Chapter 15

Mitophagy and Mitochondrial Balance

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Abstract

Mitochondria are highly dynamic organelles, with a morphology ranging from small roundish elements to large interconnected networks. This fine architecture has a significant impact on mitochondrial homeostasis, and mitochondrial morphology is highly connected to specific cellular process. Autophagy is a catabolic process in which cell constituents, including proteins and organelles, are delivered to the lysosomal compartment for degradation. Autophagy has multiple physiological functions and recent advances have demonstrated that this process is linked to different human diseases, such as cancer and neurodegenerative disorders.

In particular, it has been found that autophagy is a key determinant for the life span of mitochondria through a particularly fine-tuned mechanism called mitophagy, a selective form of autophagy, which ensures the preservation of healthy mitochondria through the removal of damaged or superfluous mitochondria. Mitophagy has been found to be altered in several pathologies and aberrant or excessive levels of this process are found in common human disorders. Thus, the measurement of the mitophagy levels is of fundamental relevance to elucidate the molecular mechanism of this process and, most importantly, its role in cellular homeostasis and disease.

In this review, we will provide an overview of the current methods used to measure mitophagic levels, with particular emphasis on the techniques based on fluorescent probes.

Key words Mitochondria, Autophagy, Mitophagy, Fluorescent probes, GFP-LC3

1 Introduction

In the presence of oxygen, cells are able to metabolize glucose by oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid (TCA) cycle. At the end of this cycle, the NADH (nicotinamide adenine dinucleotide, reduced) is used by oxidative phosphorylation to boost ATP production. Due to this, it was possible to coin the popular term that identifies mitochondria as the "powerhouse of the cell."

A growing body of recent evidence suggests that mitochondria are part of a more complex cellular signalling network and play a central role in several physiological processes (such as cell proliferation, autophagy, and apoptosis) [1–3], in cellular processes like metabolism [4], during the stress response [5], and in the regulation

Carlos M. Palmeira and Anabela Pinto Rolo (eds.), *Mitochondrial Regulation*, Methods in Molecular Biology, vol. 1241, DOI 10.1007/978-1-4939-1875-1_15, © Springer Science+Business Media New York 2015

of the homeostasis of second messengers, such as calcium (Ca^{2+}) and reactive oxygen species (ROS) [6, 7].

In short, it is clear that the mitochondrial compartment drives essential processes for a correct cell physiology and cell fate. As demonstration of this, alteration of the normal homeostasis of mitochondria is always correlated to common human pathologies [8, 9].

However, even though mitochondria are critical and indispensable elements, the unchecked existence of mitochondria within the cell could become very dangerous. Aged or damaged mitochondria could induce an excessive ROS production, which leads to several mitochondrial dysfunctions, prompting the release of apoptosis-promoting factors and the consequent damage to neighboring mitochondria. As a result, the well-being of the cell could be deeply undermined.

Based on this knowledge, it is so easy to conceive that cells have developed finely tuned mechanisms to supervise mitochondrial quality and quantity. Mitophagy, a catabolic process for lysosome-dependent degradation, has been recently described as a mechanism for the elimination of damaged and unwanted mitochondria [10].

In the last decade, three distinct mechanisms of mitophagy have been identified: (1) during the transformation from reticulocyte to a mature erythrocyte, all the internal organelles, including mitochondria, are eliminated. This particular form of mitophagy involves a Bcl-2-related mitochondria outer membrane protein Nix (also known as BNIP3L) and the microtubule-associated protein light chain 3 (LC3; also called MAP1LC3 or LC3B, the ortholog of yeast ATG8), the principal autophagosome-associated protein [10]; (2) the second mitophagic mechanism was observed in yeast, where the autophagy-related gene 32 (ATG32) protein, localized on the mitochondrial outer membrane, ensures selective sequestration of mitochondria by the recruitment of the canonical effectors of the autophagic machinery [11]; (3) the third (and also the most studied) pathway for the elimination of damaged mitochondria by mitophagy in mammals is a fine-tuned mechanism mediated by two Parkinson Disease (PD)-associated genes: PINK1 (PTEN-induced putative protein kinase 1) and PARK2/PARKIN [12]. When a subset of mitochondria suffer a collapse of Ψ_m (mitochondrial membrane potential), induced by stressors or uncoupler agents, PINK1 and Parkin cooperate together for the removal of damaged mitochondria [13]. Normally, when the Ψ_m is intact, low levels of the serine/threonine PINK1 are found in mitochondria, because it is constantly imported (probably via the TIM/TOM complex) and cleaved by mitochondrial proteases by the inner membrane presenilin-associated rhomboid-like protease (PARL) and the mitochondrial-processing protease (MPP) [14, 15]. Upon loss of Ψ_m, these mechanisms are affected and PINK1 rapidly accumulates on the outer mitochondrial surface and acts as a marker for

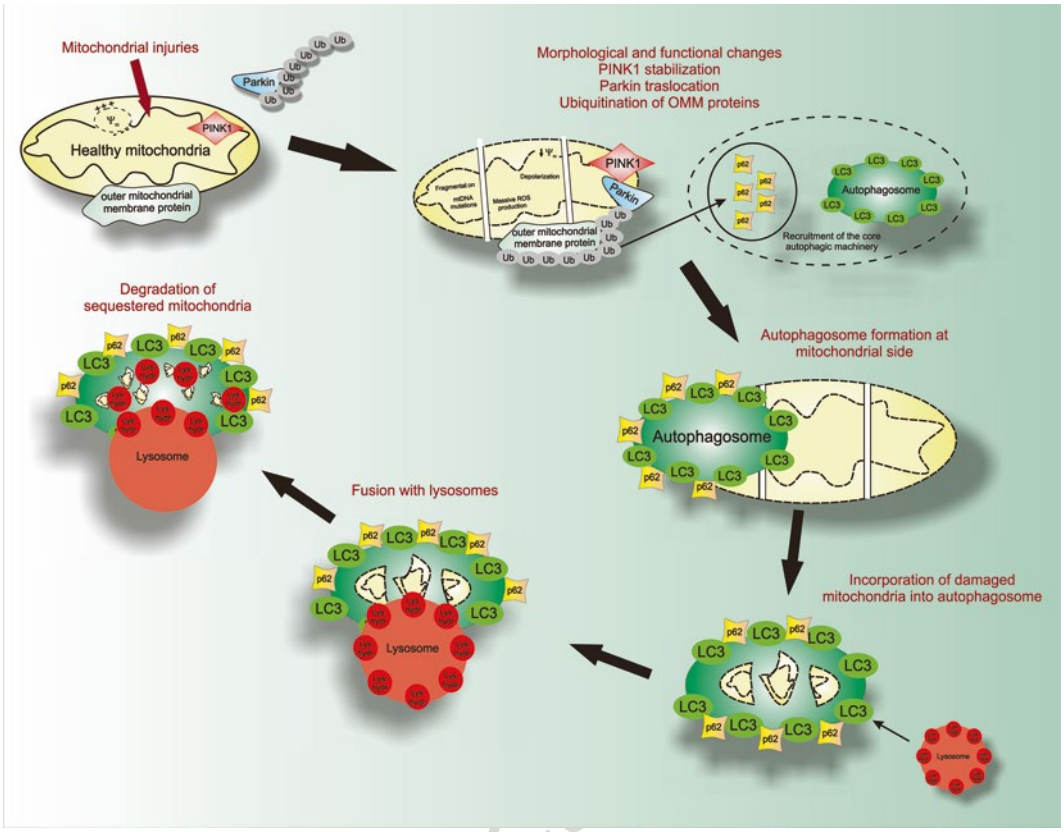

Fig. 1 Mechanism of mitophagy. Following mitochondrial injuries the kinase PINK1 accumulates on the OMM, where it recruits the E3 ubiquitin ligase Parkin to mitochondria. Parkin then promotes the ubiquitination of OMM proteins inducing the recruitment of p62 to clustered mitochondria. Finally, p62 accumulates on mitochondria, binds to parkin-ubiquitylated mitochondrial substrates, mediates clumping of mitochondria and links ubiquitinated substrates to LC3 to facilitate the autophagic degradation of ubiquitinated proteins. *Ub* ubiquitin, Ψ_m mitochondrial membrane potential, *OMM* outer mitochondrial membrane, *LC3* microtubule-associated protein light chain 3, *Lys.hydr* lysosomal hydrolase, *PINK1* PTEN-induced putative protein kinase 1

mitochondrial damage. As a consequence, PINK1 leads to the recruitment of Parkin from the cytosol to mitochondria, where it mediates the ubiquitination of numerous outer mitochondrial membrane proteins [16]. In this way, the docking site for the Ub (ubiquitin)-binding adaptor SQSTM1/p62 is created, which then accumulates in mitochondria and facilitates the recruitment of damaged mitochondria to autophagosome by binding the LC3-interacting region (LIR) motif of LC3 [17] (Fig. 1).

1.1 Methods to Monitor Mitophagy

As outlined above, the removal of damaged mitochondria is a critical aspect for the well-being of cells. Alterations of mitophagy pathways are increasingly recognized in a number of human diseases, including cancer and neurodegeneration [18]. To better understand the role and mechanism of mitophagy in these settings,

in the last decade several methods are been developed to monitor and visualize this catabolic process (*see* **Notes 1** and **2**). Like conventional autophagy, also for mitochondrial autophagy it is possible to assess the incorporation of "wasted" mitochondria into the autophagosome by electron microscopy or the release of radioactively labelled cellular proteins [19, 20]. These techniques provide confirmation of mitochondrial autophagy or clearance, but present some caveats and limitations when it comes to the quantification of mitophagy.

Another useful method to quantify the loss of the mitochondrial pool due to the mitophagic process is the measurement of expression levels of mitochondrial proteins employing immunoblot assays [21]. Since protein synthesis and protein degradation are critical and typical aspects of the mitochondrial pools of protein, and also considering that intermembrane space proteins are frequently lost after permeability transition, it is recommended to analyze the total cellular levels of proteins linked to different mitochondrial subcompartments. Furthermore, to ensure that the protein loss is limited to the mitochondrial compartment, it is suggested to perform an immunoblot analysis against non mitochondrial protein (e.g., endoplasmic reticulum proteins).

A robust way to investigate mitophagy is through the use of fluorescent probes, which may be used to visualize sequestered mitochondria in the autophagosome and the subsequent delivery to the lysosomal compartment [22].

Below, we describe the most used methods to quantify the mitophagic process by fluorescent microscopy and we present a detailed protocol using fluorescent probes to evaluate the selective removal of damaged mitochondria by mitophagy.

1.2 Monitoring Mitophagy Using Fluorescent Probes

At the moment, the main methods to measure mitophagic activity using fluorescent microscopy are based on the simultaneous visualization of mitochondria and autophagosomes with the autophagosome-specific marker LC3. Like autophagy, mitophagy is a dynamic and multistep process that can be modulated at different steps. Based on these observations, researchers have developed novel fluorescent-based techniques to monitor the activity of the mitophagic machinery. Some of them are designed to monitor the delivery of mitochondria to the lysosome. To visualize the amount of fused organelles, mitochondria may be labelled with a mitochondrial marker without significant membrane potential dependence (MitoTracker® dyes) (*see* **Note 3**) and the lysosome may be stained with fluorescent probes (such as the lysosomotropic LysoTracker® dyes) (*see* **Note 4**) or a lysosomal marker (e.g., LAMP1-GFP) [23, 24]. A similar approach, to evaluate the incorporation of mitochondria into the autolysosome makes use of a mitochondria-targeted version of the pH-dependent Keima protein, named mito-Keima. When mitochondria are sequestered into

the lysosomal compartment, the peak of excitation of this modified protein shifts from 440 nm (high pH) to 586 nm (acidic pH) [25].

One key role of mitophagy is the removal of dysfunctional and aged mitochondria so as to maintain mitochondrial turnover and cellular homeostasis. Thus, monitoring these mitochondrial changes is of great importance. A novel fluorescent tool, named "MitoTimer," is a mitochondria-targeted mutant of the red fluorescent protein Fluorescent Timer, known as DsRed1-E5, which shows a fluorescence shift from green to red as the protein matures. As such, this mitochondrial fluorescent protein may be a very useful method to visualize real-time mitochondrial exchange in living cells [26].

Other methods are based on the peculiarity that MAP1LC3 is not the only marker for autophagy, and several other proteins are also involved in this catabolic process. Importantly, a number of these markers are available to perform fluorescent imaging. As reported above, PINK1 and Parkin associate together to label damaged mitochondria, which will be marked for selective degradation via autophagy. In particular, a key aspect for the execution of mitophagy is the translocation of Parkin from cytosol to mitochondria, an event that can be recognized through the use of fluorescent microscopy based on the simultaneous visualization of mitochondria (using mitochondrial fluorescent probes, such as MitoTracker Green, or a mitochondrial marker, such as mitochondria-targeted GFP, mtGFP) and the Parkin protein (using fluorescent recombinant chimeras, such as mCherry-Parkin) [27]. Another light-based method to recognize the specific initiation of Parkin-mediated mitophagy uses a genetically encoded mitochondria-matrix targeting photosensitizer "KillerRed-dMito" and the fluorescent recombinant Parkin-chimera YFP-Parkin. KillerRed is a photosensitizer which produces ROS when illuminated with 599-nm light; as result, mitochondria in the selectively illuminated area become impaired. This mitochondrial damage is recognized by Parkin which translocates to the mitochondrial surface and induces mitochondrial clearance by the LC3-coated autophagic structures [28]. Thanks to this new method, it will be possible to monitor the Parkin-mediated mitophagy in a temporally controlled fashion and it could be useful to identify the high-tuned (but still unknown) molecular mechanism in Parkin-mediated mitophagy (*see* **Note 5**).

Even though different methods and several markers for selective autophagy are been unveiled, the final step of the mitophagic process always implies the incorporation of damaged mitochondria in LC3-coated vesicles (*see* **Note 6**). To date, the simultaneous visualization of mitochondria and autophagosome remains of fundamental importance for a correct mitophagic analysis [21, 29].

In next section, we describe in detail the direct method to provide confirmation of the incorporation of damaged mitochondria into autophagosome by fluorescent microscopy using a mitochondria-targeted RFP (mtDsRed) in combination with the autophagosomal marker GFP-MAP1LC3B (GFP-LC3).

2 Materials

2.1 Cell Culture and Reagents

1. Cell lines: Rat oligodendrocytes precursor cell (OPCs).
2. Culture medium for OPCs: Dulbecco's modified Eagle's medium (DMEM), 4 mM l-glutamine, 1 mM sodium pyruvate, 0.1 % bovine serum albumin (BSA), 50 mg/ml Apotransferrin, 5 mg/ml insulin, 30 nM sodium selenite, 10 nM d-biotin, 10 nM hydrocortisone, 100 U/ml penicillin, 100 mg/ml streptomycin.
3. 10 μg/ml PDGF (platelet-derived growth factor)-AA: stock prepared in distilled water or media, filtered and stored at −20 °C; use at 10 ng/ml.
4. 10 μg/ml bFGF (basic fibroblast growth factor): stock prepared in distilled water or media, filtered and stored at −20 °C; use at 10 ng/ml.
5. 10 μM FCCP (Carbonyl cyanide 4-trifluoromethoxyphenyl hydrazone), dissolved in ethanol and stored at −20 °C; use at 100 nM for OPCs.
6. Lipofectamine 2000 Reagent (Invitrogen, Life Technologies, Carlsbad, CA).
7. Glass coverslips (24 mm in diameter).
8. Poly-L-Ornithine (10 mg/ml stock); use at 100 μg/ml.
9. Six-well culture plates.

2.2 Image (Fluorescence)-Based Analysis of Mitophagy

1. Inverted Nikon LiveScan Swept Field Confocal Microscope (SFC) Eclipse Ti equipped with NIS-Elements microscope imaging software, an Andor DU885 electron multiplying charge-coupled device (EM-CCD) camera (Andor Technology Ltd, Belfast, Northern Ireland) and a CFI Plan Apo VC60XH objective (numerical aperture, 1.4) (Nikon Instruments, Melville, NY).
2. Autophagosomal marker GFP-MAP1LC3B (GFP-LC3). For further detailed technical descriptions about these plasmids, please refer to ref. [3].
3. Mitochondria-targeted RFP (mtDsRed). For further detailed technical descriptions about these plasmids, please refer to ref. [3].

3 Methods

3.1 Measuring Mitophagy with GFP-LC3 and mtDsRed

In order to obtain an optimal measurement of the degree of colocalization, it is necessary to analyze high-quality images captured at high magnification (60× or 100×). We use an inverted Nikon LiveScan Microscope (SFC) Eclipse Ti equipped with NIS-Elements microscope imaging software, an Andor DU885 electron multiplying charge-coupled device (EM-CCD) camera

and a CFI Plan Apo VC60XH objective (numerical aperture, 1.4). To improve resolution and the quality of images acquired, we use a 1.5× amplifier.

3.2 Sample Preparation and Transfection

Cells are seeded on glass coverslips (24 mm in diameter) and allowed to grow until 50 % confluence. After seeding the cells, wait for at least 24 h. The cells are then transfected using appropriate transfection methods (Ca^{2+}-phosphate, lipoamines, electroporation, or adenoviral vectors) with a mitochondria-targeted RFP (mtDsRed) in combination with the autophagosomal marker GFP-MAP1LC3B (GFP-LC3).

Conventionally cancerous and immortalized cell lines may be easily transfect with Ca^{2+}-phosphate or lipoamines transfection methods. Primary cultures, which are notably "hard-to-transfect cells," request electroporation, lipoamines or adenoviral vectors. After transfection, wait for 48 h and perform live fluorescence imaging (*see* **Note 7**). In alternative, it is possible to fix cells with a 2 % formaldehyde solution (*see* **Note 8**).

As certain cell lines possess too low levels of organelle clearance, it may useful to pretreat cells with the potassium ionophore valinomycin or with uncoupling agents [as like CCCP (Carbonyl cyanide m-chlorophenylhydrazone) or FCCP (Carbonyl cyanide 4-trifluoromethoxyphenyl hydrazone)] to recognize their mitophagic activity [30]. These chemical compounds provoke the dissipation of Ψ_m, accumulation of PINK1 on depolarized mitochondria and, finally, Parkin translocation to the mitochondrial outer membrane [31]. Consequently, depolarization of mitochondria by protonophores is highly associated with colocalization of GFP-LC3 on RFP-labelled mitochondria. Another important aspect to keep in mind is that maturation and lysosomal fusion is a very rapid process and the assessment of mitochondrial compartment into autophagosome may be arduous. Treatments with lysosomotropic agents (such as chloroquine, bafilomycin A1, and ammonium chloride) or with lysosomal protease inhibitors (e.g., E64d or pepstatin A), which block the degradation of LC3 with the consequent accumulation of autophagosomes, may facilitate the identification of the cargo of the autophagic vesicles (*see* **Note 9**) [32].

3.3 Sample Preparation and Transfection of Oligodendrocytes Cells

Oligodendrocyte cells were obtained following the protocol developed by Chen et al. [33] and maintained in culture medium for OPCs supplemented with 10 ng/ml PDGF-AA and 10 ng/ml bFGF for a week. The OPCs isolated can be studied in their undifferentiated state or induced to differentiate into immature oligodendroblasts and then into mature oligodendrocytes, replacing PDGF-AA and bFGF with triiodothyronine .

At ~7 days, OPCs obtained were plated at a density of 2×10^4/cm^2 on glass coverslips (24 mm in diameter) in six-well culture plates. Cover glasses were previously coated with 100 μg/ml per

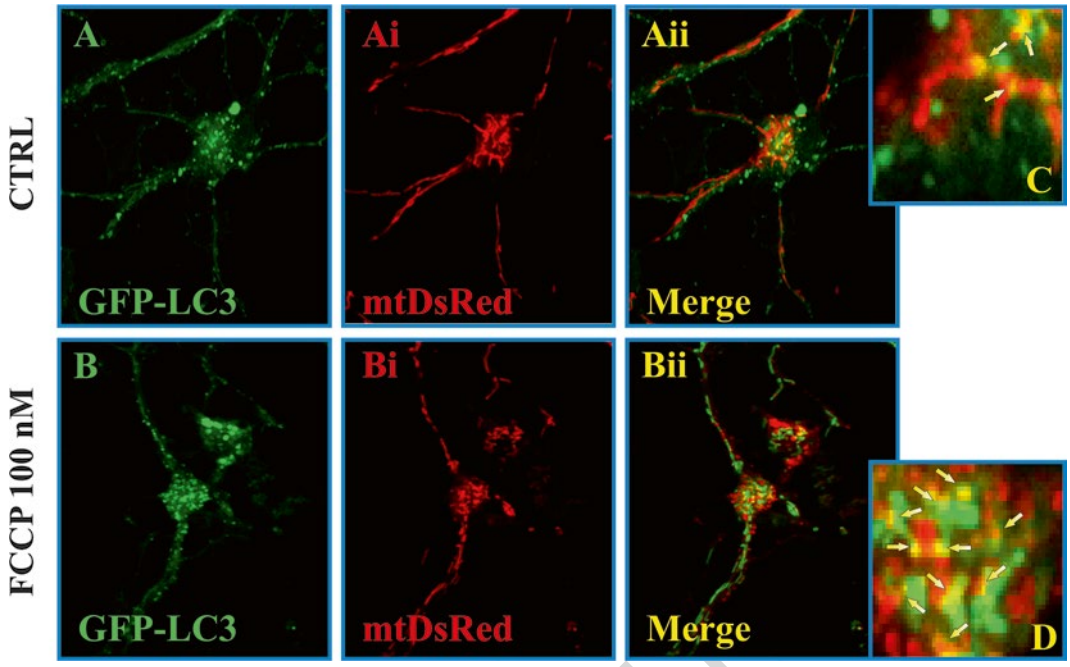

Fig. 2 Analysis of mitophagic sequestration by dual fluorescence. OPCs were transfected with GFP-LC3 and a mitochondrial-targeted red fluorescent protein. Forty-eight hours after transduction, cells were treated with vehicle (*CTRL*) or 100 nM FCCP for 1 h. Confocal images show a significant increase in GFP-LC3 puncta that colocalize with mitochondria in FCCP-treated cells (*arrows*). The images visualize in *blue boxes* (**c** and **d**) are enlargements in **aii** and **bii**, respectively. Cells were imaged on a Nikon LiveScan SFC Microscope Eclipse Ti equipped with VC60XH oil immersion objective with a 1.5× amplifier and appropriate filter sets. Scale bar: 10 μm (**a–aii** and **b–bii**) and 2 μm (**c** and **d**)

[AU1]

well of poly-D-ornithine as follow. Add sufficient quantity of 1× coating solution to glasses and incubate for 1–2 h at 37 °C. Remove solution, wash two times with distilled water and air-dry in a tissue culture hood.

After 24 h from seeding, cells were transfected with 1 μg of plasmid DNA for well (0.5 μg mtDsRed + 0.5 μg GFP-LC3), using the appropriate transfection method (Lipofectamine 2000 reagent). After 4–6 h the transfection medium were replaced with warm medium culture medium for OPCs supplemented with 10 ng/ml PDGF-AA and 10 ng/ml bFGF, and after 48 h the cells were imaged as outlined in Subheading 3.4. Prior to imaging, parallel oligodendrocytes cultures transfected as reported above were treated with the uncoupler agent FCCP 100 nM for 1 h to induce the sequestration of mitochondria into autophagosomes (Fig. 2).

3.4 Measurements

Coverslips (24-mm in diameter) were placed in a stage incubator (Okolab) of the inverted Nikon LiveScan SFC Microscope Eclipse Ti equipped with VC60XH oil immersion objective. Experiments were carried out in basal chemically defined medium supplemented with 10 ng/ml PDGF-AA and 10 ng/ml bFGF. Excitation at

488 nm used a solid state laser (Spectra-Physics, Newport) and fluorescence emission was measured through a 520/30 filter. Laser excitation at 546 nm used a solid state laser (Spectra-Physics, Newport) and fluorescence emission was measured using a 600/20 filter. Laser excitation was attenuated 100- to 1,000-fold to minimize photobleaching and photodamage. In order to obtain statistical significance for measuring the degree of colocalization, we recommend to acquire at least 25–30 images per condition.

3.5 Data Handling/ Processing

Following the experiment, the images acquired can be analyzed directly on the microscope imaging software or by the use of open-source software programs developed to help the interpretation of multidimensional images, such as ImageJ, the Open Microscopy Environment, or VisBio.

Preprocessing of images: Fields of images acquired are not illuminated in a homogeneous fashion; thus, it is recommended to correct for uneven illumination. This image processing may be achieved by correcting the image of the sample using a bright image of an empty field. The correction may be performed using the microscope imaging software or, alternatively, with open-source software programs.

Visualizing colocalization: Conventionally, the most used method to visualize colocalization is the simple merge of the different channels. In our case, GFP-LC3 (green) and mtDsRed (red) give rise to yellow hotspots where the two molecules of interest are present in the same pixels. Thus, to reach a very fast and easy quantification of colocalization, it is possible to perform the simple count of yellow dots and identifying the number of mitochondria sequestered into the autophagosome (Fig. 3a). Alternatively, it is possible to evaluate the intensity profile for the red and green channel of a region of interest, which potentially includes an autophagosome (Fig. 3ci, cii).

The simple overlay of the channels of interest possesses, however, some limits. For example, the presence of merged staining (yellow in our case) is highly dependent on the signal intensity of each single channel: to have a reliable colocalization, both images should have similar grey level dynamics. Many software tools and algorithms have been developed to try to address this issue. In particular, to evaluate colocalization events, algorithms performing the so-called Intensity Correlation Coefficient-Based analyses (ICCB; such as Pearson's or Manders' coefficients) and object-based approaches (such as the centroid or intensity center calculation) have been engineered. Recently, a simple public domain tool has been developed for the open-source software ImageJ, named JACoP, which integrates current global statistic methods to evaluate the degree of the colocalization of two channels (Fig. 3bi, bii). Using a novel object-based approach, this tool is capable of processing single and composite images and enables an automated colocalization analysis [34].

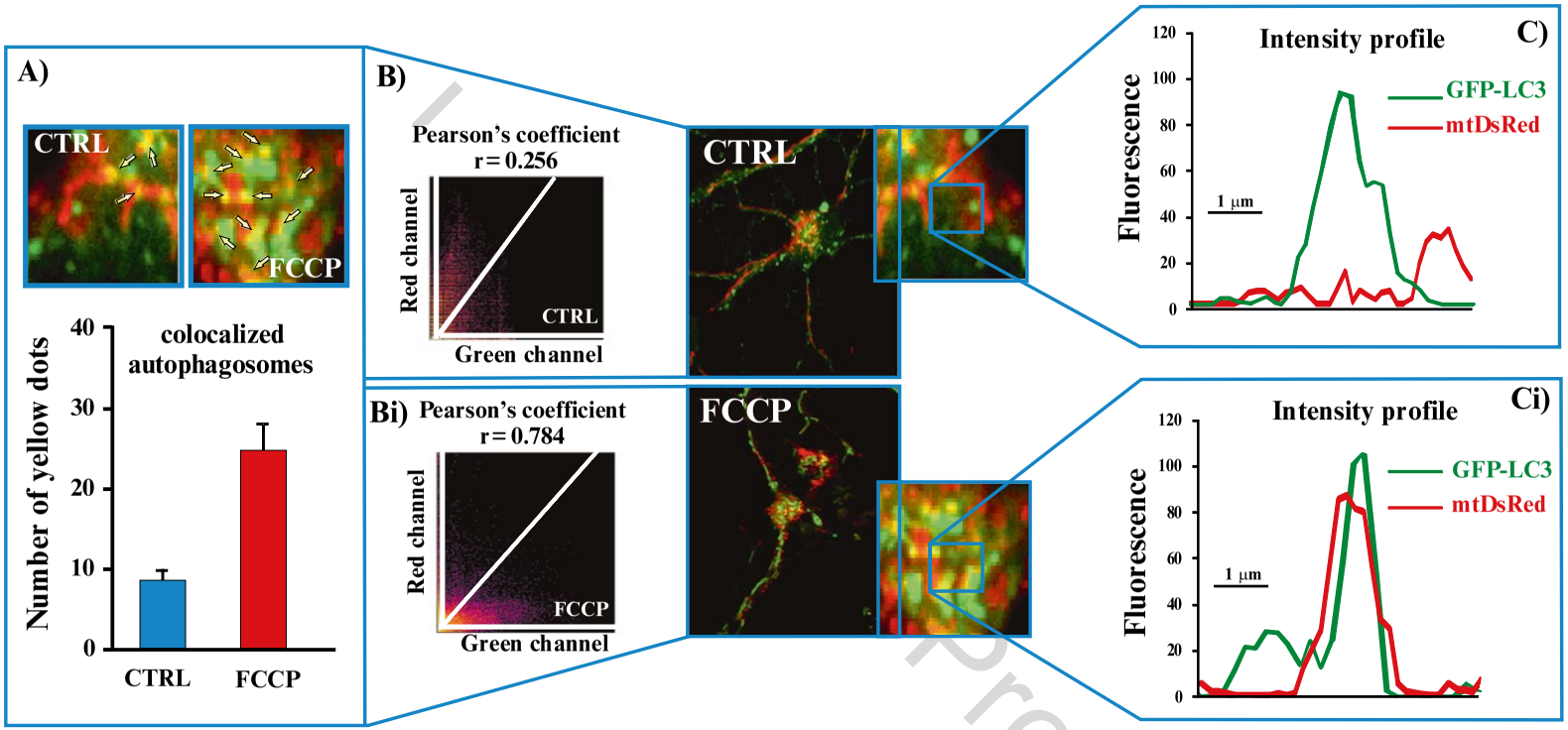

Fig. 3 Methods to quantify colocalization events. (**a**) The most used method to visualize colocalization is the merge of the *red channel* (mitochondria) with the *green one* (autophagosome). As result, it is possible to determine the number of *yellow hotspots* (*arrows*) by manual count or using microscope imaging or open-source software programs. (**b**) Alternatively, it is possible to use Pearson's correlation coefficient as a measure of colocalization of RFP signals with GFP signals. Correlation [AU2] plot (**bi** and **bii**) is reported corresponding to the left of images. (**ci** and **cii**) Intensity profile of a selected region represents a powerful analytical tool to examine the presence of a possible colocalization. Cells were left for 48 h after plasmids transfection, treated with FCCP 100 nM (*FCCP*) or vehicle (*CTRL*) and imaging. Scale bar: 10 μm (*CTRL* and *FCCP*) and 2 μm (*small blue boxes*)

4 Notes

1. Due to the dynamic nature of mitophagy and the multiple potential factors which regulate this process, it is suggested to perform multiple techniques (rather than a single technique) to evaluate the autophagic removal of damaged mitochondria. For example, immunoblot analysis of the mitochondrial proteins levels is useful to validate data from microscopy studies.
2. It is important to keep in mind that mitochondrial turnover could be promoted by other degradation systems (such as proteosomal degradation) and that several mechanisms are responsible for the disappearance of mitochondrial markers. Based on these observations, it is suggested to evaluate the mitochondrial clearance also with inhibitors of the other major degradation systems.
3. The retention and accumulation of the fluorescent probes, especially for MitoTracker dyes, depend on cell type. It is recommended to determine empirically their optimal concentration in a given cell type under the experimental conditions.
4. Because lysosomotropic agents disrupt the lysosomal acidic pH, these compounds cannot be used with LysoTracker fluorescent probes.
5. Defective or excessive mitophagy is frequently found in several human diseases. Consequently, a novel and accessible method capable of recognizing the alteration of mitophagy and, at the same time, the rapid screening of hypothetical compounds is necessary. Coupling the reported methods with high-content microscopy may be a solution. In this way, it will possible to screen large compound libraries to identify small-molecule modulators of mitophagy. As a demonstration of this theoretical approach, the first mitophagic study performed using high-throughput screening has been recently published. In this work, Hasson and colleagues characterized specific regulators of the PINK1-Parkin-mediated mitophagy. To attain their goal, cells stably expressing GFP-Parkin and a mitochondria-targeted red fluorescent protein were used. The cells were transfected with siRNA duplex in 384-well plates, treated with a chemical mitochondrial uncoupler and the translocation of Parkin to the mitochondrial surface was evaluated by high-content microscopy and automated image analysis [35].
6. In addition to colocalization between mitochondrial labels and markers for autophagy, it may be useful to perform studies in order to quantify the morphological and functional changes associated to the mitophagic removal of mitochondria. To assess these aims, it is possible to evaluate changes in mitochondrial structure (it should be noted that mitochondrial

fragmentation precedes mitophagy) through the employment of MitoTracker, whereas the loss of Ψ_m, a common trigger for mitophagy, can be measured by the use of potentiometric probes (such as JC-1 and TMRM) [36].

7. It is important to consider that certain plasmid DNA and transfection methods may modify the levels of autophagy. To start, it is important to use contaminant-free plasmid DNA. Next, to avoid negative effects induced by transfection, it is suggested to leave the cells for at least 48 h after the transfection. The use of stable cell lines could solve this concern, but: (a) stable cell line are generated by immortalization, a process that modifies markedly the normal physiology of the cell, and (b) it is not always possible or easy to obtain a stable cell line of certain cell types (i.e., nerve cells).
8. The fixation procedure may produce autofluorescent puncta or a reduction for GFP-LC3 staining. To avoid artifacts, it is suggested to compare results of live imaging with imaging after fixation.
9. The optimal lysosomotropic agents or lysosomal protease inhibitors concentration and timetable treatment are highly linked to cell-type. Before perform experiments, one should search for the ideal concentration and time course in order to prevent cytotoxic effects and avoid saturation of the observed LC3-II accumulation.

Acknowledgements

This study was supported by: the Italian Association for Cancer Research (AIRC); Telethon (GGP11139B); local funds from the University of Ferrara; the Italian Ministry of Education, University and Research (COFIN, FIRB, and Futuro in Ricerca); and the Italian Ministry of Health to Paolo Pinton. Simone Patergnani was supported by a FISM (Fondazione Italiana Sclerosi Multipla) research fellowship (2012/B/11).

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