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Evaluating Autophagy Levels in Two Different Pancreatic Cell Models Using LC3 Immunofluorescence

Felipe J. Renna[1], **Malena Herrera Lopez**[1], **Maria Manifava**[2], **Nicholas T. Ktistakis**[2], **Maria I. Vaccaro**[1]

[1] Instituto de Bioquimica y Medicina Molecular Prof Alberto Boveris (IBIMOL), Universidad de Buenos Aires, CONICET [2] Signalling Programme, The Babraham Institute

Corresponding Authors

Felipe J. Renna
fjrenna@ffyb.uba.ar

Maria I. Vaccaro
mvaccaro@ffyb.uba.ar

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Abstract

Autophagy is a specialized catabolic process that selectively degrades cytoplasmic components, including proteins and damaged organelles. Autophagy allows cells to physiologically respond to stress stimuli and, thus, maintain cellular homeostasis. Cancer cells might modulate their autophagy levels to adapt to adverse conditions such as hypoxia, nutrient deficiency, or damage caused by chemotherapy. Ductal pancreatic adenocarcinoma is one of the deadliest types of cancer. Pancreatic cancer cells have high autophagy activity due to the transcriptional upregulation and post-translational activation of autophagy proteins.

Here, the PANC-1 cell line was used as a model of pancreatic human cancer cells, and the AR42J pancreatic acinar cell line was used as a physiological model of highly differentiated mammalian cells. This study used the immunofluorescence of microtubule-associated protein light chain 3 (LC3) as an indicator of the status of autophagy activation. LC3 is an autophagy protein that, in basal conditions, shows a diffuse pattern of distribution in the cytoplasm (known as LC3-I in this condition). Autophagy induction triggers the conjugation of LC3 to phosphatidylethanolamine on the surface of newly formed autophagosomes to form LC3-II, a membrane-bound protein that aids in the formation and expansion of autophagosomes. To quantify the number of labeled autophagic structures, the open-source software FIJI was utilized with the aid of the "3D Objects Counter" tool.

The measure of the autophagic levels both in physiological conditions and in cancer cells allows us to study the modulation of autophagy under diverse conditions such as hypoxia, chemotherapy treatment, or the knockdown of certain proteins.

Introduction

Macroautophagy (commonly referred to as autophagy) is a specialized catabolic process that selectively degrades cytoplasmic components, including proteins and damaged organelles[1,2]. Autophagy allows cells to physiologically respond to stress stimuli and, thus, maintain cellular homeostasis[3]. During autophagy, a double membrane vesicle is formed: the autophagosome. The autophagosome contains the cargo molecules and drives them to the lysosome for degradation[1,4].

Autophagosomes are decorated by the autophagic protein microtubule-associated protein light chain 3 (LC3)[5]. When autophagy is not induced, LC3 is diffused in the cytoplasm and nucleus in the LC3-I conformation. On the other hand, when autophagy is induced, LC3 is conjugated with a phosphatidylethanolamine in the membrane of the autophagic structures[6]. This new LC3 conformation is known as LC3-II[1]. The LC3 conformation shift causes changes in its cellular localization and its dodecyl sodium sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) migration, which can be detected by techniques such as immunofluorescence and western blot[5,7]. In this way, LC3 conjugation is a key event in the autophagic process that can be used to measure autophagic activity.

The pancreatic acinar cell is a highly differentiated cell that, under healthy conditions, has a low rate of autophagy. However, in different physiological conditions or under pharmacological stimulation, they can activate autophagy. Therefore, the determination of autophagic levels in this cell line is useful for studying the potential direct or indirect effects of different pharmacological or biological agents on autophagy[8,9].

Ductal pancreatic adenocarcinoma is one of the deadliest types of cancer, given its late diagnosis and its high chemotherapy resistance[10]. Pancreatic cancer cells have high autophagy activity due to the transcriptional upregulation and post-translational activation of autophagy-related proteins[11]. Pancreatic cancer cells may adjust their autophagy levels in response to unfavorable conditions like hypoxia, nutrient deprivation, or chemotherapy-induced damage[11]. Hence, analyzing the autophagy levels in pancreatic cancer cells can help understand how they adapt to varying environments and evaluate the effectiveness of autophagy-modulating treatments.

This study shows a method to perform LC3 immunofluorescence in two distinct pancreatic cellular models. The first model, PANC-1 cells, served as a model for pancreatic ductal adenocarcinoma. These cells were treated with gemcitabine, a chemotherapy agent that has previously been shown to induce autophagy, specifically in pancreatic cancer cells carrying the oncogenic Kirsten rat sarcoma virus gene (KRAS)[12,13]. The second model, AR42J cells, served as a more physiological model of exocrine pancreatic cells. These cells were differentiated with dexamethasone to become more similar to acinar pancreatic cells[14]. In these cells, autophagy was pharmacologically induced through the use of PP242, which is a potent mTOR inhibitor[15]. In this study, we demonstrate the applicability of the protocol described with two different pancreatic models and its ability to discriminate between states of low and high autophagy.

Protocol

1. Cell preparation

1. Soak 12 mm round coverslips in absolute ethanol, and place them vertically in the wells of a 24-well plate.
2. Remove the cover, and expose the multi-well plate to ultraviolet radiation for 15 min.
3. Position the coverslips horizontally, and wash them with Dulbecco's Modified Eagle Medium (DMEM).
4. Seed a low passage number of pancreatic cells. The amount should be adjusted to obtain 50%-75% confluency on the day of fixation[16].

   NOTE: It is recommended to seed 2.5 × 10^4 PANC-1 or 4 × 10^4 AR42J cells per well to fixate the cells after 3 days.
5. Culture the cells in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in an incubator at 37 °C under a humidified atmosphere with 5% carbon dioxide (CO_2).

   NOTE: For PANC-1 cells, it is recommended to incubate the cells for 2 days between cell seeding and the following steps. After this time, the cells can be transfected, treated, or fixated. This protocol exemplifies the treatment with gemcitabine in non-transfected PANC-1 cells and the differentiation and PP242 treatment for non-transfected AR42J cells.

2. Treating the cells

1. Gemcitabine treatment for PANC-1 cells
   1. Prepare a solution of 1 µg/µL gemcitabine in DMEM 2 days after seeding. Treat each well with 2.6 µL of the 1 µg/µL gemcitabine solution to achieve a final dilution of 20 µM.
   2. Incubate the cells for 24 h in the incubator.
2. AR42J differentiation and PP242 treatment
   1. Prepare a solution of 4 µg/mL dexamethasone in DMEM.
   2. Treat each well with 4.9 µL of 4 µg/mL dexamethasone solution to obtain a final dilution of 100 nM.
   3. Incubate the cells for 48 h in the incubator.
   4. Remove the medium, and treat each well with 0.5 µL of 1 mM PP242 to obtain a final dilution of 1 µM.
   5. Incubate the cells for 2 h in the incubator.

3. Fixing and permeabilizing the cells

1. Prepare a 24-well plate with cold methanol and a 6-well plate with cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4). Maintain them on ice.
2. Take each coverslip with tweezers, wash it twice in PBS, and incubate for 6 min in methanol.

4. Blocking the cells

1. Wash each coverslip twice in PBS, and incubate for 1 h in 10% fetal bovine serum in PBS (blocking solution).

   NOTE: In this step, the protocol might be paused. The coverslips can be stored overnight in the fridge in the blocking solution, and the protocol can be continued the following day.

5. Incubating the coverslips with the primary antibody

1. Prepare a 1:1,000 solution of anti-LC3 in the blocking solution, and maintain it on ice.
2. Place a piece of laboratory sealing film over the multi-well lid.
3. Place one drop (25 µL) per coverslip of anti-LC3 solution over the sealing film.
4. Take each coverslip with tweezers, and place it over the primary antibody drop, taking care that the cell side is in contact with the solution.
5. Prepare a humid chamber by placing a humid piece of paper into a flat-bottom plastic box.
6. Place the multi-well plate into the humidity chamber, cover it with foil, and incubate overnight in the fridge.

6. Incubating the coverslips with the secondary antibody

1. Remove the multi-well plate from the humidity chamber, and place the coverslips back in the multi-well plate.
2. Perform three washes with PBS.
3. Prepare a solution of fluorescently labeled anti-rabbit with a dilution of 1:800 in the blocking solution, and maintain it on ice protected from light.
4. Place a sealing film piece over the multi-well lid.
5. Place a drop (25 µL) per coverslip of anti-rabbit solution over the sealing film.
6. Take each coverslip with tweezers, and place it over the primary antibody drop, taking care that the cell side is in contact with the solution.
7. Incubate the multi-well plate in the humidity chamber for 2 h at room temperature (RT) protected from light.

7. Staining the cells with 4′ ,6-diamidino-2-phenylindole (DAPI)

1. Remove the multi-well plate from the humidity chamber, and place the coverslips back in the multi-well plate.
2. Perform three washes with PBS.
3. Prepare a 300 nM solution of DAPI in PBS (protected from light).
4. Incubate each coverslip with the DAPI solution for 10 min.
5. Perform three washes with PBS. Maintain the multi-well plate protected from light.

8. Montage

1. Prepare two beakers with water and a piece of paper.
2. Place one drop (10 µL) per coverslip of a polyvinyl alcohol-Bis(trimethylaluminum)-1,4-diazabicyclo[2.2.2]octane adduct (PVA-DABCO) solution on a slide.
   NOTE: PVA-DABCO is prepared by combining 0.25 M DABCO, 10% W/V PVA, 20% glycerol, and 50% Tris HCl (1.5 M, pH 8.8) in ultrapure water.
3. Take each coverslip with tweezers, wash it in each water beaker, dry it off in the paper, and place it over the PVA-DABCO drop (with the cells in contact with the solution).
4. Let it dry overnight, protected from light.

9. Confocal microscopy viewing and image capture

1. Visualize the coverslips in an inverted confocal microscope using an objective of around 63x[17].

2. Capture representative images of the labeled cells.

10. Quantifying the LC3 dots

1. Drag and drop each image file containing the captured channels, such as ".czi", into the ImageJ (FIJI) screen to open. Click on **Ok** in the dialog box, and close the **Console** window.
2. From the **Image** tab, select **Color > Split Channels**.
3. Close the images corresponding to the channels other than the LC3 image.
4. From the **Image** tab, select **Adjust > Color Balance**
5. Move the **Maximum** slider to the left until the image is saturated to visualize the cell contours.
6. Draw the cell outline with the **Freehand Selection** tool.
7. Click on the **Reset** button to reset the color adjustment.
8. From the **Edit** tab, select **Cut** to cut the selected item.
9. Close the image without saving it.
10. From the **Edit** tab, select **Paste**.
11. In the **Analyze** menu, choose the tool **3D Objects Counter**.
12. Set the threshold. In the example provided in this study, the threshold is set at 2,000.
13. Set the size filter. In this study, it is set between 50 and 500.
14. Be sure that the boxes **Objects** and **Summary** are marked.
15. Click on **Ok**. The number of dots will be described as **Objects Detected** in the **Summary**.

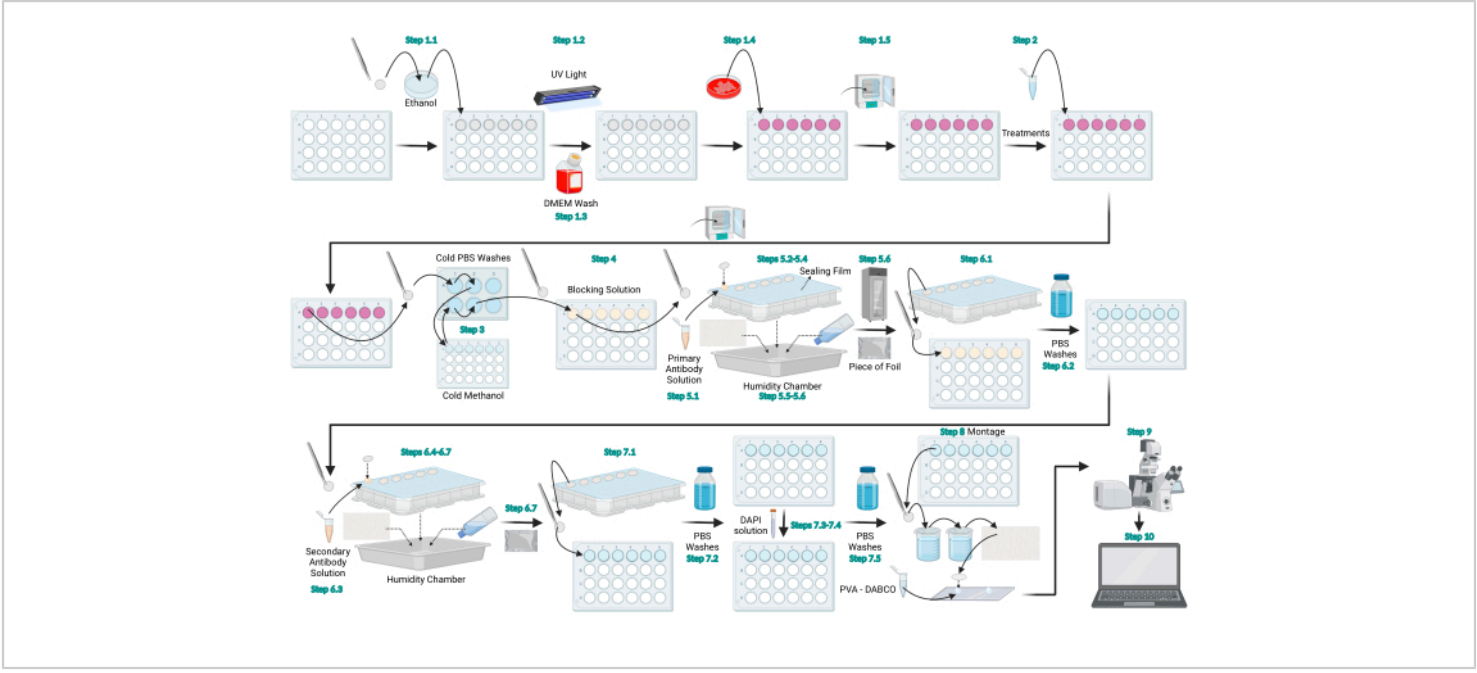

Figure 1: Schematic diagram of the LC3 immunofluorescence protocol. Schematic diagram that represents the general protocol provided for LC3 immunofluorescence. Figure created with BioRender.com. Please click here to view a larger version of this figure.

Representative Results

This protocol performs immunofluorescence of LC3 in pancreatic cell lines to determine the autophagy levels in different conditions. The outcome of this experiment was the obtention of cellular images from the red and blue channels, corresponding to LC3 and DAPI. The LC3 images indicate the cellular distribution of this protein, whereas the DAPI shows the nuclear localization. **Figure 2A** shows a representative image of the immunofluorescence of LC3 and its merge with DAPI staining in PANC-1 cells under basal or gemcitabine treatment conditions. A set of images of LC3 staining was analyzed using the tool **3D Objects Counter** in FIJI. Using this software, the amount of LC3 dots per cell was quantified. The bar graph in **Figure 2B** shows the results of LC3 dot quantification in PANC-1 cells under basal versus gemcitabine treatment conditions. In this graph, the LC3 dots significantly increased under gemcitabine treatment, with the number of LC3 dots directly indicating the autophagic activity. We also previously demonstrated that gemcitabine triggers autophagy in pancreatic cancer cells[12]. Overall, the method presented in this article allows the detection of the level of increase in autophagy activation induced by gemcitabine in these cells.

While this protocol focuses on using LC3 immunofluorescence to determine autophagic activity in pancreatic cancer cells, it could potentially be applied to other cell lines, including more physiologically relevant models. To test the method's efficacy in assessing physiological responses, the AR42J cell line was used. Although these cells are derived from a rat exocrine pancreas tumor, they can be differentiated into exocrine cells with glucocorticoid stimulation, thus making them a suitable pancreatic model[8,14,18]. The AR42J cells were differentiated with 100 nM dexamethasone treatment for 48 h, followed by treatment with the mTOR inhibitor PP242 to induce autophagy[15]. The obtained results are presented in **Figure 3**, which shows a significant increase in the number of LC3 dots per cell under the PP242 treatment.

Thus far, we have demonstrated that the presented method is effective for assessing autophagic activity in both cancer cells and a more physiological model. However, it is important to note that minor deviations from the presented protocol could result in uninterpretable results.

Figure 4A shows a representative image from a suboptimal experiment in which too many cells were seeded on the coverslips, and excessive confluence was obtained. This kind of experiment might be uninterpretable for diverse reasons. Firstly, the cellular types mentioned in this work are derived from the exocrine pancreas, where the cells are grouped in acini. Under an excessive confluence, these cells tend to pile up and grow on top of each other (such as cell 1 and cell 2 in **Figure 4A**, which are above cell 3 and cell 4). This phenomenon makes it practically impossible to know which cell the LC3 dots belong to, thus making it very difficult to estimate the number of dots per cell. On the other hand, cells at high confluency tend to be stressed, which triggers autophagy. As a result, the differences in autophagic levels between the control and treated cells might decrease due to an increase in the background autophagic activity.

In **Figure 4B**, a representative image from another kind of suboptimal experiment is shown in which the cells were fixed with paraformaldehyde instead of methanol. While this fixation method is generally effective for preserving a variety of proteins, it is not suitable for LC3, as the resulting image does not accurately reflect its true distribution. This technical

mistake might make it impossible to find differences between low and high autophagic levels.

Generally, cell lines can be treated, transfected, or fixed 1 day after seeding. Nevertheless, it is crucial to mention that, in the case of PANC-1 cells, it is necessary to wait for 2 days after seeding to ensure the complete adherence of the cells to the glass coverslips before proceeding with the subsequent steps of the experiment. **Figure 4C** shows a representative image from an experiment in which the cells were treated with gemcitabine just 1 day after seeding. From the figure, it can be observed that the cells in this experiment had a round shape. This morphology decreased the relation between the cytoplasm and nucleus, making it difficult to understand the intracellular distribution of LC3 and to discriminate between low and high autophagic levels. It is important to note that AR42J does not have this problem, and they are ready to be treated or fixed on the day following the seeding.

Another suboptimal outcome could be obtained when the time of methanol fixation is varied. Shorter times of fixation might cause incomplete fixation, as represented in **Figure 4D**, where the cells were fixed for 3 min. Incomplete fixation can interfere with proper LC3 immunolabeling, leading to unclear images and suboptimal quantification.

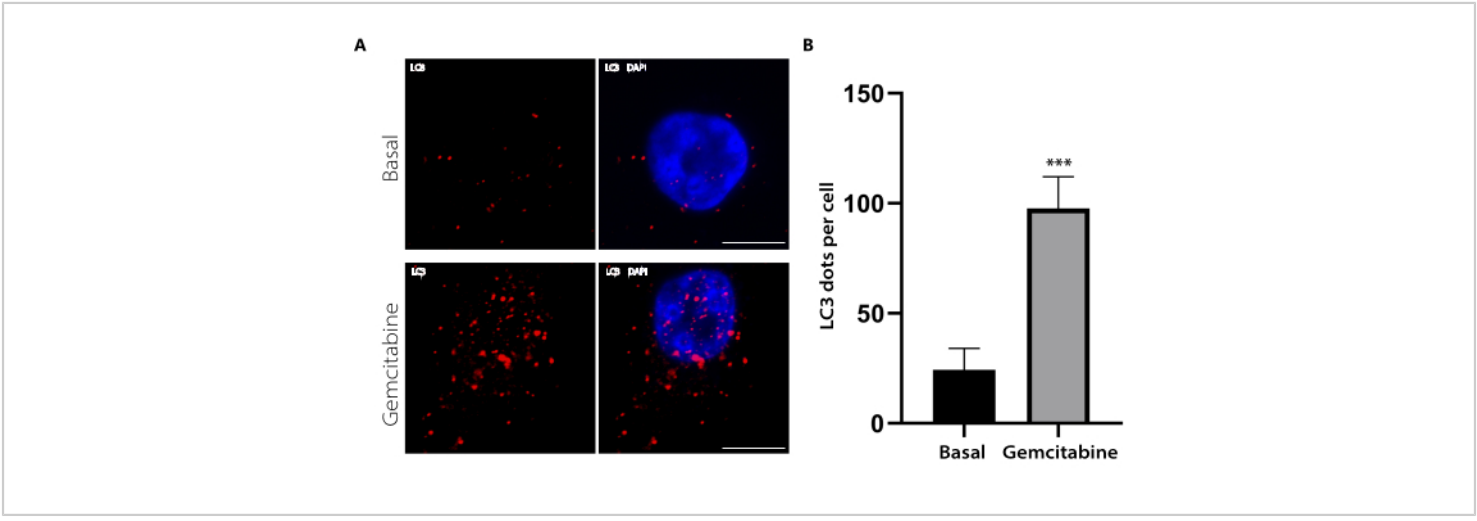

Figure 2: LC3 immunofluorescence in PANC-1 cells under basal or gemcitabine conditions and LC3 dot quantification. The PANC-1 cells were either treated with 20 µM gemcitabine for 24 h or left untreated and then immunolabelled with anti-LC3. (**A**) Representative images of each condition are shown. Scale bar: 10 µm. (**B**) The bar graph represents the means and standard errors of the means (SEM) of the LC3 dots per cell for each condition. N = 10 cells per condition from three independent experiments. *** $p < 0.001$ by a Student's t-test. Please click here to view a larger version of this figure.

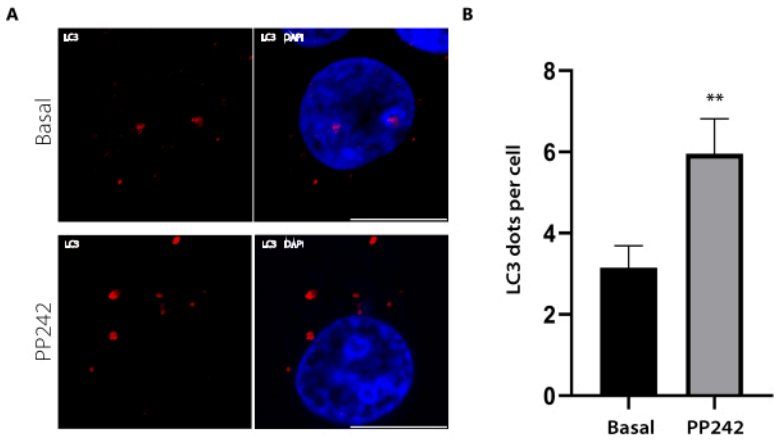

Figure 3: LC3 immunofluorescence in AR42J cells under basal or PP242 conditions and LC3 dot quantification. The AR42J cells were differentiated with 100 nM dexamethasone for 48 h and then either treated with 1 µM PP242 for 2 h or left untreated, followed by immunolabelling with anti-LC3. **(A)** Representative images of each condition are shown. Scale bar: 10 µm. (**B**) The bar graph represents the means and standard errors of the means (SEM) of the LC3 dots per cell for each condition. N = 10 cells per condition from three independent experiments. ** $p < 0.01$ by a Student's t-test. Please click here to view a larger version of this figure.

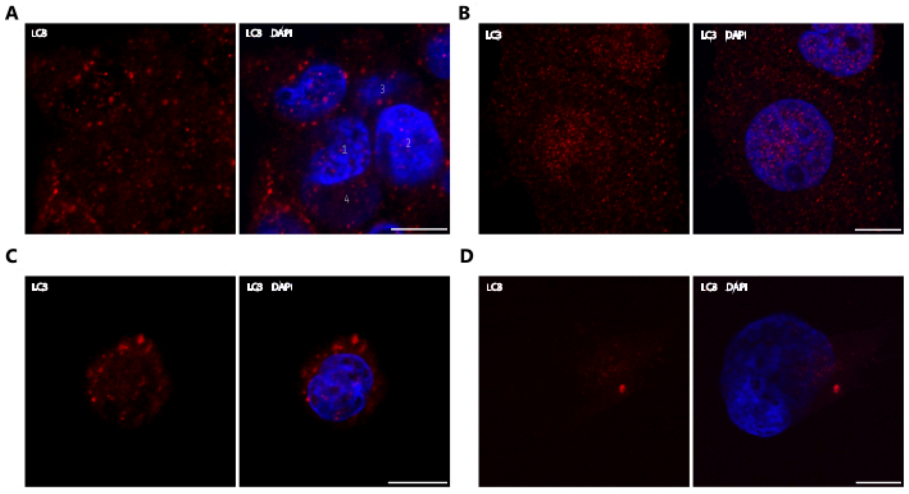

Figure 4: Suboptimal experiments. Representative images of LC3 immunofluorescence in suboptimal experiments are shown. Scale bar: 10 µm. (**A**) Excess confluence: 7 × 10^4 PANC-1 cells were seeded, treated with gemcitabine, and immunolabelled with anti-LC3. Four cells are marked to show that cell 1 and cell 2 are above cell 3 and cell 4. (**B**) PFA fixation: PANC-1 cells were treated with gemcitabine, fixated with PFA, and immunolabelled with anti-LC3. (**C**) Incomplete stretch: PANC-1 cells were treated with gemcitabine the day after seeding and immunolabelled with anti-LC3. (**D**) Incomplete fixation: PANC-1 cells were treated with gemcitabine, fixated with methanol for 3 min, and immunolabelled with anti-LC3.

Please click here to view a larger version of this figure.

Discussion

The method described in this protocol allows for visualizing the endogenous LC3 distribution in the cell and quantifying the autophagic levels under different conditions. Another similar method used to analyze the LC3 distribution and determine autophagy activation involves fluorescence-labeled LC3 transfection (such as RFP-LC3)[19]. RFP-LC3 transfection has the advantages of not needing fixation (which allows for applying this method in live cell imaging[20]), being cheaper, and not depending on LC3 antibody reactivity. On the other hand, the immunofluorescence of LC3 has the advantage of providing an image of the endogenous LC3, thus avoiding possible issues related to LC3 overexpression, such as the formation of protein aggregates that are independent of autophagy[21]. Moreover, this method does not depend on the ease of transfecting the cells, meaning it is applicable to diverse cell lines. However, depending on the LC3 antibody that is used and its reactivity, there are some cellular lines in which it might not work. Some antibodies may work well for certain species but not for others, even when they are theoretically compatible with different species. In the case of the antibody used in this protocol (LC3B D11), we have found that it works perfectly for human cells (PANC-1, HEK293T, HeLa) and rat cells (AR42J). However, it does not work for mouse cells (MEF cells), as we observed nonspecific nuclear staining. It is worth noting that the quantification of LC3 spots, whether endogenous or overexpressed, has limitations in distinguishing between changes in autophagy

activation, which may indicate the increased production of LC3-II, and changes in LC3-II degradation, which may indicate an autophagic flux state. Additional methods can be used to comprehensively assess autophagy activity. For instance, the use of RFP-GFP-LC3 expression can provide an accurate assessment by distinguishing between LC3-II inside or outside of the lysosome[22,23].

As shown in **Figure 4**, there are some critical steps in the protocol that must not be modified, given that their modification can lead to suboptimal outcomes. First, it is important to set the correct number of cells to be seeded. When not enough cells are seeded, they tend not to resist transfection or treatments and remain rounded. On the contrary, when cells are seeded in excess, they tend to grow on top of the neighboring cells, making it challenging to focus on individual cells and distinguish between their LC3 dots. Notably, in situations where cells are in close proximity but not overlapping as depicted in **Figure 4A**, it may be helpful to use immunostaining with specific membrane markers, such as EGFR, to distinguish between the positive markers belonging to each cell[24]. However, it is important to note that certain markers like E-cadherin and EpCAM are not suitable for this purpose in PANC-1 cells due to their reduced membrane expression, which results from the typical epithelial-mesenchymal transition process associated with this cell type[25,26,27]. Secondly, when working specifically with PANC-1 cells, it is essential to wait at least 1 day between the seeding and the subsequent steps of the experiment. Conversely, when one does not wait for the right time, the cells can be rounded, making it difficult to interpret the results. Thirdly, fixation is a critical step in this protocol. As we have shown, the method only works with an adequate methanol fixation. Paraformaldehyde fixation does not work correctly for LC3 immunolabelling, while the methanol fixation time should not be modified, given that shorter times might lead to an incomplete fixation. We tested methanol fixation times up to 1 h and did not observe differences in LC3 labeling. Nevertheless, we advise against the use of prolonged fixation times, as methanol fixation can lead to the loss of soluble cellular proteins and free fluorescent molecules[28]. Therefore, it is recommended to adhere to the standard fixation time to ensure precise and dependable results.

Some modifications might be accepted in the described protocol. For example, a 12-well plate can be used instead of the 24-well plate, as well as growing the cells over 15 mm round coverslips instead of 12 mm coverslips. In this case, it should be considered that the number of cells to be seeded, as well as the volumes of reagents used, will be greater than those described in this protocol. In this case, 4×10^4 PANC-1 and 6.5×10^4 AR42J should be seeded. Additionally, the used blocking solution can be replaced with others, like 1% BSA in PBS, and this would lead to similar results. In the same way, the blocking time could be increased up to 24 h without significantly altering the results. The antibody incubation times and concentration might be adjusted and can change, for example, if another LC3 antibody is used. Although PVA-DABCO solution is prepared in this study, commercial montage solutions can also be used. On the other hand, the quantification method can be modified. For example, applying some filters or masks to the images is possible, and an alternative tool can be used for dot quantification.

In this work, we directed the application of the immunofluorescence of LC3 to study the behavior of pancreatic cancer cells. In these cells, autophagy is basally activated and might be modulated as a response to diverse stressful situations, such as chemotherapy,

hypoxia, or nutrient deficiency[11,12]. The determination of autophagy in these cells may be applicable to studying the cellular response to chemotherapy, radiotherapy, or other treatments that modulate autophagy. As shown in **Figure 3**, the presented method can be applied in physiological models. Although, in this work, we focused on the quantification of autophagic levels, the immunofluorescence of LC3 might also allow the evaluation of the colocalization between LC3 and diverse proteins. It can serve, for example, as a mechanistic approach to mark proteins at different points in the autophagic process and evaluate whether the colocalization with LC3 is affected by some treatments or by the downregulation of some proteins. In this way, it could be determined, for example, whether some autophagy inhibitor interrupts the autophagic flux before or after LC3 conjugation. Finally, the method can also be adapted to tissue samples to determine autophagy activation in animal models or human biopsy samples.

Disclosures

No conflicts of interest were declared.

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