different particle-to-cell ratios.

4. At the desired time-point(s), aspirate medium from the wells and wash three times with PBS.

5. Aspirate PBS and fix with Image-iTTM Fixative Solution or equivalent for 10 min at room temperature.

A solution of 3% (v/v) paraformaldehyde (from 16% stock, EMS, cat. no. 15710) in PBS (pH 7.2) can also be used as a fixative.

6. Aspirate fixative and wash three times with PBS.

7. Optional: Stain fixed cells with 1 µg/ml DAPI in PBS or other nucleic acid dye for 1 min. Then, aspirate dye solution and wash three times with PBS.

Store chamber slides (containing PBS) at 4°C in the dark at this point or continue to step 8.

8. Aspirate PBS and permeabilize cells in 0.15% Triton X-100 in PBS for 10 min at room temperature.

9. Aspirate Triton X-100 solution and block with 2% BSA in PBS for 30 min at room temperature.

To reduce background fluorescence, block with Image-iTTM FX Signal Enhancer (Invitrogen, cat. no. I36933) for 30 min according to the manufacturer's instructions.

10. Aspirate blocking solution and wash three times with 2% BSA in PBS.

11. Prepare primary antibody staining solution by diluting anti-LC3B antibody at 1:2000 in 2% BSA in PBS.

A dilution of 1:2000 is recommended by the manufacturer, but the dilution may need to be optimized by the user.

Other antibodies with different reactivity (such as mouse polyclonal antibodies) can also be used at this time.

12. Aspirate final wash from step 10 and incubate cells with the primary antibody staining solution for 1 hr at room temperature.
13. Aspirate antibody solution and wash cells three times with 2% BSA in PBS.
14. Prepare secondary antibody staining solution by diluting goat anti-rabbit antibody conjugated to Alexa Fluor 488 at 1:250 in 2% BSA in PBS.

A dilution of 1:250 is recommended by the manufacturer, but the dilution may need to be optimized by the user.

Other secondary antibodies with different reactivity (such as anti-mouse secondary antibodies) can also be used at this time.

15. Aspirate final wash from step 13 and incubate cells with secondary antibody staining for 45 min at room temperature.
16. Aspirate antibody solution and wash cells three times with PBS. Add more PBS to chamber slides.

Store chamber slides (containing PBS) at 4°C in the dark at this point or continue to image capture (step 17).

17. For imaging, use an LSCM equipped with confocal system, an argon-ion laser at 488 nm (LC3), and DPSS lasers at 561 or 594 nm (zymosan A).

Other lasers may be required for additional staining.

18. Acquire images at desired intervals using an oil-immersion 40× 1.3 N.A. objective with phase-contrast optics, following the manufacturer's instructions.

As depicted in Figure 4, LC3-GFP translocates to the zymosan A-containing LAPosome rapidly upon engulfment. LC3-GFP localization can occur as quickly as 15 min after engulfment, so it is important to perform a time-course analysis of LAP with your cells and stimuli of interest.

DETECTION OF LAP USING FLOW CYTOMETRY OF LC3-GFP-EXPRESSING CELLS

**BASIC
PROTOCOL 3**

Although immunofluorescent imaging can provide real-time analysis of LAP kinetics as well as vital information on localization, interpretation and analysis can be subjective. Recent studies have used flow cytometry (adapted from Shvets, Fass, & Elazar, 2008; Zappavigna, Lombardi, Misso, Grimaldi, & Caraglia, 2017) to measure the amount of membrane-bound LC3-II associated with cells that have phagocytosed fluorescent particles. This technique allows for a quantitative analysis of LAP by measuring the mean fluorescent intensity of LC3-GFP during LAP. This technique, however, cannot convey direct localization of LC3 with the LAPosome and should be coupled with confocal imaging (Basic Protocol 2) for verification.

Materials

Cell line or primary cells expressing LC3-GFP in cell culture medium
200 μM rapamycin (see recipe; make fresh)
10 mM chloroquine (see recipe; make fresh)
Zymosan A–Alexa Fluor 594 [Zymosan A (*Saccharomyces cerevisiae*)
BioParticles™, Alexa Fluor™-594 conjugate, Invitrogen, cat. no. Z23374] or
equivalent LAP stimulus
PBS, 4°C

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20 $\mu\text{g/ml}$ digitonin (from 20 mg/ml digitonin; see recipe), 4°C
FACS buffer (see recipe), 4°C

6-well plates (Corning™, cat. no. 3516, or equivalent)

Conical tubes

Standard tabletop centrifuge

FACS tubes

Flow cytometer with 488-nm and 561-nm lasers

1. Plate cell line or primary cells expressing LC3-GFP in cell culture medium at the desired concentration in 6-well plates in (≤ 3 ml/well) and incubate for 4 hr to overnight at 37°C and 5% CO₂ to allow cells to adhere.

Primary cells isolated from LC3-GFP^{+/-} animals are suitable for experimentation.

Plate extra wells for single-color compensation controls. Wells for no fluorescence, GFP, and Alexa Fluor 594 should be used.

2. Add 200 nM rapamycin (200 μM stock) and 10 μM chloroquine (10 mM stock) to single-color compensation control compensation-control well and incubate for 4 to 18 hr at 37°C and 5% CO₂.

This well will also serve as a positive control.

3. Add zymosan A–Alexa Fluor 594 or an equivalent LAP stimulus at desired particle-to-cell ratio. Incubate at 37°C and 5% CO₂.

For zymosan A, a ratio of 8:1 is recommended. Other LAP stimuli may require different particle-to-cell ratios.

4. At the desired time-point(s), aspirate medium from the wells, collect cells into conical tubes, and wash two times with 5 ml cold PBS with centrifugation for 5 min at 500 $\times g$.

5. Aspirate final wash, resuspend cells in 1 ml cold 20 $\mu\text{g/ml}$ digitonin, and incubate on ice for 10 min.

Check for permeabilization with trypan blue (>90% of cells should be trypan blue⁺).

6. Pellet permeabilized cells with centrifugation for 5 min at 500 $\times g$ and wash cells two times with 5 ml cold PBS.

7. Resuspend in ~ 200 μl cold FACS buffer in FACS tubes.

8. Acquire samples on a flow cytometer with 488-nm and 561-nm lasers.

ALTERNATE PROTOCOL 2

DETECTION OF LAP USING ANTIBODY STAINING AND FLOW CYTOMETRY

Detection of LAPosome-associated LC3-II can also be achieved with antibody-mediated immunofluorescent staining rather than flow cytometry (Basic Protocol 3). This protocol can be adapted to observe the localization of other proteins with the LAPosome.

Additional Materials (also see Basic Protocol 3)

4% (v/v) paraformaldehyde (from 16% stock, EMS, cat. no. 15710) in PBS
(optional), 4°C

Anti-LC3A/B antibody conjugated to Alexa Fluor 488 [LC3A/B (D3U4C) XP®
rabbit antibody, Alexa Fluor® 488 Conjugate, Cell Signaling, cat. no. 13082]

1. Plate cell line or primary cells of interest in cell culture medium at the desired concentration in 6-well plates (≤ 3 ml/well) and incubate for 4 hr to overnight at 37°C and 5% CO₂ to allow cells to adhere.

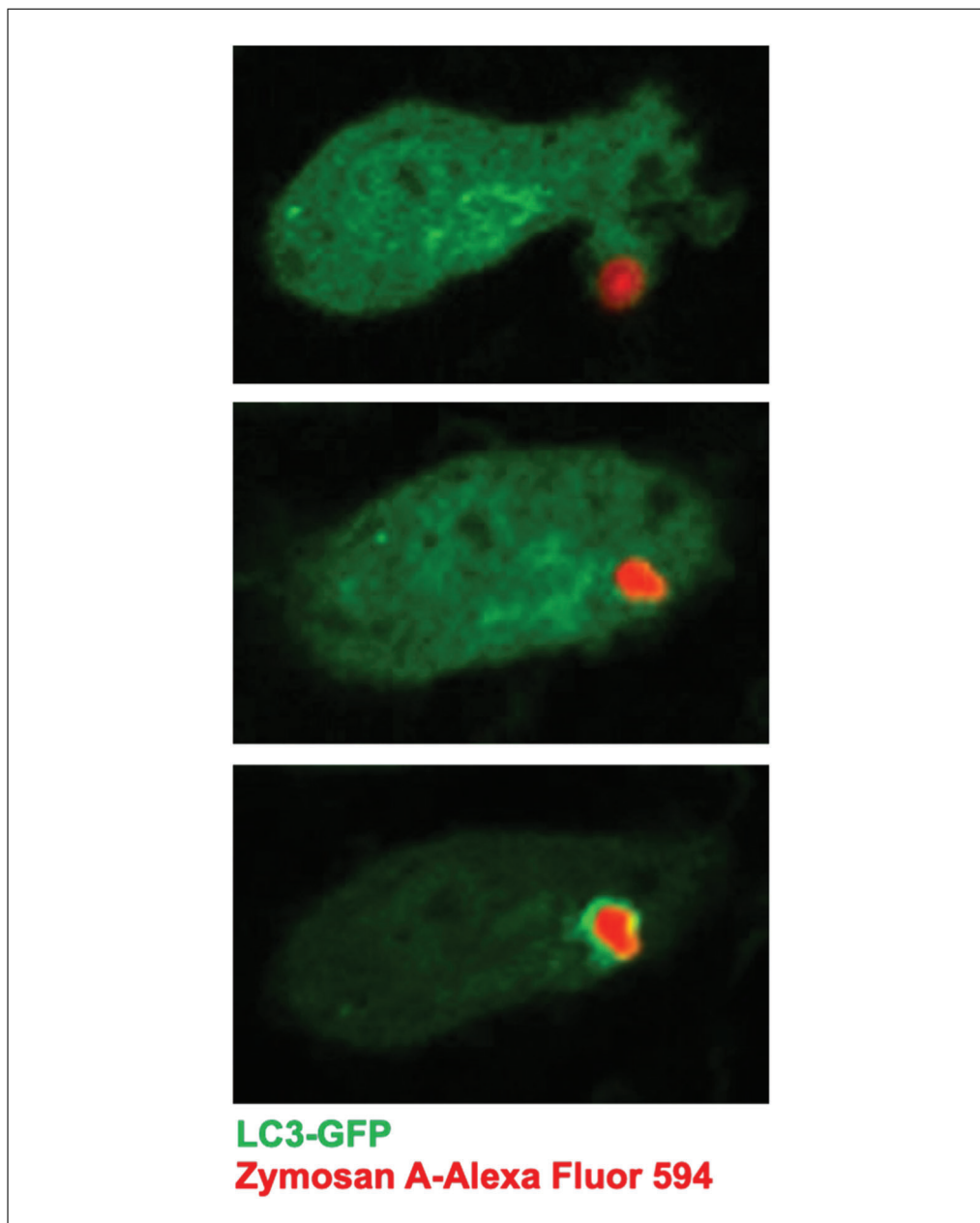


Figure 4 Visualization of LC3-GFP recruitment to a LAPosome containing zymosan A–Alexa Fluor 594 by confocal imaging. The still images are from time-lapse imaging of LC3-GFP⁺ macrophages' phagocytosis of zymosan A–Alexa Fluor 594, followed by recruitment of LC3-GFP to the zymosan-containing LAPosome. Top, $t = 10$ min; middle, $t = 20$ min; bottom, $t = 30$ min.

Plate extra wells for single-color compensation controls. Wells for no fluorescence, GFP, and Alexa Fluor 594 should be used.

2. Add 200 nM rapamycin (200 μ M stock) and 10 μ M chloroquine (10 mM stock) to single-color GFP compensation control well and incubate for 4 to 18 hr at 37°C and 5% CO₂.

This well will also serve as a positive control.

3. Add zymosan A–Alexa Fluor 594 or an equivalent LAP stimulus at desired particle-to-cell ratio. Incubate at 37°C and 5% CO₂.

For zymosan A, a ratio of 8:1 is recommended. Other LAP stimuli may require different particle-to-cell ratios.

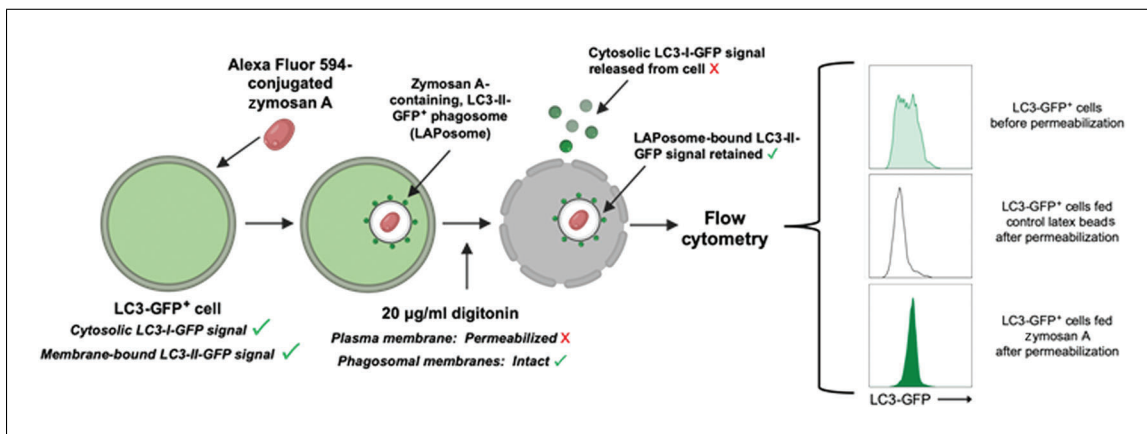


Figure 5 Use of flow cytometry to measure LAPosome-associated LC3-GFP. The workflow and example data are shown for the flow cytometric method for measuring LAPosome-associated LC3-GFP.

- At the desired time-point(s), aspirate medium from the wells, collect cells into conical tubes, and wash two times with 5 ml cold PBS with centrifugation for 5 min at $500 \times g$.

- Aspirate final wash, resuspend cells in 1 ml cold 20 $\mu\text{g/ml}$ digitonin, and incubate on ice for 10 min.

Check for permeabilization with trypan blue (>90% of cells should be trypan blue⁺).

- Pellet permeabilized cells with centrifugation for 5 min at $500 \times g$ and wash cells two times with 5 ml cold PBS.

- Optional: Fix cells with 1 ml cold 4% paraformaldehyde in PBS on ice for 10 min. Wash twice with 5 ml cold PBS.

If desired, store in PBS at 4°C.

- Aspirate final wash and incubate with anti-LC3A/B antibody conjugated to Alexa Fluor 488 diluted at 1:50 in cold FACS buffer (500 μl total volume) for 15 min on ice.

A dilution of 1:50 is recommended by the manufacturer, but the dilution may need to be optimized by the user.

Non-conjugated LC3A/B antibodies may be used at this step, followed by incubation with species-specific, secondary fluorescent antibodies.

- Wash once with 5 ml cold FACS buffer and centrifugation for 5 min at $500 \times g$.

If needed, incubate with species-specific, secondary fluorescent antibody for 15 min on ice. Wash two times with cold FACS buffer and centrifugation for 5 min at $500 \times g$.

- Resuspend in $\sim 200 \mu\text{l}$ cold FACS buffer in FACS tubes.

- Acquire samples on a flow cytometer with 488-nm and 561-nm lasers.

- Measure mean fluorescent intensity of LC3-GFP (488 nm) of events positive for Alexa Fluor 594 (561 nm).

Although use of flow cytometry to quantify LC3-GFP association with the LAPosome (Fig. 5) represents a quantitative measure of LAP, users should always test wild-type cells, autophagy-deficient but LAP-sufficient cells (such as ULK1-, FIP200-, ATG13-, or ATG14-deficient cells), and LAP-deficient but autophagy-sufficient cells (such as RUBCN-deficient cells). Comparison of these three phenotypes will allow for a more robust interpretation of flow cytometry data.

DETECTION OF LAP BY WESTERN BLOT OF PURIFIED LAPOSOMES

Purification of latex bead-containing phagosomes, adapted from Desjardins, Huber, Parton, & Griffiths (1994), is a useful tool for analyzing the protein composition of vesicles. By comparing proteins associated with an uncoated latex bead to proteins associated with a latex bead coupled to a LAP stimulus, such as TLR2 agonist Pam3csk4 or FcR agonist IgG, one can determine the proteins specifically and uniquely associated with the LAPosome. Because of the relatively small amount of material obtained during purification, it is necessary to purify phagosomes from $>10^7$ cells.

Materials

Cell line or primary cells of interest in cell culture medium

Uncoated latex beads (Polybead[®] Microspheres 3.00 μm , Polysciences, cat. no. 17134)

LAP beads: mouse IgG-coated polystyrene particles, crosslinked (Spherotech, cat. no. MsGPX-50-5), or Pam3csk4-coated beads (see recipe)

PBS, 4°C

Homogenization buffer (see recipe), 4°C

10%, 25%, 35%, and 62% (w/v) sucrose solutions (see recipe), 4°C

RIPA buffer (Sigma, cat. no. R0278-50ML, or equivalent)

10-cm dishes (100 \times 21 mm Dish, Nunclon[™] Delta, Thermo Fisher, cat. no. 172931, or equivalent) or other appropriately sized vessels

Cell lifter (Corning[™] cat. no. 3008, or equivalent)

Conical tubes

4°C refrigerated centrifuge

1-ml syringe with 27-G needle (Becton Dickinson, cat. no. 309623, or equivalent)

Ultra-centrifuge tubes (e.g., Open-Top Thinwall Ultra-Clear Tube, 14 \times 89 mm, 13.2 ml, Beckman Coulter, cat. no. 344059)

4°C refrigerated ultra-centrifuge with swinging-bucket and fixed-angle rotors

Additional reagents and equipment for western blotting (see Current Protocols article; Ni, Xu, & Gallagher, 2017)

1. Plate cell line or primary cells in \sim 25 ml cell culture medium at the desired concentration in 100-cm dishes or other appropriately sized vessels and incubate for 4 hr to overnight at 37°C and 5% CO₂ to allow cells to adhere.
2. Add uncoated latex beads (control) or LAP beads (IgG-coated beads or Pam3csk4-coated beads) at desired bead-to-cell ratio (recommended: 10:1). Incubate at 37°C and 5% CO₂.
3. At the desired time-point(s), aspirate medium and wash three times with 10 ml cold PBS on ice to remove unphagocytosed beads.
4. Aspirate final wash and add 10 ml cold PBS to each dish.
5. With each dish still on ice, use a cell lifter to remove cells. Collect cells in PBS in a conical tube.
6. Pellet cells with centrifugation for 10 min at 500 \times g, 4°C.
7. Aspirate PBS and wash cell pellet in 10 ml cold homogenization buffer with centrifugation for 10 min at 500 \times g, 4°C.
8. Remove supernatant and resuspend pellet in 1 ml cold homogenization buffer.
9. With each tube on ice, homogenize cells by drawing up and expelling the cell suspension multiple times using a 1-ml syringe with a 27-G needle.

Check for cell disruption using a light microscope (>90% of cells should be disrupted yet have an intact nucleus).

10. Isolate bead-containing phagosomes (BCPs) via flotation on a sucrose step gradient as follows, with all procedures on ice:

- a. Add an equal volume of 62% sucrose solution (~1.45 ml with cell pellet volume) to cells in homogenization buffer (~1.45 ml, including cell pellet volume), to a final concentration of 40% sucrose.

The final volume of the solution (cells in 40% sucrose) should be ~2.9 ml.

- b. Pipet 1.45 ml of 62% sucrose solution into an ultra-centrifuge tube.
- c. Carefully and slowly pipet cells in 40% sucrose on top of the 62% sucrose layer.
- d. Carefully and slowly pipet 2.9 ml of 35% sucrose solution on top of layer of cells in 40% sucrose.
- e. Carefully and slowly pipet 2.9 ml of 25% sucrose solution on top of 35% sucrose layer.
- f. Carefully and slowly pipet 2.9 ml of 10% sucrose solution on top of 25% sucrose layer.

The volumes listed above are appropriate for a 13.2-ml ultra-centrifuge tube. Other sizes of ultra-centrifuge tubes require different volumes, and the protocol should be adjusted accordingly.

11. Centrifuge sucrose gradients in a refrigerated ultra-centrifuge with a swinging-bucket rotor for 1 hr at $100,000 \times g$, 4°C.
12. Carefully collect BCP band using a 1000- μ l pipet tip and transfer to a new ultra-centrifuge tube.

The BCP band will be visible at the interface of the 10% and 25% sucrose solutions.

13. Wash BCP with cold PBS and centrifugation in the refrigerated ultra-centrifuge with a fixed-angle rotor for 15 min at $40,000 \times g$, 4°C.
14. Remove supernatant and resuspend pellet in ~50 μ l RIPA buffer for western blot analysis (see Current Protocols article; Ni et al., 2017).

The BCP pellet may be very small, so be careful not to disturb it when removing the supernatant.

If other proteomics procedures are performed with the purified BCP fraction, resuspend the pellet in the appropriate solution and proceed.

*Although purification is based on the density of the latex beads, users should ensure that the protein fraction that they are analyzing is phagosomal by using markers such as *UNC93B* in addition to LAP-specific proteins, such as *RUBCN* (Fig. 6).*

REAGENTS AND SOLUTIONS

Aqueous uranyl acetate, 3%

1. Add 0.3 g uranyl acetate (Polysciences, cat. no. 21447-25, or equivalent) to 10 ml distilled water.
2. Sonicate in a water bath until in solution.
3. Store ≤ 1 month at room temperature and protect from light.
4. Let freshly made solution sit for ≥ 18 hr prior to use.

Chloroquine, 10 mM

1. To make a 1000 \times stock of chloroquine, add 5.1586 mg chloroquine (CAS no. 50-63-5) to 1 ml deionized water under sterile conditions.
2. Incubate in a 37°C water bath and invert to mix.

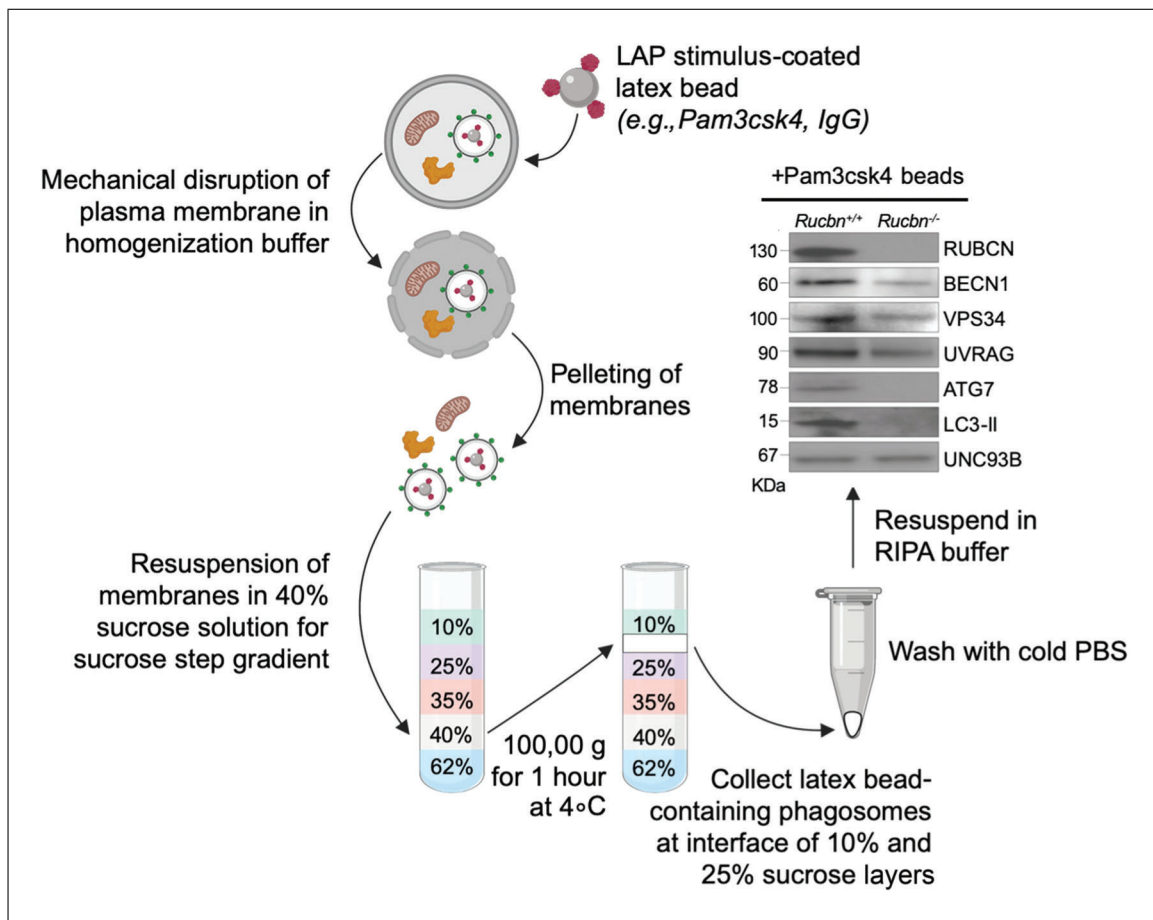


Figure 6 Phagosome purification to assess protein association with LAPosomes. The workflow and example data (reprinted from Martinez et al., 2015) are shown for phagosome purification to analyze protein association.

3. Store ≤ 2 years at -20°C and protect from light.
4. To make a $1\times$ working stock of chloroquine solution (prepare fresh immediately before use), dilute $1000\times$ stock at 1:1000 (final working concentration $10\ \mu\text{M}$) in deionized water under sterile conditions.
5. Invert to mix.
6. Dispose of working solution after use.

Digitonin, 20 mg/ml

1. Add 20 mg digitonin (Sigma, cat. no. D141) to 1 ml deionized water under sterile conditions.
2. Heat to 95°C to dissolve.
3. Once dissolved, return to room temperature before use or store ≤ 1 week at 4°C .

FACS buffer

1. Add 1 g BSA (CAS no. 9048-46-8) to 100 ml PBS.
2. Add $100\ \mu\text{g}$ sodium azide (CAS no. 26628-22-8) to BSA-PBS solution.
3. Use a magnetic stir bar and plate to mix and dissolve. Apply heat if needed.
4. Store ≤ 2 years at 2° to 8°C .

Homogenization buffer

1. Add 42.7875 g sucrose (CAS no. 57-50-1) to 100 ml of 3 mM imidazole (pH 7.4; see recipe).
2. Use a magnetic stir bar and plate to mix.
3. Use HCl to adjust pH to 7.4, if needed.
4. Store ≤ 2 years at 2° to 8°C .

Imidazole (pH 7.4), 3 mM

1. Add 102.12 μg imidazole (CAS no. 288-32-4) to 500 ml deionized water.
2. Use a magnetic stir bar and plate to mix.
3. Use HCl to adjust pH to 7.4.
4. Store ≤ 2 years at 2° to 8°C.

Lead citrate, 2.66%

1. Add 30 ml distilled water to a volumetric flask.
2. Add 1.33 g lead citrate (Polysciences, cat. no. 25350-100, or equivalent) to above flask.
3. Add 1.76 g sodium citrate dihydrate (CAS no. 6132-04-03) to above flask.
4. Insert a stopper into flask and shake vigorously for 1 min.
5. Let solution stand for 30 min with occasional shaking.
6. Add 8 ml of 1 N NaOH (CAS no. 1310-73-2) to flask.
7. Bring flask volume to 50 ml with ~ 12 ml distilled water.
9. Invert to mix.
10. Store ≤ 3 months at 2° to 8°C in a tightly stopped flask.

Pam3csk4-coated beads

1. Wash 150 μl bead suspension (Polybead Amino Microspheres, 2.1×10^8 particles/ml, Polysciences, cat. no. 19118-2) twice in PBS with centrifugation for 5 min at $12,000 \times g$, room temperature.
2. Prepare 5% glutaraldehyde by adding 1 ml of 25% aqueous glutaraldehyde (CAS no. 111-30-8) to 5 ml deionized water. Invert to mix.
3. Resuspend beads in 100 μl of 5% glutaraldehyde and vortex at maximum intensity for 1 min.
4. Add 100 μl of 1 mg/ml Pam3csk4 in PBS (Invivogen, cat. no. tlr1-pm2s-1) to bead solution. Incubate for 1 hr at room temperature with gentle mixing to allow crosslinking reaction to occur.
5. Prepare 1% glycine by adding 0.5 g glycine (CAS no. 59-40-6) to 50 ml deionized water.
6. Pellet beads by centrifugation for 5 min at $12,000 \times g$, room temperature.
7. Resuspend beads in 500 μl of 1% glycine solution. Wash twice total with 500 μl of 1% glycine.
8. Aspirate final glycine wash and wash three times with 500 μl PBS.
9. Aspirate final PBS wash and resuspend in 250 μl PBS.
10. Store ≤ 1 month at 2° to 8°C.

Poly-D-lysine solution, 50 $\mu\text{g}/\text{ml}$

1. To make a 1000 \times stock of poly-D-lysine solution, add 50 mg poly-D-lysine (Thermo Fisher, cat. no. A3890401) to 1 ml deionized water under sterile conditions.
2. Invert to mix.
3. Store ≤ 1 month at room temperature.
4. To make a 1 \times working stock of poly-D-lysine solution (prepare fresh immediately before use), dilute 1000 \times stock at 1:1000 (final working concentration 50 $\mu\text{g}/\text{ml}$) in deionized water under sterile conditions.
5. Invert to mix.
6. Dispose of working solution after use.

Rapamycin, 200 μM

1. To make a 1000 \times stock of rapamycin, add 1.8283 mg rapamycin (CAS no. 53123-88-9) to 10 ml sterile DMSO under sterile conditions.
2. Incubate in a 37°C water bath and invert to mix.

3. Store ≤ 2 years at -20°C and protect from light.
4. To make a $1\times$ working stock of rapamycin solution (prepare fresh immediately before use), dilute $1000\times$ stock at 1:1000 (final working concentration 200 nM) in DMSO under sterile conditions.
5. Invert to mix.
6. Dispose of working solution after use.

Sucrose solutions, 10%, 25%, 35%, and 62%

1. To make 10% sucrose solution, add 5 g sucrose (CAS no. 57-50-1) to 50 ml of 3 mM imidazole (pH 7.4; see recipe).
2. To make 25% sucrose solution, add 12.5 g sucrose (CAS no. 57-50-1) to 50 ml of 3 mM imidazole (pH 7.4; see recipe).
3. To make 35% sucrose solution, add 17.5 g sucrose (CAS no. 57-50-1) to 50 ml of 3 mM imidazole (pH 7.4; see recipe).
4. To make 62% sucrose solution, add 31 g sucrose (CAS no. 57-50-1) to 50 ml of 3 mM imidazole (pH 7.4; see recipe).
5. Use a magnetic stir bar and plate to mix.
6. Use HCl to adjust pH to 7.4, if needed.
7. Store ≤ 2 years at 2° to 8°C .

COMMENTARY

Background Information

The evolutionarily conserved pathway of autophagy is a catabolic cell survival mechanism activated during stress or starvation that degrades and recycles unnecessary or dysfunctional cellular components (Levine, 2005). Although this canonical form of autophagy is considered a nonspecific mechanism for bulk recycling during starvation, we now appreciate that the autophagy machinery plays roles that extend far beyond nutrient deprivation and is critical for establishing and maintaining immune homeostasis. LAP is a newly described process of non-canonical autophagy, wherein receptor engagement during engulfment recruits distinct components of the autophagy machinery to the single-membraned cargo-containing phagosome, or LAPosome (Sanjuan et al., 2007) (Fig. 1). A pathway called LC3-associated endocytosis (LANDO) has also been described, with similar molecular requirements and outcomes for cargo that is endocytosed (Heckmann et al., 2019). The activity of autophagy-associated proteins facilitates the processing of cargo via recruiting lipidated LC3 (LC3-II) to the LAPosome to enable its fusion with the lysosomal pathway. Engagement of multiple types of receptors, including pathogen recognition receptors (PRRs), Fc receptors, and phosphatidylserine (PtdSer) receptors, has been shown to trigger LAP (Florey, Kim, Sandoval, Haynes, & Overholtzer, 2011; Henault et al., 2012; Martinez et al., 2011, 2015). Previous work has demonstrated that LAP plays a criti-

cal role in the control of *Aspergillus fumigatus* (Sanjuan et al., 2007); the type I interferon response to immune complexes (Henault et al., 2012); and degradation, processing, and the anti-inflammatory response during the clearance of dying cells (efferocytosis) (Kim et al., 2013; Martinez et al., 2011, 2016).

Whereas components of the pre-initiation complex, such as ULK1, ATG13, and FIP200, are not required for LAP, Rubicon (RUBCN), a Class III PI3K- and NOX2-interacting protein, was found to be uniquely associated with LAPosomes but not required for canonical autophagy (Martinez et al., 2015). Using CRISPR/Cas9 gene-targeting technology, a *Rubcn*-deficient (*Rubcn*^{-/-}) mouse line was generated as a LAP-deficient but autophagy-sufficient model (Martinez et al., 2015; Wang et al., 2013). Use of different knockout lines is critical to understanding whether the process occurring is canonical autophagy or LAP (Fig. 2).

Although defects in the autophagic machinery have been linked with aberrant host defense, inflammation, and autoimmune disorders, it is possible that defects in LAP, rather than canonical autophagy, account for some of these phenomena. As mentioned above, previous work has demonstrated that LAP plays a critical role in the control of *A. fumigatus* (Sanjuan et al., 2007); the type I interferon response to immune complexes (Henault et al., 2012); degradation, processing, and the anti-inflammatory response during the clearance of dying cells

Table 2 Troubleshooting Guide for Detection of LC3-Associated Phagocytosis

Problem	Solution
<i>Basic Protocol 1: Detection of LAP by electron microscopy</i>	
Holes in membranes	Begin dehydration series with lower concentration of ethanol
Cell structures look small	Use alternative fixative Adjust concentration of salt to avoid hypertonic solution
<i>Basic Protocol 2: Detection of LAP by confocal microscopy of LC3-GFP-expressing cells</i>	
<i>Alternate Protocol 1: Detection of LAP by confocal microscopy using immunofluorescence</i>	
Not many cells in chamber slide	Use different extracellular matrix solution or allow cells to adhere longer
Excessive LC3-GFP puncta	Increase percentage of serum in cell culture medium
Speckled LC3-GFP	Reduce dilution of anti-LC3 antibody
<i>Basic Protocol 3: Detection of LAP using flow cytometry of LC3-GFP-expressing cells</i>	
<i>Alternate Protocol 2: Detection of LAP using antibody staining and flow cytometry</i>	
No difference in LC3-GFP fluorescence in positive control or other samples	Increase concentration of or incubation with digitonin solution Increase rapamycin concentration or incubation time
Excessive LC3-GFP fluorescence in negative control or other samples	Decrease concentration of or incubation with digitonin solution
<i>Basic Protocol 4: Detection of LAP by western blot of purified LAPosomes</i>	
No bands or signal	Increase cell number for harvest
No UNC93B bands in sample	Repeat purification to avoid contamination with proteins not from bead-containing phagosomes

(efferocytosis) (Kim et al., 2013; Martinez et al., 2011); and processing of β -amyloid in a model of Alzheimer's disease (Heckmann et al., 2019). However, an increased inflammatory response to dying cells can contribute to improved antitumor immunity (Cunha et al., 2018). Thus, further understanding of this unique pathway is required to design more effective therapies.

Critical Parameters and Troubleshooting

When assessing LAP, the most critical parameter is the health of the cell. Cellular stress can result in autophagy-mediated LC3-II lipidation, which can mask LAP-mediated LC3-II lipidation in immunofluorescence (Basic Protocol 2 and Alternate Protocol 1) and flow cytometry (Basic Protocol 3 and Alternate Protocol 2) assays. Ensure that cell culture or differentiation conditions have been optimized prior to detection of LAP using the protocols described here.

In addition, it is imperative that experiments include cells deficient in autophagy or LAP alone to determine whether or not the process being examined is truly LAP.

For all antibody-mediated detection of LAP (Alternate Protocols 1 and 2), it is imperative that antibodies be titrated prior to use. To avoid nonspecific activity, use of isotype-control antibodies is recommended. If multiple fluorophores are used, compensation for spectral overlap may be required, as described (see Current Protocols article; Cohen, Valm, & Lippincott-Schwartz, 2018).

For phagosome purification (Basic Protocol 4), a minimum of 107 cells is required for successful detection of phagosome-associated protein.

Please refer to Table 2 for a troubleshooting guide.

Understanding Results

By electron microscopy (Basic Protocol 1), the autophagosome is identifiable as a

circular double-membraned structure, possibly with other cellular content inside the vesicle. The LAPosome, in contrast, is a single-membraned vesicle containing engulfed cargo. It is possible for both autophagosomes and LAPosomes to be present in a single cell.

By immunofluorescence (Basic Protocol 2 and Alternate Protocol 1), autophagy is characterized by the formation of LC3-II⁺ puncta throughout the cytosol of the cell, which appear >4 hr after autophagy induction. LAP, in contrast, is characterized by rapid translocation of LC3-II to cargo-containing phagosomes and can occur as quickly as 45 min post-engulfment.

Flow cytometry (Basic Protocol 3 and Alternate Protocol 2) can be a useful tool to quantify the level of LC3-II associated with a cargo-containing phagosome, though users must ensure that they have achieved digitonin-mediated rupture of the plasma membrane and are quantifying phagosome-associated LC3-II, rather than any autophagosome-associated LC3-II. Use of fluorescent cargo and autophagy-only controls will help guide this analysis.

Time Considerations

The multi-stage protocol for electron microscopy (Basic Protocol 1) can take up to 5 days, though multiple stopping points are built into the steps for experimental ease. All other protocols can be performed in <8 hr, with options for stopping points indicated in the steps.

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