Chapter 8

LC3-II Tagging and Western Blotting for Monitoring Autophagic Activity in Mammalian Cells

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Abstract

The autophagosome-associated protein LC3-II is commonly used as a marker of autophagic activity within cells, but its levels are affected by both formation and degradation of autophagosomes. This can make the significance of altered LC3-II levels ambiguous. Here we describe the method of Bafilomycin A_1 blotting, in which the degradation of autophagosomes is prevented in cultured cells, allowing the causes of altered LC3-II levels to be determined.

Key words Autophagy, LC3, Assay, Flux, Bafilomycin A1

1 Introduction

The process of macroautophagy, whereby a portion of cytosol is engulfed into double-membraned autophagosomes and transported to the lysosome for degradation [1], has potential as a therapy for delaying the onset of neurodegenerative diseases. By clearing aggregate-prone proteins from the cytosol, the formation of aggregates within cells can be reduced [2]. This has been demonstrated in experimental models involving mutated huntingtin [3], alpha synuclein [4] and tau [5].

As a specific marker of autophagosomes, LC3-II is a useful and versatile tool in accurate measurement of autophagic flux. LC3 (microtubule-associated protein 1 light chain 3, MAP1-LC3) is the mammalian homologue of the yeast autophagy protein Atg8 [6]. The C-terminus of LC3 is cleaved by the action of Atg4 to give the LC3-I form of LC3, which is then conjugated to the lipid phosphatidylethanolamine to give the membrane-associated LC3-II form [7]. This is found on the inner and outer membranes of autophagosomes. The pool associated with the inner membrane is degraded in the autolysosomes (formed by the fusion of autophagosomes and lysosomes), while that associated with the outer

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membrane can be cleaved by Atg4 and recycled. LC3-II levels in a cell therefore give an indication of the number of autophagosomes in the cell at that particular time and hence the autophagy state of the cell.

The level of LC3-II in cells, however, needs to be interpreted with care, as it is affected by both formation and degradation processes. An increase in LC3-II levels can be due to increased formation or decreased degradation, while decreased LC3-II could be due to increased degradation or decreased formation. In order to distinguish between these criteria, Bafilomycin A_1 blots of LC3-II levels are used. Bafilomycin A_1 is a drug which inhibits the V-ATPase responsible for acidification of the lysosome, and prevents fusion of autophagosomes and lysosomes [8, 9]. Comparison of LC3-II levels in the absence and presence of Bafilomycin A_1 (or other inhibitors of LC3-II degradation) allows the effects of formation and degradation to be uncoupled [10, 11].

As well as studying the LC3-II levels of cells, it is useful to look at clearance of autophagy substrates such as p62 in order to gain a more comprehensive idea of the autophagy state of the cells. This can be done by Western blotting, though similarly to LC3-II levels, the effect of formation of new p62 (i.e. translation) should be taken into consideration when interpreting the results.

Here, the procedures for carrying out Bafilomycin A_1 blots to determine the autophagic activity of mammalian cells are detailed. Cells in culture are treated with Bafilomycin A_1 and harvested, and the LC3-II levels of the lysates analysed by Western blotting. Interpretation of the blots will be explained.

2 Materials

Make up reagents in distilled water unless otherwise stated.

1. HeLa cells in culture (*see* **Notes 1** and **2**).
2. Treatments being investigated (drugs, culture conditions etc) (*see* **Note 3**).
3. 100 μM Bafilomycin A_1 stock solution in DMSO (*see* **Note 4**).
4. Phosphate-buffered saline (PBS) buffer: 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4 at pH 7.4 (*see* **Note 5**).
5. Radioimmunoprecipitation assay (RIPA) buffer: 150 nM NaCl, 1 % NP40, 0.5 % sodium deoxycholate (NaDoC), 0.1 % SDS, 50 mM Tris pH 7.4, 1× protease inhibitor cocktail (Roche) (*see* **Note 6**). Make up fresh each time and use on ice.
6. Standard protein assay kit (e.g. Bio-Rad DC™ Protein Assay).
7. 100 mg/mL BSA solution.

8. 2× Laemmli buffer: 65 mM Tris–HCl pH 6.8, 25 % (w/v) glycerol, 2 % SDS, 0.01 % (w/v) bromophenol blue, 5 % (v/v) β-mercaptoethanol.
9. 30 % Acrylamide/bis-acrylamide solution (37.5:1).
10. Resolving gel buffer: 1.5 M Tris pH 8.8.
11. 10 % SDS solution.
12. 10 % ammonium persulfate solution in H_2O.
13. Stacking gel buffer: 1 M Tris pH 6.8.
14. TEMED (*N*, *N*, *N′*, *N′*—tetramethylethylenediamine).
15. Isopropanol.
16. Pre-stained molecular weight markers (e.g. Invitrogen SeeBlue® Plus2 Pre-Stained Standard).
17. Gel running buffer: 25 mM Tris pH 8.3, 0.192 M glycine, 0.1 % SDS (*see* **Note 7**).
18. Wet transfer buffer: 25 mM Tris pH 8.3, 0.192 M glycine, 20 % methanol (*see* **Notes 7** and **8**).
19. Ponceau S stain solution: 5 % (w/v) in 5 % acetic acid.
20. Milk: 5 % (w/v) milk powder in PBS.
21. PBS-Tween: 0.1 % Tween-20 in PBS.
22. Novus Biologicals rabbit anti-LC3 primary antibody (NB100-2220) (*see* **Note 9**).
23. Sigma rabbit anti-actin primary antibody (A2066) (*see* **Note 9**).
24. IR dye-conjugated anti-rabbit secondary antibody (*see* **Note 10**).
25. Western blot and wet transfer equipment (e.g. PVDF membranes).
26. Licor Odyssey equipment or equivalent (*see* **Note 11**).

3 Methods

The instructions here test the effect of a hypothetical drug ("Drug A") on autophagy in HeLa cells following a 4 h treatment. For assistance on adapting the protocol for other experimental setups, relevant notes are referred to at the appropriate stages.

1. Seed HeLa cells, using approximately 2×10^5 cells per well of a 6-well plate (*see* **Note 12**) to ensure that they will be approaching confluence when harvested. Allow the cells to settle overnight.
2. Treat the cells with Drug A and 400 nM Bafilomycin A_1 for 4 h. This concentration of Bafilomycin A_1 treatment is saturating (*see* **Note 13**). Control conditions should include an appropriate volume of DMSO to control for the DMSO added

in the Bafilomycin A_1 stock solution. The conditions required are: no Drug A; Drug A; Bafilomycin A_1 and no Drug A; Bafilomycin A_1 and Drug A.

3. Harvest the cells. Remove the media from the cells and wash once with PBS (allow approximately 1.5 mL of PBS per well in a six-well plate). Pipette 100 μL of ice-cold RIPA buffer into each well (*see* **Note 14**), and use a cell scraper to lift cells from the bottom of the well. Pipette the lysed cells into labelled Eppendorf tubes and incubate on ice for 10 min. Centrifuge for 10 min at 13,000 × *g* and transfer the post-nuclear supernatant to fresh tubes on ice.
4. Carry out a protein assay. Make standards by making serial dilutions of BSA in the range 5–0 μg/μL in RIPA buffer. Make up Reagent A' by mixing 20 μL of Reagent S with 1 mL of Reagent A. In a 96-well plate, mix 1 μL of sample/standard, 20 μL of Reagent A', and 200 μL of Reagent B. Allow blue color to develop in a shaded place for 15 min. Scan the plate on a plate reader at a wavelength of 750 nm, using the standards to make a standard curve from which to determine the protein concentration in the samples.
5. Adjust the volumes of the samples using RIPA buffer to give the same protein concentration in all samples. Add 2× Laemmli buffer to each sample so that the protein concentrations remain equal between samples (*see* **Note 15**). Boil the tubes for 5 min on a hot block set to 100 °C (*see* **Note 16**). Use a bench-top centrifuge to briefly spin down condensation in the tubes. Samples may be frozen at –20 °C.
6. Mix 3.3 mL of distilled H_2O, 4 mL of 30 % acrylamide/bis acrylamide solution, 2.5 mL of resolving gel buffer, 100 μL of SDS and 100 μL of ammonium persulfate. Add 4 μL of TEMED, mix well, and pour the gel in a 1.5 mm × 10.1 cm × 7.3 cm cast. Leave space for a 1 cm depth of stacking gel (*see* **Note 17**), and pipette approximately 250 μL of isopropanol on top of the gel (*see* **Note 18**). Allow to set (around 15–20 min, depending on room temperature).
7. Mix 3.4 mL of distilled H_2O, 830 μL of 30 % acrylamide/bis acrylamide solution, 630 μL of stacking gel buffer, 50 μL of SDS and 50 μL of ammonium persulfate. Pour off the isopropanol from the top of the resolving gel, and rinse with distilled water. Use blotting paper to soak up residual water. Add 5 μL of TEMED to the stacking gel mixture and pour the gel immediately. Insert a 10-well comb to form the wells. Allow the gel to set for approximately 1 h (*see* **Note 19**).
8. Remove the comb from the gel, and set up the gel-running tank and fill with the appropriate volume of running buffer. Ensure the buffer is not leaking from the gel chamber. Load the gel with 20 μL of each of the samples (*see* **Note 20**) and 3 μL

of molecular weight markers. Run the gels slowly at 0.2 mA per gel, until the blue dye front is 3–5 mm from the end of the gel (approximately 95 min). Do not run further, as this may result in losing the small LC3 proteins from the gel.

9. Assemble a cassette for wet transfer of proteins to the polyvinylidene difluoride (PVDF) membrane (*see* **Note 21**). Soak the PVDF membrane in methanol for 1 min, rinse in H_2O, and then soak in transfer buffer before use. Working in a tray containing a shallow depth of transfer buffer, soak sponges and blotting paper in transfer buffer and place in a cassette. Prise apart the gel casting plates, and trim off the stacking gel. Carefully place the gel on the blotting paper, taking care not to stretch or damage the gel. Place the PVDF membrane on top of the gel, and then another layer of blotting paper. Ensure that no bubbles are trapped in the layers by smoothing the stack with a test tube. Add pre-soaked sponges and close the cassette. Ensure it is loaded into the frame with the membrane between the gel and the positive electrode. Keep the apparatus cool with an ice pack while transferring for 1 h at 90 V.
10. Disassemble the cassette and check for successful transfer of protein markers to the PVDF membrane. Stain the membrane with Ponceau S stain to confirm transfer of proteins to the membrane (*see* **Note 22**), agitating for 10 min at room temperature. Wash in water to develop the bands. Cut the PVDF membrane at around 30 kDa, using the markers as a guide. Block the membrane in milk, agitating for 1 h at room temperature (*see* **Note 23**).
11. Add 10 μL of anti-LC3 primary antibody to 10 mL of milk (giving a 1 in 1,000 dilution), and do the same for anti-actin antibody. Incubate the upper part of the membrane in anti-actin primary antibody, and the lower half in anti-LC3 primary antibody overnight, agitating at 4 °C. Primary antibody in milk can be frozen at –20 °C and reused.
12. Wash the membrane three times for 5 min each time in PBS-Tween. Make up secondary antibody against the primary antibodies used at a concentration of 1 in 3,000 (3 μL of secondary antibody in 9 mL of milk) (*see* **Note 24**). Incubate the membrane parts in secondary antibody for 1 h, agitating at room temperature. Wash the membrane three times for 5 min each time in PBS-Tween.
13. View membranes on the Licor Odyssey imager, placing them face down to get the best signal (*see* **Note 25**). Quantify the bands, and normalize the LC3-II signal using the actin bands as a loading control. The actin levels should be fairly even between the lanes, so this should only be a slight adjustment.

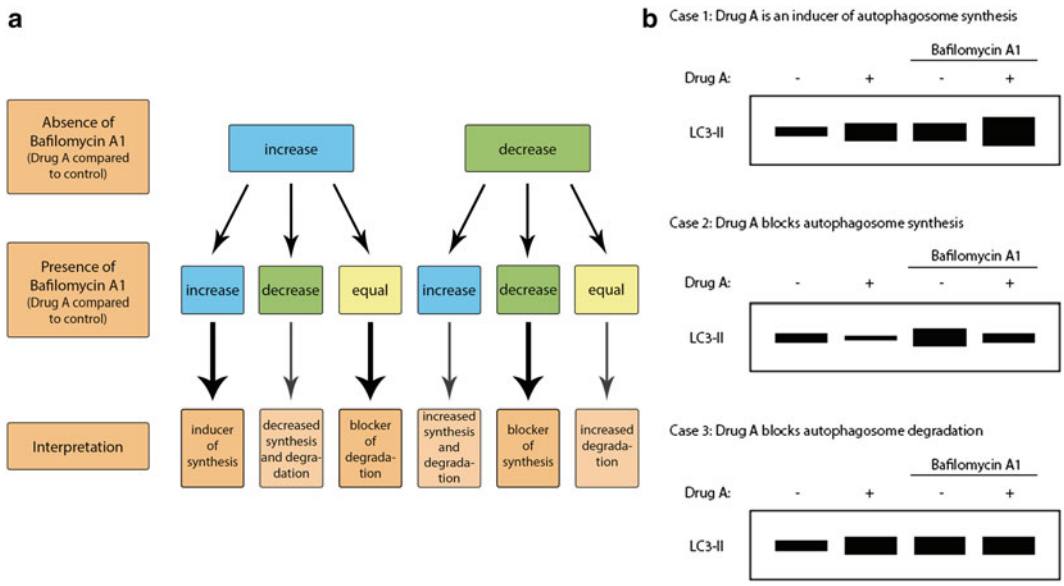

Fig. 1 Interpretation of Bafilomycin A_1 western blots. (**a**) Flow-chart indicating interpretations of LC3-II levels in blots. More common scenarios are indicated by *bold arrows*. (**b**) Schematics of LC3-II levels in Bafilomycin A_1 blots. Protein loading, as measured by actin, is assumed to be even across the lanes. Situations are shown in which hypothetical treatment Drug A is an inducer of autophagosome synthesis (Case 1), a blocker of autophagosome synthesis (Case 2), or a blocker of autophagosome degradation (Case 3)

14. Interpret the results of the blot (Fig. 1).
    - Compare cells treated with Drug A and untreated control cells to see the effect of Drug A on the size of the LC3-II pool in the cells.
    - Compare the cells treated with Drug A and Bafilomycin A_1 with control cells treated with Bafilomycin A_1 to see the contribution of LC3-II formation. If the LC3-II levels increase with Drug A in the presence of Bafilomycin A_1, then Drug A has increased formation of LC3-II (Case 1 in Fig. 1b). A decrease in LC3-II levels with Drug A in the presence of Bafilomycin A_1, together with a decrease with Drug A in the absence of Bafilomycin A_1, indicates that Drug A blocks autophagosome synthesis.
    - Compare LC3-II levels between cells treated with Drug A and cells treated with both Drug A and Bafilomycin A_1 to see the effect of Drug A on degradation of LC3-II. If Drug A blocks degradation of LC3-II, there will be no increase in LC3-II levels on treatment with Bafilomycin A_1, as Bafilomycin A_1 will not exert an additional effect on LC3-II levels (Case 3 in Fig. 1b). If Drug A does not affect degradation of LC3-II, there will be an increase in LC3-II levels when cells are treated with Bafilomycin A_1.

4 Notes

1. A wide variety of cells may be used for Bafilomycin A_1 blots. The method described here looks at the effect of a hypothetical drug ('Drug A') on autophagy in HeLa cells, but is easily adapted for other cell types and treatments.
2. Comparing LC3-II levels directly between cell types or lines is not advised, as levels of LC3-I and LC3-II vary widely between cell types, and the extent of the response to autophagy-modulating treatments can differ significantly. Therefore, we recommend that treated cells are compared to untreated controls of the same cell line.
3. The treatment of cells will be determined by the interests of the researcher. Bafilomycin A_1 blots are compatible with many types of treatment, but the incubation of cells with Bafilomycin A_1 will not necessarily occur for the entire length of time that the investigated treatment is being carried out.
4. Bafilomycin A_1 is made up into 100 μM stock solution by adding 1.61 mL of DMSO to 100 μg of powder (available from Enzo Sciences). This stock should be made into aliquots and stored in the dark at −20 °C.
5. For 1 L of PBS, use 8.1 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 (final concentration 10 mM) and 0.24 g of KH_2PO_4 (final concentration 1.76 mM), at pH 7.4.
6. We use RIPA buffer for harvesting cells, but in theory other buffers can be used instead.
7. We have a 10× solution consisting of 250 mM Tris pH 8.3, 1.92 M glycine. To make up 1 L of running buffer, take 100 mL of 10× solution, add 10 mL of 10 % SDS and make up to 1 L with distilled water. When adding the water, run it down the side of the container to avoid excessive amounts of bubbles being formed by the SDS, or add the SDS last. To make up 1 L of transfer buffer, take 100 mL of 10× solution, and add 200 mL of methanol and 700 mL of distilled water.
8. Methanol in the transfer buffer improves the transfer of small proteins to the PVDF membrane by giving a charged surface the protein can bind to. It slightly fixes the gel however, which can limit the transfer of large proteins. As LC3-I and LC3-II are small (running at 18 kDa and 16 kDa, respectively), methanol transfer buffer is used in this protocol.
9. Other antibodies can be used, but the concentrations used here are specific to the named antibodies and may require adjustment when using other brands.
10. The details in this protocol are for visualization of the Western blot using Licor Odyssey equipment, which visualizes infrared

dye-conjugated secondary antibodies and allows quantification of the signal. This means that LC3-II signal can be normalized to actin and the results viewed quantitatively rather than qualitatively. Quantification should not be used as a replacement for even loading. It is important to normalize LC3-II to actin rather than to LC3-I, as LC3-I levels vary relative to LC3-II depending on cell types and treatment, and the dynamics of the pool are poorly understood. In addition, the affinity of anti-LC3 antibody to LC3-I and LC3-II is different [7].

11. Western blots can be developed using enhanced chemiluminescence (ECL) instead of the Licor system. We prefer to use the Licor system because it allows quantification of protein across a wider linear range than is possible with ECL. If ECL is being used, use HRP-conjugated secondary antibodies. For more information on Licor equipment, visit: http://www.licor.com/bio/applications/quantitative_western_blots/.
12. The volumes of cell suspension given here are for HeLa cells which are to be subjected to a 4 h treatment with a hypothetical drug. With different cell lines and treatments, the seeding density will need to be varied accordingly, ensuring that the cells will be approaching confluence when harvested. For example, a longer treatment time means that fewer cells need to be seeded. The rate of growth of the cells will also affect the number of cells which are seeded. For very long treatments, such as some small interfering RNA (siRNA) knockdown procedures, it may be necessary to split the cells part-way through the treatment.
13. Bafilomycin A_1 causes a defect in lysosomal acidification, which causes a block in fusion with autophagosomes [9]. Prolonged treatment with Bafilomycin A_1 can affect other protein degradation mechanisms [12]. We use 400 nM Bafilomycin A_1 (4 μL of stock solution in 1 mL final volume) for 4 h, or reduced concentrations if the treatment needs to be longer. Bafilomycin A_1 treatments longer than 16 h are best avoided where possible. Where the conditions being investigated require longer times in culture, we recommend adding Bafilomycin A_1 for the final 4 h only.
14. In order to optimize LC3 stability, cells can be lysed directly in 200 μL of Laemmli buffer in each well. This however means that a protein assay cannot be carried out. If, by eye, there appear to be similar numbers of cells per well, then the protein assay is not essential and this method can be used instead. With longer treatments or ones that affect cell survival or growth rates, the number of cells in each well can vary quite significantly, so we recommend that a protein assay is used.
15. For example, if a sample has been lysed in 100 μL of RIPA buffer, and 15 μL of RIPA has been added to adjust the concentration to match the other samples, add 115 μL of 2× Laemmli buffer.

16. If the samples have a high DNA content, making them hard to load accurately into gels (particularly an issue when lysing directly into Laemmli buffer), boiling for slightly longer (e.g. 7 min) at this stage can reduce the viscosity. Care should be taken not to damage the samples by boiling for too long however. Alternatively, samples can be sonicated briefly using a probe sonicator.
17. Having a reasonably sized stacking gel layer improves band resolution.
18. The layer of isopropanol prevents contact with the air, which inhibits polymerization of the acrylamide. In addition, this layer bursts bubbles and gives an even top to the resolving gel.
19. The stacking gel will be set after approximately 10 min, but in our experience allowing longer gives a better quality Western blot, with improved band resolution.
20. Aim to load approximately 15 μg of protein per well. When using a 15-well comb we load around 10 μg in each well. Even protein loading is very important, as analysis of the blots involves comparison of the lanes. Actin is used as a loading control in this procedure. Loading an equal (or at least similar) volume of sample in each lane, as achieved by ensuring equal protein concentrations in the samples, is important to make sure that the gel runs evenly.
21. Semi-dry transfer can be used instead of wet transfer; in our experience, the wet transfer method gives slightly better transfer results for LC3.
22. With practise, an idea of whether a transfer has been successful can be obtained by holding the membrane at an angle to the light and seeing if the lanes can be seen. This method also shows whether there were bubbles in the cassette.
23. Blocking is a very flexible step, and can last between 30 min and several hours.
24. If the primary antibodies used were both raised in the same animal, then the parts of the membrane can be incubated together in secondary antibody. With the Licor system, it is possible to color the bands red or green, thus distinguishing them by color as well as size, by using IR680- or IR800-conjugated secondary antibody respectively.
25. To view the membranes using enhanced chemiluminescence (ECL), mix equal volumes of ECL reagents, allowing 1 mL of solution per membrane. Pipette ECL reagents onto the surface of the membrane which was against the gel during transfer, ensuring the solution is evenly spread over the membrane. Allow to develop for approximately 30 s. Using forceps, touch the edge of the membrane against tissue to remove excess ECL

reagent. Develop the blot using photographic film. Take care not to overdevelop the blot. The bands should be clearly seen, but writing should be visible through the bands when the film is held against text. If films are overdeveloped, the sensitivity of the blot is reduced as differences in band intensity are harder to observe.

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