Chapter 20

Analysis of Relevant Parameters for Autophagic Flux Using HeLa Cells Expressing EGFP-LC3

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Abstract

Macroautophagy (called just autophagy hereafter) is an intracellular degradation machinery essential for cell survival under stress conditions and for the maintenance of cellular homeostasis. The hallmark of autophagy is the formation of double membrane vesicles that engulf cytoplasmic material. These vesicles, called autophagosomes, mature by fusion with endosomes and lysosomes that allows the degradation of the cargo. Autophagy is a dynamic process regulated at multiple steps. Assessment of autophagy is not trivial because the number autophagosomes might not necessarily reflect the real level of autophagic degradation, the so-called autophagic flux. Here, we describe an optimized protocol for the analysis of relevant parameters of autophagic flux using HeLa cells stably expressing EGFP-LC3. These cells are a convenient tool to determine the influence of the downregulation or overexpression of specific proteins in the autophagic flux as well as the analysis of autophagy-modulating compounds. Western blot analysis of relevant parameters, such as the levels of EGFP-LC3, free EGFP generated by autophagic degradation and endogenous LC3·I–II are analyzed in the presence and absence of the autophagic inhibitor chloroquine.

Key words Autophagy, Autophagosome, LC3, HeLa

1 Introduction

Autophagy is a degradative pathway in which cytoplasmic constituents are degraded in the lysosomes. The process comprises the formation of structures, called phagophores, which sequester the cytoplasmic cargo and elongate to form closed double membrane vesicles, the autophagosomes. Subsequently, they fuse with compartments of the endolysosomal pathway to form autolysosomes, where the inner membrane of the vesicle and its content are degraded [1]. It occurs constitutively at basal levels but it is further induced in response to starvation and to other circumstances that could threaten cellular homeostasis, such as the presence of defective organelles or accumulated protein aggregates [2, 3].

Although numerous proteins participate in the constant formation and degradation of autophagic structures [4–6], only

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Atg8—and its homologs in other eukaryotes—remains associated to all those structures (phagophores, autophagosomes, and autolysosomes). Precisely because of its association to autophagic membranes until the end of the process, Atg8 is the quintessential protein for monitoring autophagy. In mammals, one of its homologs, microtubule-associated protein 1 light chain 3 (LC3), is the most widely used as an autophagic marker [7].

Membrane association of LC3 requires the posttranslational modification of the protein. Newly synthesized LC3 (proLC3) is cleaved by Atg4 at Gly120 at the C-terminus to form LC3-I, which is diffusely localized in the cytoplasm [8]. During autophagosome formation, LC3-I is conjugated to phosphatidylethanolamine in an ubiquitin-like reaction and the lipidated LC3 form (LC3-II) binds to the outer and the inner membranes of autophagosomes [9–11]. After autophagosome–lysosome fusion, outer membrane-bound LC3-II is again cleaved by Atg4 and dissociated from the membrane [12]. On the contrary, inner membrane-bound LC3-II is degraded within the autolysosome [8], *see* Fig. 1 for a schematic representation.

Most current assays to monitor autophagy are based on LC3 conversion and degradation. LC3 conversion can be traced by microscopy and immunoblotting techniques as LC3-II presents a punctate localization instead of a diffuse pattern and migrates faster

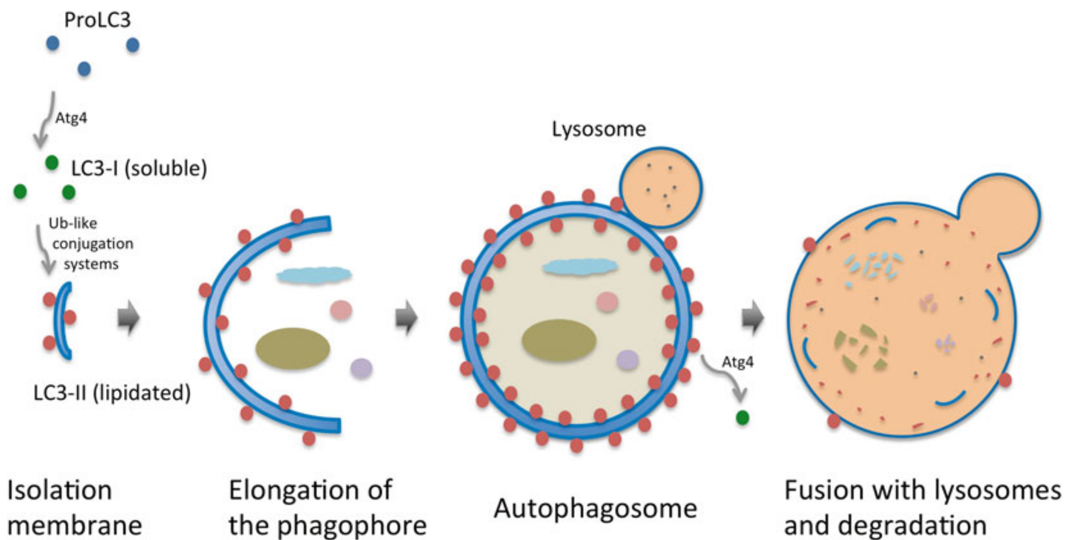

Fig. 1 Simplified representation of LC3 lipidation and turnover during autophagy. Cellular proLC3 is first cleaved by the protease Atg4 at the C-terminus to form LC3-I, also known as the soluble form. Upon autophagy induction, LC3-I is conjugated to the membrane of the nascent autophagosome and remains attached to both sides of the double membrane as the vesicle elongates, engulfs the cargo, and closes up. At that point, the outer LC3 is cleaved and recycled, the autophagosome fuses with lysosomes, and the internal LC3 is the degraded together with the inner membrane and the cargo. For cells expressing GFP-LC3, the fused protein suffers the same process as the endogenous LC3. The degradation of LC3 and GFP-LC3 in the autophagosome is the base for the methods described here, as their levels and localization change in the course of autophagic flux

than LC3-I on SDS-PAGE due to its hydrophobicity. LC3-II puncta and LC3-II levels correlate with the number of autophagic structures present in the cell but they do not inform about the actual autophagic activity. The reason is that autophagy is a dynamic process, in which the number of autophagosomes at a certain moment depends on their formation and degradation. This is referred to as autophagic flux and can be conveniently inferred from the assessment of LC3-II degradation in the lysosomes [13]. For that, LC3-II accumulation is monitored by immunoblotting after the addition of compounds that inhibit lysosomal degradation such as chloroquine.

The GFP-tagged version of LC3 at the N-terminus (GFP-LC3) is widely used to visualize autophagosomes. Following autophagy induction, GFP-LC3 is lipidated and associates to forming autophagosomes, resulting in a punctate pattern of the marker. Non-autophagic LC3 puncta can also occur due to the aggregation of the overexpressed GFP-LC3 [14] but this artifact is prevented in cell lines stably expressing GFP-LC3 at moderate levels. We would like to emphasize that not only the fluorescence of the lipidated membrane-bound form (GFP-LC3-II) can be used to monitor autophagy, but that the fluorescence of the soluble form (GFP-LC3-I) can also be informative of the autophagic process. In particular, when GFP-LC3 is overexpressed, GFP-LC3-I is diffusely located in the nucleus but translocates to the cytoplasm upon autophagy induction [15]. Thus, translocation of the diffuse fluorescence reflects autophagic activity. Differences in intensity of the diffuse fluorescence pattern can give a clue about the rate of LC3 conversion and degradation. However, it should be noted that differences of intensity can be due to different expression levels of the marker, so this is only applicable when using samples expressing a relatively homogeneous level of GFP-LC3, which is achieved in cell lines stably expressing the marker.

In addition, the potential of GFP-LC3 to monitor autophagy goes beyond its use as a fluorescent label. The fusion protein undergoes the same conversion and degradation of endogenous protein, but GFP is more resistant to lysosomal proteases than LC3. Thus, the appearance of free GFP also serves as an indicator of autophagic degradation [16]. We have found that most of the published studies limit the use of GFP-LC3 in immunoblotting to the detection of the free GFP band and that lysosomal inhibitors are not always added to properly characterize autophagic flux. However, as GFP-LC3 overexpression does not affect autophagic activity [17], we consider that the detection of both forms of the overexpressed and endogenous protein (GFP-LC3-I/II and LC3-I/II) together with the detection of the free GFP fragment, in the presence and absence of lysosomal inhibitors, provides a more complete view of the autophagic flux in a given experimental condition.

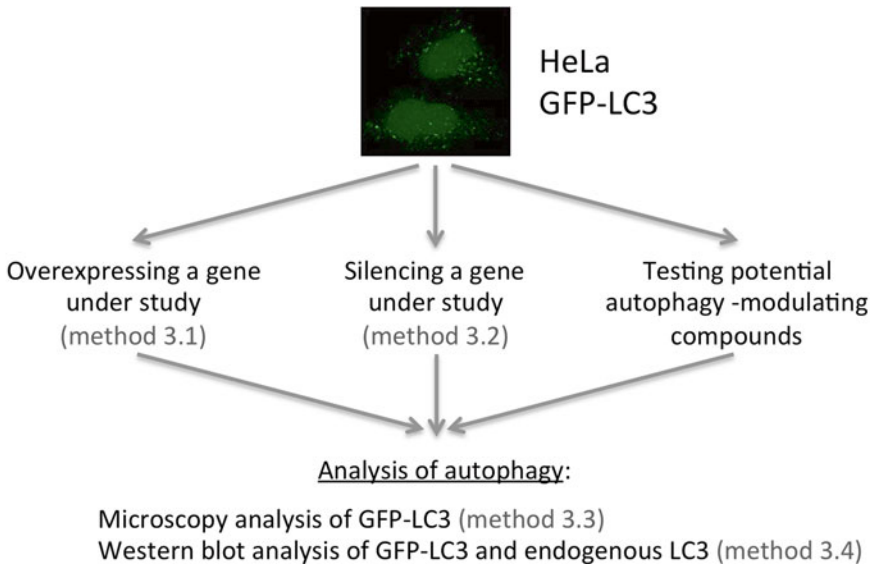

Fig. 2 Schematic representation of the methods described in this chapter. HeLa cells stably expressing the autophagosome marker GFP-LC3 can be used to determine autophagy changes upon overexpression (Subheading 3.1) or downregulation (Subheading 3.2) of genes under study as well as for testing the effect of compounds on autophagy (for example, in preclinical and high-throughput screenings). The effect of these alterations in autophagy can then be monitored by microscopy analysis (Subheading 3.3) or western blot to determine autophagic flux using the change in the levels of LC3-I and II (or GFP-LC3-I, II) and free GFP fragments upon autophagy inhibition with chloroquine (Subheading 3.4)

We describe in this chapter a series of protocols to efficiently assess autophagy using HeLa cells stably expressing EGFP-LC3 and a combination of fluorescence microscopy and immunoblotting procedures. These methods complement each other, which helps to interpret changes in the autophagic activity upon downregulation or overexpression of your favorite protein. This protocol can easily be adapted to the analysis of autophagy-modulating compounds. Figure 2 shows a diagram of the different methods described in this chapter.

2 Materials

2.1 Reagents

1. HeLa cells stably expressing EGFP-LC3 were kindly provided by Aviva M Tolkovsky (John Van Geest Center for Brain Repair, Cambridge, UK) and described previously [18].
2. Complete cell culture medium: DMEM (Dulbecco's Modified Eagle's Medium) (Sigma-Aldrich) supplemented with 10% FBS (Fetal Bovine Serum) (Gibco) and 1× penicillin-streptomycin (Gibco).

3. Starvation cell culture medium: EBSS, Earle's Balanced Salt Solution (Sigma-Aldrich).
4. PBS (phosphate-buffered saline) 1×: 133 mM NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4.
5. TrypLE Express Enzyme (Gibco) for detachment of adherent cells.
6. Lipofectamine 2000 (Invitrogen).
7. Lipofectamine RNAiMAX (Invitrogen).
8. Opti-MEM (Invitrogen).
9. Silencer Select siRNA, target and negative control (Ambion).
10. 4% Paraformaldehyde (Merk) in PBS. Prepare the solution in a hood, heating it to approximately 60 °C and stirring it gently. 1 M NaOH is added drop by drop to clear the solution, but taking care that the pH is maintained around 7.4.
11. 100 mM Glycine (Carlo Erba) in PBS.
12. DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) (Molecular Probes).
13. ProLongGold antifade mountant (Molecular Probes).
14. Chloroquine diphosphate salt (Sigma-Aldrich) stock solution at 1 mM, prepared with deionized distilled water (ddH_2O).
15. RIPA lysis buffer: 50 mM Tris–HCl pH 8, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 2 mM EDTA. Protease inhibitor cocktail (Sigma-Aldrich) is added to the lysis buffer at a 1:100 dilution just before use.
16. Pierce BCA protein assay kit (Thermo Scientific).
17. Sample loading buffer 5×: 250 mM Tris–HCl pH 6.8, 10% SDS, 25% glycerol, 0.04% bromophenol blue, 100 mM dithiothreitol (DTT).
18. SDS-polyacrylamide resolving gel (14%). Recipe for 10 ml (enough to prepare a 1.5 mm thick mini-protean gel): 3.85 ml ddH_2O, 3.5 ml 40% acrylamide/bis-acrylamide solution 37.5:1 (Bio-Rad), 2.5 ml Tris buffer 1.5 M pH 8.8, 100 μl SDS 10%, 50 μl ammonium persulfate (APS) 10% in ddH_2O (Bio-Rad, 161-0700), 5 μl *N,N,N′,N′*-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich).
19. SDS-polyacrylamide stacking gel (4%). Recipe for 4 ml (enough to prepare a 1.5 mm thick mini-protean gel): 3.04 ml ddH_2O, 0.4 ml 40% acrylamide/bis-acrylamide solution 37.5:1, 0.5 ml Tris buffer 1 M pH 6.8, 40 μl SDS 10%, 20 μl APS 10%, 4 μl TEMED.
20. SDS running buffer: 25 mM Tris Base, 192 mM glycine, 0.1% SDS in ddH_2O.
21. Transfer buffer: 25 mM Tris Base, 192 mM glycine, 20% methanol in ddH_2O.

22. Tris buffered saline containing tween (TBS-T): 0.136 mM NaCl, 20 mM Tris Base, 0.05% Tween-20 (Sigma-Aldrich). The pH should be adjusted to 7.4 with HCl.
23. Skim milk (Sigma-Aldrich).
24. Primary antibodies: anti-LC3 (Cell Signaling, 2775), anti-GFP (Sigma-Aldrich, G1544), anti-GAPDH antibody (Enzo LifeSciences, ADI-CSA-335).
25. Secondary antibodies are horseradish peroxidase (HRP)-conjugated antibodies: goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2004), goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005).
26. Amersham ECL Western Blotting detection reagent (GE Healthcare).

2.2 Other Materials and Equipment

1. Falcon 100 mm TC-treated polystyrene cell culture dishes (Corning), 6-well and 24-well clear TC-treated polystyrene multiwell cell culture plates (Corning).
2. CO_2 incubator.
3. 12 mm coverslips (Heinz Herenz) and microscope slides (VWR).
4. Inverted Zeiss LSM 710 laser confocal microscope (Zeiss) equipped with a Plan-Apochromat 63×/1.40 NA oil-immersion objective. ZEN2009 acquisition software and ImageJ processing software.
5. Mini-PROTEAN Electrophoresis System and Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad).
6. BioTrace Polyvinylidene fluoride (PVDF) transfer membrane (Pall Life Sciences).
7. CURIX RP2 Plus film (Agfa) and an X-ray film processing machine. A digital imaging system (GE Healthcare ImageQuant LAS4000) can be used instead.

3 Methods

3.1 DNA Transfection

1. The day before, detach the adherent cells on culture with the aid of a dissociating solution, such as TrypLE Express Enzyme, centrifuge the cells, and seed approximately 2×10^6 cells in a 100 mm dish to become 80% confluent at the time of transfection. Incubate the cells in complete cell culture medium at 37 °C in a CO_2 incubator.
2. Prior to transfection, replace the growing medium with 8 ml of prewarmed complete medium without antibiotics because the presence of antibiotics during transfection may increase cell death.

3. For each sample, dilute 40 μl Lipofectamine 2000 in 1 ml of serum-free medium Opti-MEM, mix gently by pipetting and incubate at room temperature for 5 min.
4. Dilute 20 μg of plasmid DNA in 1 ml Opti-MEM. Make the equivalent dilution with the same amount of a control empty vector.
5. Add the Lipofectamine 2000 dilution to the DNA dilution (2 ml of final volume for each sample), mix by pipetting, and incubate at room temperature for 20 min.
6. Add the 2 ml of the solution with the DNA-Lipofectamine 2000 complexes to the cells slowly and move the dish to evenly distribute the solution.
7. Incubate the cells with the DNA-Lipofectamine 2000 complexes at 37 °C in a CO_2 incubator for 4 h to minimize toxicity, aspirate the medium, and add fresh complete medium.
8. Incubate for about 24–30 h to allow the cells to recover from transfection. The cells are now ready for the subsequent autophagy experiments (*see* Subheadings 3.3 and 3.4) (*see* **Note 1**).

3.2 siRNA Reverse Transfection

1. The day of the transfection, detach the adherent cells on culture, count, centrifuge the cells, and dilute approximately 1×10^6 cells in 8 ml of prewarmed complete medium without antibiotics. Keep the cells in the falcon until **step 6** or alternatively **step 1** can be performed during the 20 min incubation described in **step 4**.
2. To prepare the complexes, for each sample, dilute 35 μl Lipofectamine RNAiMAX in 1 ml Opti-MEM, mix gently by pipetting, and incubate at room temperature for 5 min.
3. Dilute 100 pmol (10 μl of a 10 μM solution) of siRNA in 1 ml Opti-MEM. Make the equivalent dilution with the same amount of a negative control siRNA.
4. Add the Lipofectamine RNAiMAX dilution to the siRNA dilution (2 ml of final volume for each sample), mix by pipetting, and incubate at room temperature for 20 min.
5. Add the siRNA-Lipofectamine RNAiMAX complexes to the 100 mm dish and move back and forth to cover the surface of the dish with the mixture.
6. Add the 8 ml dilution of the cells to the dish containing the 2 ml of siRNA-Lipofectamine RNAiMAX complexes (the final siRNA concentration is 10 nM). Rock the dish gently.
7. Incubate at 37 °C in a CO_2 incubator for 48 h. Changing the medium is not necessary.
8. Repeat the siRNA reverse transfection from **steps 1–6** (*see* **Note 2**) using again 1×10^6 cells and the same volumes of every reagent for each transfection.

9. Three days after the second transfection the cells can be used for the subsequent autophagy experiments (*see* Subheadings 3.3 and 3.4).

3.3 Microscopy Analysis of GFP-LC3 Fluorescence

1. The day before the experiment, seed approximately 40,000 transfected cells on sterilized coverslips in 24-well plates. The culture should be 60–70% confluent at the time of the experiment (*see* **Note 3**).
2. For starvation treatment to induce autophagy, wash the cells twice with 1 ml of prewarmed PBS and once again with 1 ml of prewarmed starvation medium EBSS. Aspirate off the medium and add 1 ml of EBSS. Similarly, aspirate off the medium, wash the cells, and add 1 ml of prewarmed fresh complete medium (DMEM with 10% FBS) to the samples in which basal autophagy will be analyzed.
3. Incubate the cells at 37 °C and 5% CO_2 for 2 h (*see* **Note 4**).
4. Discard the medium and rinse the cells with PBS.
5. Fix the cells with 4% paraformaldehyde in PBS, pH 7.4, for 15 min at room temperature (*see* **Note 5**).
6. Wash the cells three times with PBS.
7. To quench the possible fluorescence signal from free aldehyde groups in paraformaldehyde, it can be convenient to incubate with 100 mM glycine in PBS for 30 min at room temperature.
8. At this point, permeabilization and incubation with primary and fluorescent-secondary antibodies can also be performed if the detection of additional proteins is desired. A nuclear dye such as DAPI can also be employed to stain nuclei (*see* **Note 6**).
9. Mount the samples using an antifade reagent such as ProLongGold. Put 4 μl of ProLongGold onto a microscope slide and with the aid of a forceps, place the coverslip onto the drop with the cells facing down and avoiding air bubble trapping. Leave the mounted samples in the dark at room temperature until the ProLongGold reagent is dry and then store them in the dark at 4 °C until visualization.
10. Observe the cells under the microscope. We routinely use a 63× objective. Higher magnification (100×) can be used to visualize the ring-shape of the autophagosomes.
11. Acquire the necessary captures along the *z*-axis to image the whole cell. Then, perform the montage to obtain the maximum intensity *z*-projection of the stack (*see* **Note 7**). Figure 3a shows images of the control sample of a representative experiment.
12. Count the puncta (*see* **Notes 8** and **9**). Figure 3b illustrates an example of quantification of the puncta observed in the control samples of independent experiments.

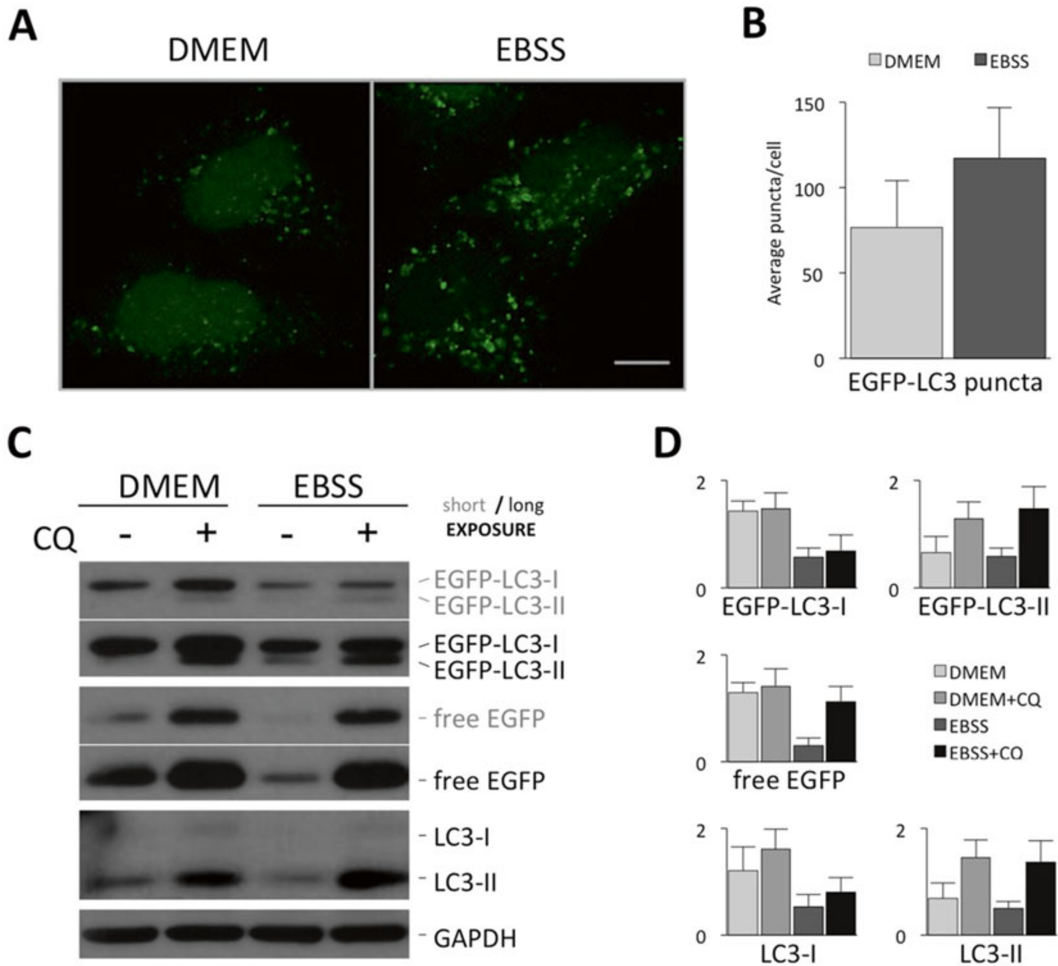

Fig. 3 HeLa cells stably expressing EGFP-LC3 and transfected with control siRNAs were incubated in complete (DMEM) or starvation (EBSS) medium for 2 h (**a**, **b**) or for 4 h in the absence or presence of 5 μM chloroquine (CQ) (**c**, **d**). Starvation causes the translocation of diffuse EGFP-LC3 fluorescence from the nucleus to the cytoplasm (**a**), an increase in the number of puncta per cell (**b**), a decrease in the amount of EGFP-LC3-I (45 kDa) and endogenous LC3-I (18 kDa) and the faster degradation of the free EGFP (27 kDa) generated by the cleavage of EGFP-LC3 (**c**, **d**). The block of the degradation caused by the presence of chloroquine shows the accumulation of the free EGFP fragment and the EGFP-LC3-II (43 kDa) and LC3-II (16 kDa) (**c**, **d**). The graphs show the mean values and standard deviations of the quantification of the puncta per cell in more than 300 cells for each condition from seven independent experiments (**b**) and of the densitometry of the protein bands observed in western blots of nine independent experiments, showing the comparison of the amounts of protein in arbitrary units (**d**). Scale bar: 10 μm

3.4 Western Blot Analysis

1. The day before the experiment, seed approximately 200,000 transfected cells in six-well plates. Cell confluence should be 60–70% at the time of the experiment (*see* **Note 3**).
2. The day of the experiment, aspirate off the medium, wash the cells twice with 2 ml of prewarmed PBS and once again with 1 ml of the prewarmed incubation medium for each experimental condition (DMEM or EBSS, without or with

chloroquine) to avoid any undesirable variation in the final composition and chloroquine concentration of the experimental incubation medium. Aspirate off the medium and add 2 ml of DMEM or EBSS medium, for incubation in rich nutrient or starvation conditions, respectively. To the samples in which autophagic degradation will be blocked, chloroquine has to be added to the medium at a final concentration of 5 μM from a prediluted stock solution (*see* **Notes 10** and **11**).

3. Incubate the cells at 37 °C and 5 % CO_2 for 4 h (*see* **Note 12**).
4. Put the plate on ice, discard the medium, and wash the cells with ice-cold PBS.
5. Add approximately 50 μl of RIPA lysis buffer with the protease inhibitor cocktail added prior to use.
6. Harvest the cells using a cell scraper and transfer them into a cooled microcentrifuge tube.
7. Leave the cells on ice during 30 min, mixing the solution each 10 min by pipetting or vortexing.
8. Centrifuge at 13,500 × *g* for 15 min at 4 °C and transfer the supernatant to a new microcentrifuge tube.
9. Take an aliquot of the sample and make a 1:4 dilution for measuring protein concentrations by the BCA protein assay. Follow the manufacturer's instructions to determine protein concentration.
10. Prepare the samples to load 7 μg of protein per well in sample loading buffer (*see* **Note 13**).
11. Boil the samples at 98 °C for 5 min. If the samples are not going to be loaded immediately, store them at −80 °C.
12. Place the previously prepared gels in the electrophoresis chamber and fill it with SDS running buffer. For preparing the gels, pour the freshly prepared resolving gel solution (APS and TEMED are added just prior pouring) in the cassette of cleaned glasses. Pour on top some milliliters of water or isopropanol to facilitate polymerization. Retire the water, pour the stacking solution, place the comb on top, and allow the gel to polymerize.
13. Load the samples and the protein marker in the polyacrylamide gel. Proceed with the electrophoresis at a constant voltage of 150 V at room temperature until the sample buffer is at the bottom of the gel.
14. Disassemble the gel cassette, discard the stacking gel, wash the gel in transfer buffer and assemble the transference sandwich (gel and methanol-activated PVDF membrane between filter papers and sponges) (*see* **Note 14**).
15. For protein transference, we routinely use a wet-type transfer system. Place the sandwich in the electrophoretic transfer cell

and fill it with transfer buffer. Transfer the proteins at 100 V constant current at 4 °C for 1 h (*see* **Note 15**).

16. Optionally, after transference the membrane can be stained with 0.1 % Ponceau S in 5 % acetic acid for 5 min to verify transference efficiency.
17. Block the membrane by incubating with 5 % skim milk in TBS-T on a shaker at room temperature for at least 1 h.
18. Incubate the membrane first with anti-LC3 antibody diluted 1:1000 in 5 % skim milk TBS-T on a shaker at 4 °C overnight (*see* **Note 16**).
19. Wash the membrane with TBS-T at room temperature five times for 10 min each.
20. Incubate the membrane with secondary antibody (goat anti-rabbit IgG-HRP) diluted 1:5000 in 2 % skim milk TBS-T on a shaker at room temperature for 1 h.
21. Wash the membrane with TBS-T at room temperature five times for 10 min each.
22. Mix the developing ECL solution, add it to the membrane and incubate for 1 min.
23. Use an X-ray film to capture the chemiluminescent signal on the membrane (*see* **Note 17**).
24. Repeat **steps 18–23** using anti-GFP antibody diluted 1:4000 and the goat anti-rabbit IgG-HRP antibody (*see* **Note 18**).
25. Repeat **steps 18–23** for detection of GAPDH as a loading control using anti-GAPDH antibody at 1:2000. In this case, use the secondary goat anti-mouse IgG-HRP antibody at 1:4000. Actin or tubulin can be also used as loading control proteins. Figure 3c shows the protein bands of the control sample of a representative experiment.
26. Scan the film and quantify bands signal intensity by densitometry using ImageJ software (*see* **Notes 19–21**). Figure 3d contains the graphs obtained from the protein bands densitometry of the control sample in independent experiments.

4 Notes

1. It is preferably to perform the procedure in a single population of cells that will be split at least 16–18 h before the planned experiment and no longer than 24 h. This is important to avoid heterogeneity in protein overexpression or depletion levels and even in confluence rates, which can alter basal autophagy and even LC3 expression. Concerning confluence, in general, cells should be maintained subconfluent but at a density that does not compromise transfection efficiency.

2. In certain cases, a large level of downregulation might be necessary to observe an effect. This can be particularly important for long-lived proteins. Longer periods of silencing can be achieved by clonal selection using expression of small hairpin RNA (shRNA); however, knockdown for prolonged time periods is not recommended for autophagy-related proteins [19]. We prefer to perform two consecutive siRNA transfections to assure the maximal depletion of the protein. However, it should be kept in mind that repetitive transfection is an additional stress to the cells. The decision to perform single or double transfections will depend on the protein of interest.
3. As stated above, confluence has an effect on basal autophagy levels. Thus, density of the cultures evaluated within and between experiments should be similar and confluence should be avoided.
4. This time period has been established for HeLa cells stably expressing EGFP-LC3. It might have to be modified if other cell lines are used as it depends on the autophagic activity of the cell [7]. In any case, it is not recommended to extend too much this incubation time when neither chloroquine nor other similar compound are added to block lysosomal degradation because it has been reported that LC3-puncta fluorescence decreases when cells are starved for longer periods [20].
5. Although the expression of the fluorescent marker allows live cell imaging, we describe here a protocol for fixing cells. Live cell imaging has the advantage that the response to the autophagy induction stimulus can be monitored along the time and that mobility of autophagosomes can also be traced. The major drawback is that the proper equipment is required to control temperature and CO_2 concentration in the environment, necessary to maintain the culture in suitable healthy conditions during the period of imaging. Another consideration is that GFP signal diminishes at an acidic pH. As a consequence, GFP fluorescence is quenched inside the autolysosomes [20]. This attenuation of fluorescence can be circumvented by fixation of the samples. Paraformaldehyde fixation maintains the sample at a neutral pH and thus, GFP fluorescence is retained. Fixed samples have the additional advantage that they can be stored and also used for immunodetection of other proteins and colocalization studies.
6. For immunofluorescence, it is important to keep in mind that certain detergents can lead to the appearance of artifactual GFP-LC3 puncta [21]. Similar cautions apply with regard to methanol, which can also be used for cell fixation and permeabilization, but might reduce GFP intensity [22].
7. To avoid misinterpretation of results that could arise from an uneven distribution of autophagosomes within the cell and the selection of random sections along the *z*-axis, we routinely

image and count the puncta present of the whole cell. For this, we capture the necessary slices to imaging the entire cell. For showing purposes, we also consider that the maximum intensity *z*-stack projection provides a more objective view of the cell.

8. Puncta can be automatically quantified by using specialized imaging software (ImageJ, Imaris, or CellProfiler). In that case, manual evaluation of the analysis is highly recommended to verify the quantification or, if necessary, adjust the parameters that define what is considered "puncta". Manual quantification might be more accurate, but similarly, uniform criteria must be applied regarding the definition of puncta. An advantage of automated analysis is that other parameters, such as area, can be monitored. In particular, the average percentage of the total area of GFP-LC3 puncta on a per cell basis can be a more appropriate index in the cases when individual puncta quantification is not possible due to autophagosome clustering.
9. GFP-LC3 overexpression results in most of the cells displaying some puncta regardless of the experimental condition. Therefore, the percentage of cells with puncta is not a good indicator of autophagic activity. To circumvent this problem, a threshold can be established to classify the population in cells with basal or induced autophagy. However, the definition of this cut-off value is rather subjective, and can be dependent on the expression levels of GFP-LC3. GFP-LC3 expression is more uniform in cell lines stably expressing the marker, but variability of autophagic activity is still significant. We think that a more appropriate index is the average number of puncta per cell. As a consequence of the variability of the autophagic activity within the same population, this parameter also considerably fluctuates across cells. Thus, for a good quantification of puncta, a large number of cells (around 100) from multiple sections should be documented for each condition in at least three independent experiments.
10. We have observed that this nonsaturated concentration of chloroquine is enough to allow the simultaneous accumulation of free GFP and LC3-II in HeLa cells stably expressing GFP-LC3. If this concentration has to be increased for other cell types, it is important to ensure that it is low enough to allow the visualization of the cleavage of the GFP-LC3, which requires a nonsaturating concentration of chloroquine as described previously [16]. It is believed that GFP is relatively resistant to lysosomal degradation and can be accumulated using low concentrations of lysosomal inhibitors. Higher concentrations (saturating concentrations) would totally inhibit lysosomal proteases hampering the GFP-LC3 cleavage, so free GFP fragments will not be generated and the GFP cleavage assay would not be applicable. Besides, high concentrations of

chloroquine could induce autophagy or could also affect other pathways independent of autophagy. This kind of side-effect has to be also taken into account if other compounds are used to raise the lysosomal pH.

11. When deciding the experimental incubation conditions, it should be kept in mind that acidity of lysosomes regulates GFP-LC3 cleavage and free GFP accumulation. This also accounts for the use of EBSS as a starvation medium to induce autophagy. EBSS lowers lysosomal pH and this provokes that, while free GFP fragments can still be detected when the cells are incubated in complete medium in the absence of lysosomal inhibitors, free GFP is further degraded and might be undetectable when EBSS is used without lysosomal inhibitors.
12. Note that we have extended the incubation period with respect to the protocol for fluorescence microscopy because accumulation of the lipidated GFP-LC3-II and LC3-II as well as free GFP will increase in a time-dependent manner due to the altered lysosomal pH and thus, the differences, in comparison to the samples in the absence of chloroquine, will be more evident. Longer incubations are not advisable because the expression of some proteins can change. One example is the autophagy substrate p62/SQSTM1 [23] but changes in other autophagy-related proteins, even in LC3, might also occur [24–26]. Moreover, a secondary autophagic response could be induced due to the accumulation of nondegraded autophagosomes if the incubation period is too long.
13. Using small amounts of protein is advisable so the chemiluminiscent signal is less saturated, and minor differences between the bands of accumulated proteins are easier visualized. In contrast, it is probably necessary to load higher amounts of protein to visualize the LC3-I band, which is difficult to detect even after long exposure periods in this cell type.
14. PVDF membranes are preferred rather than nitrocellulose membranes due to their better retention of the lipidated LC3-II.
15. Time of transfer should be short because of the low molecular weight of endogenous LC3.
16. We recommend incubating first with anti-LC3 antibody because LC3 signal is more easily lost during subsequent hybridizations than the GFP signal, due to the lesser sensitivity of the anti-LC3 antibody. This might also help in the detection of little differences in the fusion protein GFP-LC3-I and GFP-LC3-II amounts, which might be more difficult to perceive using the more sensitive anti-GFP antibody.
17. Capture the signal at different exposure times to cover all the range of signal saturation levels. Discard for densitometry those with too low or too strong signals as they might not be

in the linear range and would not represent the real differences between the samples. Short exposure times can lead to too faint bands in conditions of induced and nonblocked autophagy, while long exposure times can mask differences between bands in conditions where autophagy degradation has been blocked. Thus, for illustrating purposes, it is usually helpful to show both a short and a long exposure. Nevertheless, for a quantitative evaluation, the lanes from all the conditions have to be measured from a unique exposure time.

18. Stripping of the membrane is not necessary as the molecular weights of the proteins are different and this procedure could be aggressive and affect latter signals.

19. ImageJ software tool is useful for this purpose. As with puncta quantification, there are some practical aspects to be considered. The most important one is that the bands that are going to be subjected to densitometry must not be overexposed. This accounts, in particular, for the samples corresponding to chloroquine incubation, where the accumulation of GFP and the lipidated forms of overexpressed and endogenous LC3-II results in a strong signal. As stated above, for comparison between all the samples, the densitometry has to be done from a unique exposure; and although it might be difficult in practice, this exposure should be in the linear range for all the bands, avoiding saturation as much as possible. The data must be normalized to the loading control protein and then, the mean and the standard deviations of the independent experiments can be used to compare between samples and determine the autophagic flux in each experimental setting.

20. We would like to add some important considerations regarding data interpretation in the following notes. LC3-I levels decrease in response to autophagy induction due to the conversion to the LC3-II form and therefore, changes in the LC3-I amount in a given experimental setting compared to controls suggest that LC3-I conversion is affected in that condition. The comparison of LC3-II levels between samples in the presence and absence of lysosomal inhibitors better represents autophagic activity than the comparison of the ratio of LC3-II to LC3-I. Although the later has been employed as a measure of autophagic induction, we believe that it is not a trustworthy indicator because LC3-II is degraded within the autophagolysosomes and its levels can increase or decrease depending on the rate of conjugation and degradation if the later is not blocked. Besides, we have experienced that this ratio might change depending on the antibody used for immunodetection. Certain antibodies barely detect LC3-I although they give a strong signal for LC3-II and, inversely, LC3-I is sensitive to detection by other antibodies that might result in a

less intense LC3-II band [7]. Moreover, it should be kept in mind than LC3-I seems to be more fragile and poorly conserved in stored samples, especially when they have been repetitively frozen and thawed [15].

21. The amounts of the soluble and the lipidated forms of LC3 can also be analyzed when the protein is fused to GFP. Conversion of GFP-LC3 parallels that of endogenous LC3 [18]. Therefore, the evaluation of the fused protein, in addition to that of the endogenous one, might be helpful to interpret the autophagic activity within the cell. As in the case of endogenous protein, GFP-LC3-I amount decreases in response to autophagy induction and thus its comparison between growth and starvation conditions can be used as a reliable parameter. While the detection of the endogenous LC3-I have some drawbacks, GFP-LC3-I detection is clear due to the overexpression and the higher sensitivity of anti-GFP detection. Similarly to endogenous LC3-II, GFP-LC3-II band intensity can increase or decrease in response to starvation or other autophagy induction stimulus if the autophagic degradation is not blocked. But in reference to its accumulation in the presence of lysosomal inhibitors, it may be less evident for GFP-LC3-II, which tends to accumulate less than endogenous LC3-II in certain conditions [20]. This effect might arise from GFP-LC3-II cleavage within the lysosome when a nonsaturating concentration of lysosomal inhibitor is being used. Regarding the evaluation of free GFP, we believe that the comparison between its levels in the presence and absence of lysosomal inhibitors informs about the autophagic flux mainly in starvation conditions.

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