# Chapter 15

# Analyzing Autophagic Flux in Nerve Cultures

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## Abstract

Autophagy is a key cellular mechanism involved in the degradation of long-lived proteins and organelles. We and others have previously shown that Schwann cells are able to degrade their own myelin by a form of selective autophagy, or myelinophagy. There is now increasing evidence that myelinophagy could also be aberrantly activated in other demyelinating diseases, including hereditary or inflammatory neuropathies, implicating this pathway in the pathogenesis of these disorders. In this chapter, we describe our protocol to monitor autophagy in peripheral nerves, using the autophagy flux assay. This assay can be useful to compare basal and demyelination-induced autophagy in genetic mice models, or after treatment with specific compounds.

**Key words** Myelinophagy, Autophagy flux, LC3, Western blot, Schwann cell

## 1 Introduction

Axon myelination is essential for rapid saltatory conduction of nerve impulses in the vertebrate nervous system. Oligodendrocytes and Schwann cells are the glial cells that wrap their plasma membranes around axons to myelinate nerve fibers in the central nervous system (CNS) and peripheral nervous system (PNS), respectively. Mature Schwann cells found in adult nerves are derived from multipotent neural crest cells via three main transitions; first is the formation of Schwann cell precursors from neural crest cells. The second transition is the generation of immature Schwann cells, which then differentiate to myelinating and non-myelinating Schwann cells [1].

A striking feature of mature myelin Schwann cells is their remarkable plasticity, a potentially unique trait among adult differentiated cells in mammals. These cells can be readily destabilized leading to a switch in phenotypic states, a process referred to as Schwann cell reprogramming [2]. Even more striking is that this process can be initiated by a wide variety of distinct conditions that range from nerve injury to immune assaults, metabolic disturbances, microbial infec-

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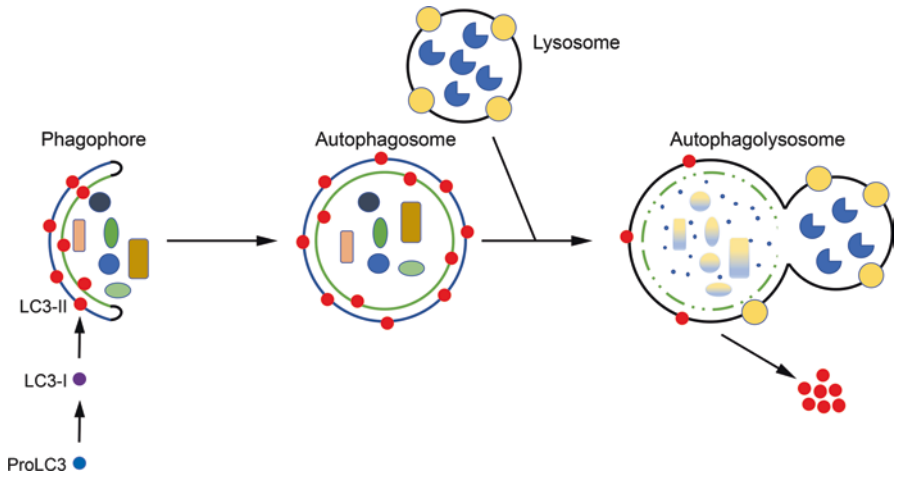



**Fig. 1** Schematic representation of autophagy. Macroautophagy is an inducible degradation system by which cells break down their own organelles and large macromolecules. Autophagy involves the formation of an isolation membrane that extends around cytoplasmic cargo to form an autophagosome, which transfers cargo to the lysosome for degradation. LC3 is initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form, LC3-I. This is finally modified into the PE-conjugated form, LC3-II. LC3-II is the only protein marker that is reliably associated with completed autophagosomes, but is also localized to phagophores

tions, or genetic defects. In all these conditions, myelin breakdown, or demyelination, is invariably a universal outcome.

In studies using nerve transection as a model of demyelination, it was shown that Schwann cells themselves have the ability to turn against their own myelin and initiate myelin breakdown, in addition to being able to call on macrophages for myelin phagocytosis [3]. We recently showed that this degradation of myelin by Schwann cells is mediated by a novel form of selective macroautophagy, or myelinophagy [4, 5]. Autophagy is an inducible degradation system by which cells break down their own organelles and large macromolecules. It involves the formation of an isolation membrane that extends around cytoplasmic cargo to form an autophagosome, which transfers cargo to the lysosome for degradation [6] (Fig. 1). Another two subsequent studies essentially confirmed our findings of a key role of autophagy in Schwann cell-mediated myelin breakdown [7, 8]. There is also increasing evidence that myelinophagy could also be aberrantly activated in other demyelinating diseases, including hereditary or inflammatory neuropathies, implicating this pathway in the pathogenesis of these disorders [4, 8–10]. Therefore, the recognition of this mechanism in Schwann cells potentially provides novel targets for manipulating demyelination in injury and disease.

Activation of autophagy can be measured using different assays in vivo, including examining expression of autophagy-related genes and proteins as we showed previously. But here, we present our method to monitor autophagy activation in vitro using a simple culture system, based on the autophagy flux assay [11] that we previously employed to demonstrate myelinophagy [4].

Autophagic flux is inferred on the basis of turnover of a key autophagosomal protein, called LC3 II in the presence and absence of lysosomal degradation. LC3 is initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form, LC3-I. This is finally modified into the PE-conjugated form, LC3-II. LC3-II is the only protein marker that is reliably associated with completed autophagosomes, but is also localized to phagophores (Fig. 1). The difference in the amount of LC3-II in the presence and absence of saturating levels of inhibitors can be monitored by Western blotting, forming the basis of the autophagy flux assay. If autophagy flux is occurring, then the amount of LC3-II will be higher in the presence of the inhibitor (Fig. 2) [12].

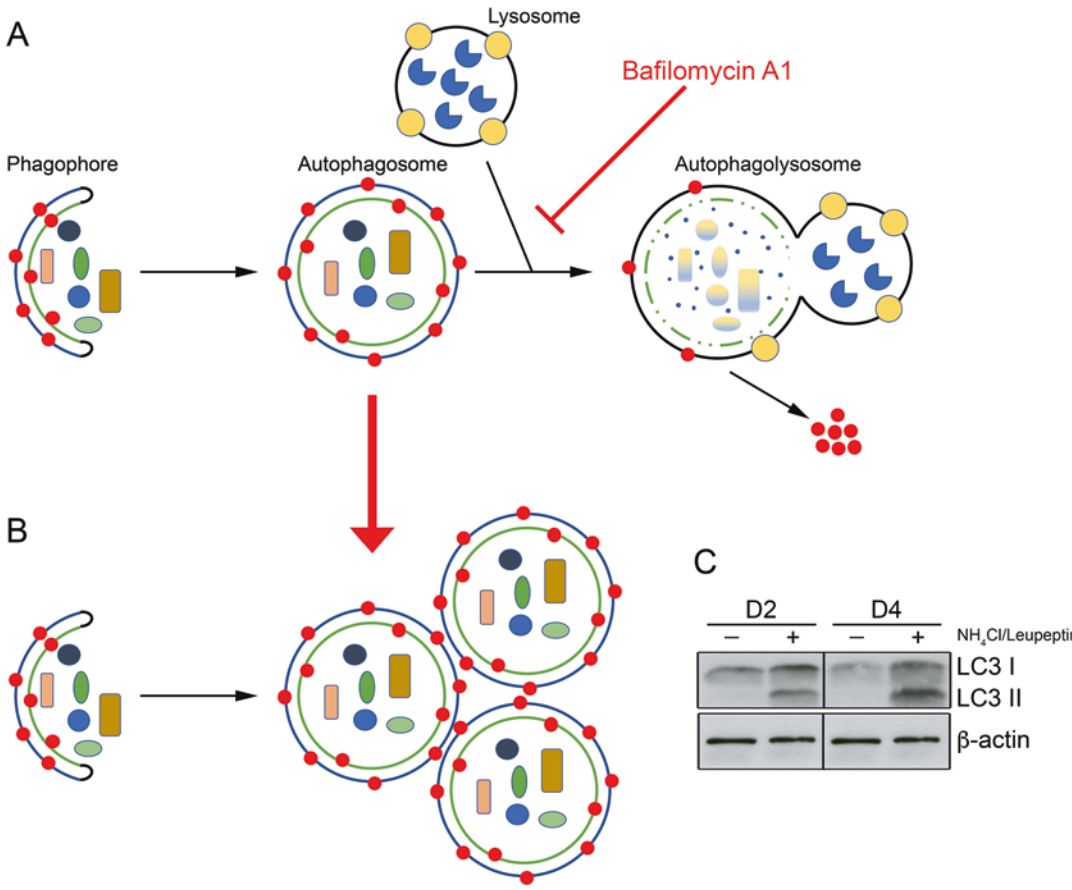



**Fig. 2** Schematic representation of autophagy flux assay. (**a**) Lysosomal degradation can be prevented through the use of protease inhibitors (e.g., pepstatin A, leupeptin, and E-64d), compounds that neutralize the lysosomal pH such as bafilomycin $A_1$, chloroquine or $NH_4Cl$, or by treatment with agents that block the fusion of autophagosomes with lysosomes such as bafilomycin $A_1$. This leads to an accumulation of autophagosomes (**b**), and consequently of LC3 II levels, which can be monitored by Western blotting (**c**). Please note the increase in LC3 II levels in cultures treated with Bafilomycin A1 (Denoted as '+') compared with untreated cultures ('–') at 2 days in culture (D2) or 4 days in culture (D4)

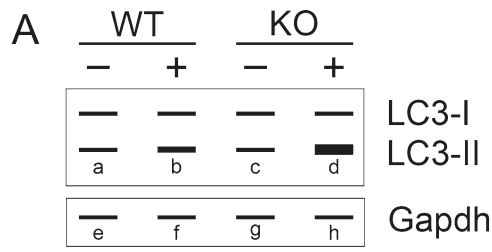
By comparing autophagy flux between different situations (e.g., after treatment with a compound), we can determine whether this compound can activate autophagy or block it, and also quantify the extent of these differences. For example, if this compound was to increase autophagy activation, we would see an increased turnover of LC3-II [12]. This would be similar to what we previously described for ceramides in myelinophagy [4]. Conversely, if this compound was to decrease autophagy activation, we would see a decreased turnover of LC3-II. This assay can also be used to compare activation of autophagy in genetic mice models. For example, we showed that there was a decreased LC3-II flux in c-Jun knockout mice (specific for Schwann cells) [4], consistent with an important role for c-Jun in myelin breakdown [13].

In this chapter, we describe two short methods for monitoring autophagy flux in Schwann cells. In the first method, the nerve segment method, sciatic nerves are isolated from adult mice, cut into 5 mm segments and cultured. Autophagy inhibitors are added for the last 2 h of culture, and the nerve segments are extracted and LC3 II levels examined by Western blotting. In the second method, the dissociated Schwann cells method, sciatic nerves are isolated from young pups, Schwann cells are dissociated and cultured. Autophagy inhibitors are added for the last 2 h of culture, and proteins extracted from the cultures and LC3 II levels examined by Western blotting. We also describe a simple method to quantify the autophagy flux, and compare it between different conditions (Fig. 3).

## 2 Materials

### 2.1 In Vitro Cultures

1. Postnatal day 3–7 (P3–7) mice/rat pups.
2. Adult mice/rats (>1 month old).
3. Nerve extraction medium: Add 1% Antibiotic/Antimycotic (A/A) to Leibovitz's L-15 medium.
4. Semken blunt forceps.
5. Two small size dissecting scissors straight.
6. One Micro-dissecting spring scissors Vannas-Tubingen.
7. One Dumont # 7 forceps and 3 Dumont # 5 forceps fine tips.
8. Round Handled Vannas straight sharp spring scissors.
9. Dissecting board.
10. 70% Ethanol.
11. Stereomicroscope.
12. Scalpel.
13. Ruler.
14. Rat nerve segment culture medium: DMEM containing 5% fetal bovine serum (FBS), 1% A/A.




Net LC3 II flux of WT nerve = **(b/f) – (a/e)**

Net LC3 II flux of KO nerve = **(d/h) – (c/g)**

B

Assume denitometric analysis for the blot above is as follows:
a=1, b=1.5, c=1, d=3, e=1, f=1, g=1, h=1

*Net LC3 II flux of WT nerve = (b/f) – (a/e) =(1.5/1)-(1/1) = 0.5*
*Net LC3 II flux of KO nerve = (d/h) – (c/g)=(3/1)-(1/1) = 3*

**> 6X more autophagy flux in KO nerves compared to WT nerves (3/0.5 = 6)**

**Fig. 3** Quantification of net autophagy flux. (**a**) Schematic representation of a Western blot of LC3-II levels in WT and KO nerves, left untreated or treated with autophagy inhibitors, and the equation for calculation of net autophagy flux is provided. (**b**) Example of the calculation of net LC3 flux in the Western blot above, and comparison between WT and KO nerves is shown (*see* [4] for an example)

15. Mouse nerve segment culture medium: DMEM containing 5% horse serum (HS), 1% A/A.
16. Rat Schwann cell purification medium: DMEM containing 10% FBS, 1% A/A, and 10 μm cytosine arabinoside (AraC) (*see* **Note 1**).
17. Mouse Schwann cell purification medium: DMEM with 5% horse serum (HS), 1% A/A, and 10 μm AraC.
18. Cytosine arabinoside (AraC) (1 mM): Dissolve 2.79 mg AraC in 2 mL sterile $ddH_2O$. Filter using a 0.2 μm filter. Make 100 μL aliquots, snap freeze in liquid nitrogen, and store at −20 °C. Use at a dilution of 1:100 (final concentration of 10 μm).
19. Petri dishes (60 mm) and 12- and 6-well plates.
20. Trypsin solution (0.25%): Make 100 μL aliquots. Snap freeze in liquid nitrogen and store aliquots at −20 °C.
21. Collagenase solution (4 mg/mL): Dissolve 100 mg collagenase Type 2 in 25 mL Hanks' Balanced Salt Solution (HBSS without calcium, without magnesium). Filter sterilize using a 0.2 μm syringe filter. Make 100 μL aliquots and store at −20 °C.

22. Poly-D-lysine solution (PDL) for rat Schwann cells: Dissolve 5 mg PDL in 11.5 mL sterile $ddH_2O$. Filter sterilize using a 0.2 μm syringe filter. Make 300 μL aliquots and store at −20 °C. Thaw one aliquot at the time and make 1:100 dilution in $ddH_2O$ before use.
23. Poly-L-lysine solution (PLL) for mouse Schwann cells: Dissolve 100 mg PLL in 100 mL sterile $ddH_2O$. Filter sterilize using a 0.2 μm syringe filter. Make 5 mL aliquots and store at −20 °C. Thaw one aliquot at the time and dilute 1:10 in $ddH_2O$ before use.
24. Laminin solution: Defrost laminin solution (1 mg/mL) on ice, and make 50 or 100 μL aliquots. Freeze down in liquid nitrogen and store at −80 °C. Thaw one aliquot at a time and make a dilution of 1:100 and 1:50 in DMEM for rat and mice Schwann cell cultures, respectively (*see* **Note 2**).
25. Ammonium chloride ($NH_4Cl$, 2 M): Add 107 mg of $NH_4Cl$ to 1 mL sterile water. Use at a final concentration of 20 mM (1:100 dilution).
26. Leupeptin (10 mM): Add 2.5 mg of leupeptin powder to 5.26 mL sterile water. Aliquot and store at −20 °C. Use at a final concentration of 100 μM (1:100 dilution).
27. Hydroxychloroquine (60 mM): Add 25 mg of hydroxychloroquine powder to 960 μL sterile water. Aliquot and store at −20 °C. Use at a final concentration of 60 μM (1:1000 dilution).
28. Bafilomycin A1 (1 μM): Add 2 μg of Bafilomycin A1 powder to 3.2 mL DMSO. Aliquot and store at −20 °C. Use at a final concentration of 1 nM (1:1000 dilution).

### 2.2 Western Blot

1. Protein Electrophoresis chamber.
2. Western Blot Transfer system.
3. Multicasting chambers.
4. Imaging System.
5. Protein Standards.
6. 30% Acrylamide/Bis Solution, 37.5:1.
7. TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine).
8. Ammonium persulfate (APS), 10% solution in distilled water.
9. Methanol.
10. Ethanol.
11. Tween-20.
12. Glycerol, 100%.
13. Phosphate Buffered Saline (PBS), 10×.

14. Deoxycholic acid.
15. Protease inhibitor cocktail.
16. Phosphatase inhibitor cocktail.
17. Tris, 1 M, pH 6.8.
18. Tris, 1 M, pH 8.8.
19. Sodium Dodecyl Sulfate (SDS), 10% solution in distilled water.
20. β-Mercaptoethanol.
21. Bromophenol Blue.
22. Blotting paper.
23. Nonfat dry milk.
24. PVDF membrane.
25. HRP-conjugated secondary antibody.
26. Rabbit LC3B Antibody (Cell Signalling, Cat. no. 2775).
27. Loading control antibody (e.g., Gapdh, β-Actin).
28. Pierce™ BCA Protein Assay Kit.
29. RIPA lysis buffer: 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton, 0.1% SDS, 5 mM EDTA. Filter and store this solution at 4 °C. At the moment of use, add deoxycholic acid (10 mg per 1 mL of RIPA lysis buffer), and supplement with protease and phosphatase inhibitor cocktail.
30. SDS-PAGE sample buffer 5×: For 20 mL, add 5 mL of 1 M Tris, pH 6.8, 690 μL of β-Mercaptoethanol, 5 mL of 40% SDS, 10 mL of 100% glycerol, 100 μL of saturated bromophenol blue.
31. Resolving gel (15%): Add 1.5 mL of distilled water, 3.3 mL of 1 M Tris, pH 8.8, 5 mL of 30% Acrylamide/Bis Solution 37.5:1, 100 μL of 10% SDS, 100 μL of 10% APS, 10 μL of TEMED.
32. Stacking gel (4%): Add 3.65 mL of distilled water, 0.62 mL of 1 M Tris pH 6.8, 0. 62 mL of 30% Acrylamide/Bis Solution 37.5:1, 50 μL of 10% SDS, 50 μL of 10% APS, 5 μL of TEMED.
33. Running Buffer (10×): 0. 25 M Tris base, 1.92 M glycine, 1% SDS, pH 8.5.
34. Transfer Buffer (10×): Add 140 g glycine (final concentration 1.9 M) and 30 g Tris base (0.25 M). Make up to 1 L distilled water.
35. Transfer buffer (1×): For 1 L, add 100 mL 10× Transfer buffer, 700 mL MilliQ Water and 200 mL Methanol.
36. Tris-buffered saline (TBS; 20×): 3 M NaCl, 0.2 M Tris–HCl, pH 8.

37. TBS containing 0.1% Tween-20 (TBST).
38. Blocking milk solution: 5% Nonfat dry milk in TBST.

## 3 Methods

Cell culture experiments subsequent to tissue dissociation should be performed under sterile conditions and all procedures should be carried out at room temperature unless otherwise specified.

### 3.1 Nerve Segment Culture

1. Sterilize dissecting area and dissecting equipment with 70% ethanol.
2. Sacrifice adult mice/rat (>1 month old) by cervical dislocation or $CO_2$ euthanasia (*see* **Note 3**).
3. Pin the body to a dissecting board with the dorsal side up.
4. Spray bodies with 70% ethanol (*see* **Note 4**).
5. Remove skin from lower part of the body.
6. Dissect out sciatic nerves, and place in 60 mm dish containing 5 mL nerve extraction medium (Keep dishes on ice) (*see* **Note 5**).
7. Pin the body of the same animal this time with the ventral side up.
8. Remove skin from upper part of the body.
9. Dissect out brachial plexus, and place in a separate 60 mm dish above containing 5 mL nerve extraction medium (Keep dishes on ice) (*see* **Note 6**).
10. Once nerves have been extracted from all animals, proceed to clean the nerves from muscle and fat.
11. Using a scalpel and a ruler, cut the sciatic nerves and brachial plexus into 5 mm segments and place in separate dishes containing nerve extraction medium.
12. Once all segments have been obtained, place 1–2 segments in a 24-well plate containing 500 μL nerve segment culture medium (*see* **Note 7**).
13. Culture at 37 °C and 5% $CO_2$ (*see* **Note 8**).
14. Add autophagy inhibitors to cultures (*see* **Note 9**) for the last 2 h of culture (*see* **Note 10**).
15. Remove nerve segments and place in eppendorf tubes.
16. Add 200 μL of ice-cold RIPA lysis buffer containing inhibitors, homogenize (e.g., using a mechanical tissue disruptor or dounce homogenizer), and snap freeze in liquid nitrogen.
17. Defrost vials and incubate on ice for 15 min, vortexing every 5 min.

18. Centrifuge cell lysates at 17,200 *g* for 20 min at 4 °C, discard the pellet and aliquot the supernatant in 10–50 μL aliquots and snap freeze in liquid nitrogen (*see* **Note 11**).
19. Store at −80 °C until use (Proceed to Subheading 3.3).

### 3.2 Dissociated Schwann Cell Cultures

#### 3.2.1 PDL-Coated Cell Culture Dishes (for Rat Schwann Cells)

1. Dissolve one aliquot (300 μL) PDL solution in 30 mL $ddH_2O$.
2. Add enough volume of PDL solution to completely cover dishes (e.g., 1.5 mL in 60 mm dishes). Leave at RT for 2 h.
3. Remove the solution and allow to air-dry.
4. Store desiccated at RT (*see* **Note 12**).

#### 3.2.2 PLL-Coated Dishes (for Mouse Schwann Cells)

1. Dilute 5 mL of 1 mg/mL PLL solution 1:10 to give 100 μg/mL solution.
2. Add enough volume of diluted PLL solution to completely cover dishes (e.g., 1.5 mL in 60 mm dishes). Leave at RT for 2 h (*see* **Note 13**).
3. Remove the solution and wash three times with $ddH_20$.
4. Store desiccated at room temperature (*see* **Note 12**).

#### 3.2.3 Laminin Coating

PLL- and PDL-coated dishes must be coated with laminin at least 1 h prior to plating cells.

1. Dilute the stock solution of laminin in DMEM to a final concentration of 20 μg/mL (1:50 dilution) for PLL-coated dishes, and to a final concentration of 10 μg/mL (1:100 dilution) for PDL-coated dishes.
2. Add enough volume of laminin solution to completely cover dishes (e.g., 1.5 mL in 60 mm dishes), and leave the solution on for at least 1 h.
3. Remove laminin solution immediately prior to plating cells (*see* **Note 2**).

#### 3.2.4 Peripheral Nerves Dissection

1. Sterilize dissecting area and dissecting equipment with 70% ethanol.
2. Sacrifice mice/rat pups (P3–7 days old) by decapitation with scissors (*see* **Note 3**).
3. Pin the body to a dissecting board with the dorsal side up.
4. Spray bodies with 70% ethanol (*see* **Note 4**).
5. Remove skin from lower part of the body.
6. Dissect out sciatic nerves, and place in 60 mm dish containing 5 mL nerve extraction medium (Keep dishes on ice) (*see* **Note 5**).
7. Pin the body of the same animal this time with the ventral side up.

8. Remove skin from upper part of the body.
9. Dissect out brachial plexus, and place in 60 mm dish above containing 5 mL nerve extraction medium (Keep dishes on ice).
10. Once nerves have been extracted from all animals, proceed to clean the nerves from muscle and fat, and then remove epineurium from nerve (the outermost layer of connective tissue surrounding a peripheral nerve) using forceps under a stereomicroscope (*see* **Note 14**).

#### *3.2.5 Nerve Digestion and Plating*

1. Place all nerves together in a 15 mL centrifugation tube containing enzymatic digestion solution (100 μL of trypsin solution and 100 μL collagenase per animal).
2. Incubate nerves at 37 °C for 30 min.
3. Triturate gently using 1 mL pipette tip (4–5 times) and continue with a 200 μL pipette tip (10 times) until tissue starts to dissociate (*see* **Note 15**).
4. Incubate the digested nerves at 37 °C for a further 10 min, and triturate nerves (as above) until tissue is fully dissociated.
5. Add an equal volume of L-15 medium containing 10% FBS to stop the enzyme reaction.
6. Centrifuge for 10 min at 200 *g* at 4 °C.
7. Remove supernatant carefully and discard.
8. Resuspend pellet in Schwann cell purification medium, and plate onto PDL- and laminin-coated dishes (rat) or PLL- and laminin-coated dishes (mice) (*see* **Note 16**).
9. Culture at 37 °C and 5% $CO_2$ (*see* **Note 8**).
10. Add autophagy inhibitors to cultures (*see* **Note 9**) for the last 2 h of culture (*see* **Note 10**).
11. On day of protein extraction, wash wells with ice-cold 1× PBS and scrape proteins in ice-cold RIPA lysis buffer containing inhibitors (100–200 μL per well) using cell scarper. Collect lysates in eppendorf tubes, and incubate on ice for 15 min, vortexing every 5 min.
12. Centrifuge cell lysates at 17,200 *g* for 20 min at 4 °C, discard the pellet and aliquot the supernatant in 10–50 μL aliquots and snap freeze in liquid nitrogen (*see* **Note 11**).
13. Store at −80 °C until use (Proceed to Subheading 3.3).

### *3.3 Western Blot*

1. Use one of the frozen protein lysate aliquots to quantify total protein content by BCA protein assay (*see* **Note 17**).
2. To a new frozen aliquot of protein lysate, add 4× SDS-PAGE buffer to give a final concentration of 1× (*see* **Note 18**). Add SDS-PAGE buffer just immediately prior to boiling.

3. Boil the samples at 95 °C for 5 min.
4. Load the samples and protein standards into 4–15% SDS-PAGE gel and run them at 100 V (*see* **Note 19**).
5. Transfer the proteins in the gel into a PVDF membrane (*see* **Note 20**).
6. Wash membranes with distilled water.
7. Block the membranes with blocking milk solution for 1 h at room temperature.
8. Incubate membranes overnight with LC3 antibody (1:1000 dilution in blocking milk solution).
9. Wash the membranes with TBST three times, each wash for 5 min.
10. Incubate membranes with secondary antibody, diluted in blocking milk solution, for 1 h at room temperature.
11. Wash the membranes with TBST three times, each wash for 5 min.
12. Detect immunoreactive proteins by chemiluminescent substrate and capture the signal exposing to X-ray films or using an imaging system.
13. Wash the membrane with TBST.
14. Incubate overnight with loading control antibody (e.g., Gapdh or ß-actin) in blocking milk solution.
15. Wash the membranes with TBST three times, each wash for 5 min.
16. Incubate membranes with secondary antibody, diluted in blocking milk solution, for 1 h at room temperature.
17. Wash the membranes with TBST three times, each wash for 5 min.
18. Detect immunoreactive proteins by chemiluminescent substrate and capture the signal exposing to X-ray films or using an imaging system.

### 3.4 Quantification of LC3 Flux

1. Measure densitometric values of LC3-II bands and loading controls using Image J analysis software.
2. Calculate net LC3 II flux using the following equation (adapted from [12]):

   *Net LC3 II flux = Densitometry value of LC3 II in sample treated with inhibitors (normalized to GAPDH) − Densitometry value of LC3 II in untreated sample (normalized to GAPDH).*

   *See* Fig. 3 as an example.
3. Calculate net flux for all samples and represent in a graph (*see* **Note 21**).

## 4 Notes

1. We add AraC in cultures to kill contaminating fibroblasts. AraC is a pyrimidine antimetabolite that kills proliferating cells by inhibiting DNA synthesis. Contaminating fibroblasts found in these cultures are killed over this period of 3 days by AraC, since they are the only proliferative cells (Schwann cells do not proliferate in this medium).
2. Laminin solution can be reused for up to four times. After use, keep at 4 °C. Reuse within 1 month after first use.
3. All experiments using mice must be approved by the respective institutional animal ethic committee. The criteria may change between institutions and countries, and also over time, so please refer to the respective guidelines.
4. It is important to maintain sterile conditions to avoid contamination of cultures.
5. Use different sets of materials to remove skin, and to remove nerves.
6. When we perform autophagy flux assays with nerve segments, we keep sciatic nerves and brachial plexus separate. We never compare nerve segments from sciatic nerves to nerve segments from brachial plexus, because of different thickness of nerves, and consequently rates of myelin breakdown.
7. For autophagy flux assays, you need to compare untreated cultures with cultures treated with autophagy inhibitors. Thus, for each condition, two sets of nerve segments are required (untreated and treated).
8. We culture the nerve segments/dissociated Schwann cells at different time points depending on the experiments. For example, if you want to compare basal level of autophagy between a wild-type and a mutant mouse strain, culture the nerve segments/dissociated Schwann cells for only 2 h. If you want to compare autophagy levels between a wild-type and a mutant mouse strain during demyelination, culture the nerve segments/dissociated Schwann cells for 3–5 days before treatment with autophagy inhibitors. If you want to examine the effects of different compounds on autophagy levels in demyelinating nerves, you can culture nerve segments/dissociated Schwann cells over a time course of 0–5 days.
9. We routinely use $NH_4Cl$ solution (20 mM) (freshly prepared) and leupeptin (100 μM) to inhibit autophagy. Hydrochloroquine (60 μM) or Bafilomycin A1 (1 nM) can also be used (Fig. 2).
10. This can be extended to 4 h depending on the requirements of the experiment.

11. Due to the low stability of LC3-I, fresh samples should be run into gels as soon as possible, avoiding repeated freeze-thaw cycles [11].
12. Best not to use within 24 h. Can be kept for many months.
13. PLL solution can be re-frozen and used for up to three times.
14. It is important to remove as much as possible the epineural sheaths, since this is the greatest source of contaminating fibroblasts in Schwann cell cultures.
15. Do this gently to avoid making bubbles.
16. We routinely plate 100, 000 cells per well of 12-well plates or 250, 000 cells per well of 6-well plates.
17. As mentioned before, LC3 is an unstable protein. Therefore, it is highly recommended to aliquot sample extracts to avoid repeated freeze-thaw cycles, and use independent aliquots for protein determination and for Western blotting.
18. LC3-I is sensitive to degradation in SDS-containing sample buffer. A quick boiling and loading into the gel after SDS-PAGE sample buffer addition is highly recommended. It is also recommended not to store the samples once they have been mixed with SDS-PAGE sample buffer [11].
19. Since in Schwann cells the LC3-I levels are much higher than LC3-II, the intensity of LC3-I band may interfere with the detection of LC3-II band. It is therefore important to run a 4–15% gel completely to properly resolve both bands [11].
20. Since the molecular weight of LC3-I/II is 17/14 KDa, long transfer times are not recommended. Semidry transfer at 150 mA during 1.5 h is recommended.
21. You need at least three different biological replicates for statistical analysis.

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