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Protocols for assessing mitophagy in neuronal cell lines and primary neurons

Ruben K. Dagda* and Monica Rice

Department of Pharmacology, University of Nevada, Reno School of Medicine, NV, 89557

Abstract

Mitochondria are organelles that regulate essential eukaryotic functions including generating energy, sequestering excess calcium, and modulating cell survival. In order for neurons to thrive, mitochondria have to be continuously replenished by maintaining autophagic-lysosomal mediated

*To whom correspondence is addressed to: Ruben K. Dagda, Ph.D., Assistant Professor, Department of Pharmacology, rdagda@medicine.nevada.edu, Phone: 775-784-4121, Fax: 775-784-1620.

Note 1: It is important to freeze down a high number of vials containing early passages of SH-SY5Y cells that respond well to neurotrophic/differentiation factors (cyclic AMP and retinoic acid). Approximately 1.0×10^6 cells are frozen in 1ml of cell-freezing media containing 10% DMSO (e.g. Cryostor, CS10, Stem Cell Technologies).

Note 2: It is important to isolate embryonic primary neurons and dissociate them preferably through mechanical means (e.g. passing cells through Pasteur glass pipettes with decreasing bore sizes) without the need to enzymatically digest with papain or trypsin in the tissue as these treatments tend to significantly reduce neuronal viability.

Note 3: It is critical to routinely test different stocks and each lot of RA for its differentiation potential in SH-SY5Y cells as RA tends to get inactivated by light. SH-SY5Y cells should respond well to RA by spreading out on plastic dishes, adopting fusiform neuronal cell bodies, and typically send out neurites that are as long as two cell body lengths within 24 hours of exposure to RA.

Note 4: Materials transfer agreement needs to be processed prior to purchasing reagents from AddGene (www.addgene.com).

Note 5: One Lipofectamine 2000 reagent should be purchased for transfecting DNA plasmids in neuronal cells/primary neurons whereas a second vial of Lipofectamine 2000 should be purchased for transfecting cells with siRNA to avoid cross-contaminating siRNA reagents with RNAases.

Note 6: The anti-TOM20 and anti-cytochrome C oxidase antibodies can label mitochondria in both SH-SY5Y cells and primary cortical neurons.

Note 7: Unlike cells grown on plastic, we have noticed that growing SH-SY5Y cells on coverslips pre-coated with poly-L-lysine can facilitate the removal of the Epon-embedded cell monolayer by exposing the Epon capsules through sequential freeze/thaw cycles (Agarwal A, Rice M., and Dagda RK, 2014, unpublished observations).

Note 8: It is important to test the epoxy resin, at least three days prior to processing the experimental samples, as the quality of the epoxy resin decreases with time. Therefore, it is advisable to freeze a large working stock (~50mL) of epoxy resin at -20°C . Also, to remove the epoxy resin from plastic for cells seeded on 6-well plastic dishes, it is important to snap off the epoxy capsule from the bottom using a small pliers to prevent shattering the capsule if not handled correctly.

Note 9: To analyze mitophagy in neurons, it is imperative to obtain high resolution and high quality images by confocal microscopy as it is conceivable that a cluster of several overlapping bright fluorescent GFP-LC3 or RFP-LC3 puncta can be misinterpreted as a single LC3 granule in low quality epifluorescence micrographs, a very common but avoidable pitfall.

Note 10: It is important to keep in mind that a decrease in the protein levels of one mitochondrial protein is not sufficient evidence for mitophagy. Hence, it is highly advisable to immunoblot for several mitochondrial markers at different time points in order to determine whether a decrease in mitochondrial levels is caused by mitophagy as opposed to impaired mitochondrial biogenesis or ubiquitin-mediated degradation of OMM-localized mitochondrial proteins. A final word of caution is to avoid immunoblotting for apoptogenic factors (e.g. cytochrome C) when assessing mitophagy as cytochrome C is released from mitochondria in response to apoptotic stimuli.

Note 11: Depending on the level of transient expression of GFP-LC3, an important caveat to consider when overexpressing GFP-LC3 or RFP-LC3 is that these LC3 chimeras can associate with large ubiquitinated protein aggregates or aggresomes, a phenomenon that does not reflect autophagosome formation or flux and likely represents an artifact of overexpression [53]. Hence, it is conceivable that a small percentage of GFP-LC3 puncta is not associated with AVs in any experimental context. To overcome this issue, cells can be transfected with the lipid binding deficient version of GFP-LC3 (G120A) to determine the percentage of GFP-LC3 puncta that is not associated with AVs.

degradation of mitochondria (mitophagy) and mitochondrial biogenesis. While a plethora of image- and biochemical-based techniques have been developed for measuring autophagy (macroautophagy) in eukaryotic cells, the molecular toolbox for quantifying and assessing mitophagy in neurons continues to evolve. Compared to proliferating cells, quantifying mitophagy in neurons poses a technical challenge given that mitochondria are predominantly present in neurites (axons and dendrites) and are highly dynamic.

In this chapter, we provide a brief overview on mitophagy and provide a list of validated fluorescence- and biochemistry-based techniques used for assessing mitophagy in neuronal cells and primary neurons. Secondly, we provide comprehensive guidelines for interpreting steady-state levels of mitophagy and mitophagic flux in neurons using modern fluorescence- and biochemistry-based techniques. Finally, we provide a comprehensive list of common pitfalls to avoid when assessing mitophagy and offer practical solutions to overcome technical issues.

Keywords

Autophagy; mitophagy; GFP-LC3; RFP-LC3; lysosome; electron microscopy; mitochondrial trafficking; oxidative stress; lysosome; neurites

1. Introduction

Mitochondria are the main energy hubs of eukaryotic cells. To produce energy in the form of adenosine triphosphate (ATP), mitochondria employ oxygen, water, tricarboxylic cycle intermediates and the electron transport chain. Other physiological functions of mitochondria include regulating cell death pathways that converge at the outer mitochondrial membrane (OMM), lipid synthesis (e.g. cardiolipin, steroids and phosphatidylethanolamine), sequestering calcium, heat production, and generating and sequestering reactive oxygen species (ROS).

The mitochondrion is a highly specialized organelle divided into four subcompartments: the OMM, the intermembrane space (IMS), inner mitochondrial membrane (IMM), and the matrix. The OMM has emerged as a crucial platform by which a myriad of protein signaling pathways converge to regulate mitochondrial structure, metabolism, and oxidative phosphorylation [1–5].

There are several mitochondrial quality control and repair pathways that serve to maintain mitochondrial structure and function. At the OMM, oxidatively-damaged proteins are targeted for localized degradation by the ubiquitin-proteasome system. In addition, the OMM serves as the “dumping ground” for IMS/IMM-localized proteins that were shuttled to the OMM so they can be tagged for degradation by E3 ubiquitin ligases [6]. The mitochondrial matrix has intrinsic quality control mechanisms for sensing and degrading damaged or unfolded proteins. Several proteases, including LON1, sense and proteolytically degrade damaged matrix-localized proteins. Therefore, the turnover of damaged matrix-localized proteins permits the efficient *de novo* import of new mitochondrial proteins [7].

On the other hand, when the whole organelle is extensively damaged, for instance through exposure to ROS or through normal aging, mitochondria are sequestered by autophagic vacuoles (AVs) and targeted for lysosomal degradation through a catabolic process termed mitophagy.

Autophagy, or also known as macroautophagy, is a catabolic process by which oxidatively-damaged and aged organelles are directed for lysosome-mediated degradation by acid hydrolases [reviewed in ([8,9]). Autophagy is a highly coordinated physiological process requiring several steps orchestrated by autophagy-related proteins that were initially identified in yeast as ATG proteins. Autophagy is initiated by the formation of isolation membranes (phagophores) that are derived from the endoplasmic reticulum (ER), mitochondria and other organelles [10,11] and are subsequently molded into double membrane organelles termed AVs. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a *bona fide* marker of AVs and the mammalian homolog of ATG8. ATG8 is posttranslationally processed by the protease ATG4 and it is subsequently C-terminally lipidated by ATG7 through the addition of phosphatidylethanolamine in a C-terminal glycine (G120 in the rat cDNA). This posttranslational modification permits ATG8/LC3 to physically associate with AVs. In cooperation with ATG8, the association of ATG5-ATG12 complexes – as formed through the sequential conjugation of ATG12 to ATG5 – regulate the expansion, remodeling and the successful closure of nascent AVs [12]. While ATG5-ATG12 complexes dissociate from the outer membrane of AV during the maturation phase of AVs, LC3 remains bound. The mature AVs eventually fuse with lysosomes to deliver the cargo for degradation by acidic hydrolases, a physiological process which allow for the eventual recycling of necessary nutrients in eukaryotic cells [8].

In mammalian tissues, basal autophagy is critical for development, differentiation of tissues (e.g. neurons, lymphocytes and adipocytes), and for maintaining immunity [13,14]. On the other hand, stimulated autophagy, such as induced by starvation, is critical for providing the necessary energy to allow a stressed eukaryotic cell to thrive. Autophagy was once believed to be dispensable in the central nervous system (CNS) given that neurons have privileged access to nutrients *in vivo*. However, this concept was challenged seven years ago when two landmark studies demonstrated that specific gene deletions of ATG5 and ATG7 produce widespread degeneration in the brain. More importantly, these seminal studies suggested that autophagy predominantly plays a neuroprotective developmental role in the CNS [15,16]. In other instances, autophagy is a double-edged sword that can be either beneficial or detrimental. During physiological conditions, autophagy contributes to the removal of large protein aggregates when the ubiquitin-proteasome system is overwhelmed. During neuronal development, autophagy participates in the pruning and remodeling of neurites [13,17,18]. On the other hand, high chronic levels of oxidative stress as induced by many neurodegenerative diseases – including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Huntington's disease (HD), prion-related diseases (PRD), and Parkinson's disease (PD) – lead to early dysregulation of autophagy [19,20,13,21–23]. While physiological mitophagy, as induced by starvation or by treating cells with the mTOR inhibitor rapamycin, permits the efficient recycling of nutrients for the formation of new mitochondria, pathological stimuli that tend to overactivate mitophagy in the absence of

mitochondrial biogenesis – a concept coined “autophagic stress” – promotes neurodegeneration [24].

Once thought to be an unregulated physiological process, the signaling pathways and molecular players that govern mitophagy are beginning to be unveiled. In yeast, 36 autophagy-related genes (ATG) and 41 other non-ATG proteins initially identified to regulate autophagy. So far, only a few yeast proteins (Atg32 and AUP1P) are known to regulate mitophagy [25–27]. Three types of mitophagy have been well characterized in eukaryotic cells. In yeast, the OMM-localized Atg32p interacts with the Atg11p/Atg8p complex, leading to the recognition and engulfment of dysfunctional mitochondria by AVs. In mammalian cells, PINK1 and Parkin, two enzymes whose mutations are associated with familial forms of PD, link the ubiquitin-proteasome and autophagy machineries to promote mitophagy of severely damaged mitochondria [28]. In red blood cells, the OMM-localized protein NIX associates with LC3 to initiate mitophagy and allows for the maturation of red blood cells by eliminating mitochondria. In summary, mitophagy is now regarded as a highly selective physiological process that requires specific signals to trigger the highly coordinated temporal and spatial recruitment of a set of intermediate players and mitophagy effectors to the OMM [29–34].

In neurons, some mitochondrial toxins can concomitantly activate autophagy and mitophagy. For instance, treating neuronal cells or primary midbrain dopaminergic neurons with the Parkinsonian toxins 1-methyl-4-phenylpyridinium (MPP+), 6-hydroxydopamine (6-OHDA), or rotenone not only activates macroautophagy, but also promotes mitophagy in a concentration and time-dependent manner [30,31,35]. Other toxins such as staurosporine can also enhance the flux of mitochondria through the lysosomes (mitophagic flux) [30]. Recently, one landmark study showed that the externalization of cardiolipin (CL) from the IMM to the OMM leads to the recruitment of ATG8/LC3 to mitochondria by associating with CL. The externalization of CL in neurons appears to be a generalized mechanism for initiating mitophagy as induced by multiple mitochondria-directed toxins and apoptotic stimuli, a physiological process that does not require the recruitment of PINK1 and Parkin [30].

While neuronal mitophagy has been predominantly characterized *in vitro* (e.g. primary neurons), it is worth noting that a few protocols have been recently developed for analyzing mitophagy *in vivo* and *ex vivo* (e.g. brain slices) [36]. However, mitophagic flux *ex vivo* is challenging to assess given the poor permeation of reagents to manipulate autophagic flux (e.g. bafilomycin, chloroquine and leupeptin) into tissue slices. Moreover, studying mitophagy *in vivo* requires sophisticated equipment to fluorescently monitor mitophagy. Moreover, there is a lack of reagents to induce flux *in vivo*. Given these aforementioned issues, this chapter provides a comprehensive list of fluorescent- and biochemical-based protocols for interpreting mitophagy in cultured neuronal cells and in primary neurons. Finally, we provide a thorough set of guidelines for interpreting mitophagy in neurons and troubleshooting various technical issues.

Key concepts: There are several key words and concepts to bear in mind in order to fully comprehend the mitophagy techniques presented in this chapter. **Steady-state autophagy**

refers to rate of formation and turnover of AVs under physiological conditions whereas **induced autophagy** refers to the increased steady-state autophagy beyond baseline levels that occurs in response to an exogenous stimulus, chronic or acute stress. **Flux** is the fraction of the total pool of AVs that fuse with lysosomes to promote autophagic-lysosomal mediated degradation of cargo. **Mitophagic flux** is defined as the fraction of the total pool of AVs containing mitochondria that fuse with lysosomes to promote their degradation.

2. Materials

2.1 Key fluorescently tagged reagents

Due to its intrinsic ability to bind to early AVs, ATG8/LC3 is considered a *bona-fide* marker for autophagy. Unlike Atg5-Atg12 complexes, the fact that LC3 remains associated to AVs until it is degraded by acid hydrolases in an autolysosome (autophagosomes fused to lysosomes) makes it an ideal AV marker [8]. Moreover, ever since the GFP fusion chimera of LC3 (GFP-LC3) was engineered by Dr. Tomatsu Yoshimori [37], this popular molecular biology reagent has been used by neurobiologists to monitor early AVs in live and fixed cells and for assessing autophagic flux as a tandem reporter version of LC3 [36]. Whereas the GFP moiety is labile in low pH, the RFP fusion chimeric version of LC3 (RFP-LC3) specifically labels autolysosomes due to the higher fluorescence stability of RFP inside the acidic environment of lysosomes [38]. Indeed, the tandem reporter construct which expresses a version of LC3 containing both fluorophores facilitates the analyses of autophagic flux in eukaryotic cells [38]. Overall, the ability to use the three fluorescent versions of LC3 gives the neurobiologist powerful reagents to quantify the initiation (GFP-LC3), progression (RFP-LC3), and flux (tandem reporter construct) using relatively simple, but powerful fluorescent image-based techniques.

2.2 Cell Culture

1. SH-SY5Y, a human dopaminergic neuroblastoma cell line, can be purchased from American Type Culture Collection (Rockville, MD), and primary cortical neurons are derived from embryos of 14–16 day -timed pregnant C57/BL6 mice (Charles River, Wilmington, MA) as previously described [39]. These cells (low passage number) are grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l D-glucose (Life Technologies, Cat#10313021) and supplemented with the following additional components: 10% heat inactivated fetal bovine serum (SIGMA, St. Louis, MO, Cat#F2442), 10 mM HEPES, and 2 mM glutamine. Primary mouse cortical neurons are seeded in Neurobasal® medium (Life Technologies, Cat# 21103-049) containing 2% B27™ supplement (50X stock, Life Technologies, Cat#175044, stored at – 20°C until ready to use), 0.5% FBS and 2.0 mM L glutamine/L-alanyl-L-glutamine (Glutamax, ThermoFisher Scientific, Cat #35050061), preferably without serum or antibiotics. Three days after plating, 2/3 of the seeding media is replenished with maintenance media (same NB/B27 media as described above but without FBS to reduce growth of glia).

2. To study the effects of a stimulus on mitophagy in differentiated neuronal cells, SH-SY5Y cells can be differentiated with retinoic acid (RA) (10 μ M; Sigma, Cat#R2625) or with dibutyryl cyclic AMP (db-cAMP) (250 μ M, Sigma, Cat# D0627) for three to five days. Stock solutions of 10 mM retinoic acid are prepared in DMSO (Sigma, St. Louis, MO, USA, Cat#D8418), and stored at -20°C as 20 – 50 μ l aliquots for up to 4 months or at 4°C for 1 month and away from light. Db-cAMP (Sigma, St. Louis, MO, USA) is prepared as a 10 mM stock concentration in water and stored as 100 μ l aliquots in -20°C .
3. Tissue culture ready poly-L-lysine (0.01%, sterile-filtered) (Sigma, Cat# P-4707). Alternatively, poly-D-lysine hydrobromide (Sigma, Cat# P-6407) stocks can be prepared at 2 mg/ml (filter-sterilized) to coat chambered coverglasses at a final concentration of 150 μ g/ml for 1 hour prior to washing with water three times. Although poly-D-lysine is less expensive to purchase, it tends to degrade at a faster rate than poly-L-lysine which is stable at room temperature (RT).
4. 6-hydroxydopamine (1mM stock, 6-OHDA; Sigma, Cat# H4381), freshly prepared in distilled water or media. Store powder stock in -20°C in a desiccator jar and keep away from light. Use 6-OHDA at 75 μ M (LD50) for 4 hours in SH-SY5Y cells as a control to induce autophagy/mitophagy.
5. Rotenone (1mM stock, Sigma, Cat#R8875), freshly prepared in water prior to each use in cells. Rotenone can be used at 1 μ M for SH-SY5Y cells and at 100–250 nM for primary cortical neurons for 4 – 6 hours to induce autophagy/mitophagy.
6. Staurosporine (1 mM ready-made solution stock, Sigma, Cat# S5921), can be used at 1 μ M for 4 hours for SH-SY5Y cells or at 100 nM for 4 hours for primary cortical neurons to induce mitophagy.
7. E64-D (10 mM, Calbiochem, San Diego, CA, Cat# CAS 66701-25-5), stocks made in DMSO and stored at -20°C . Use at 10 μ M for 2 hours for SH-SY5Y cells and primary cortical neurons for flux studies.
8. Pepstatin-A (25 mM, Calbiochem, Cat# 516481), dissolved in methanol or DMSO. Use at 25 μ M for SH-SY5Y cells for 2 – 4 hours for flux studies.
9. Bafilomycin A1 (10 μ M, Sigma, Cat# B1793), dissolved in DMSO as a 32.1 mM stock solution. Use at 10 nM for 4 hours for both SH-SY5Y cells and primary cortical neurons for flux studies.
10. Chambered Lab-Tek II cover glasses (#1.5 German borosilicate; Nalge Nunc International, Naperville, IL, USA, Cat# 155382). Alternatively, 35mm pie-sectioned cell cultured dishes with glass bottoms (Cellview™, Greiner, Germany, Cat #627870), can also be used for analyzing autophagy/mitophagy by confocal microscopy. We have found that using 4 well chambered LabTekII coverglasses are better suited for running time-course experiments and for performing immunocytochemistry whereas Cellview™ culture dishes are optimal for confocal microscopy.

2.3. Electron Microscopy

1. 0.1 M Phosphate buffered saline (PBS), pH 7.4.
2. 2.5% glutaraldehyde (Sigma, Cat #G5882) in 0.1 M PBS, pH 7.4, frozen as 30 ml aliquots and maintained in -20°C or at 4°C for two months.
3. 1% osmium oxide (Sigma, Cat # 419494) in 0.1 M PBS.
4. 2% uranyl acetate solution (Polysciences, Warrington, PA, Cat# 21447-25), prepared in distilled water. Stored at 4°C .
5. 1% lead citrate solution (Sigma, Cat# 15326), prepared in distilled water and can be stored for 3 – 6 months at 4°C .
6. Polybed 812 epoxy resin (Polysciences, Warrington, PA, Cat# 08792-1).
7. Phillips CM10 transmission *electron microscope or equivalent electron microscope*.
8. 15 mm coverglasses (German glass, #1 thickness, Electron Microscopy Sciences, Hatfield, PA, Cat# 72228-01).

2.4. Fluorescence Imaging-based Analysis of Mitophagy

1. MitoTracker Red dye 580 (1 mM, MTR, 50 μg lyophilized pellet; Molecular Probes, Life Technologies, Cat #M22425). Pellet is dissolved in DMSO and stored at -20°C .
2. Tetramethylrhodamine methyl-ester (10 mM, TMRM, powder, Sigma, Cat#T5428) is dissolved in DMSO as 10 mM stocks and frozen down as 20 μl aliquots at -20°C . Use at 40 nM to stain mitochondria in SH-SY5Y cells and primary neurons.
3. MitoTracker Green FM dye (1 mM, Molecular Probes, Life Technologies, Cat #M7514)) the pellet is dissolved as a 1 mM stock solution, and stored in small aliquots at -20°C . Use at 250 nM to stain mitochondria in SH-SY5Y cells and primary cortical neurons.
4. LysoTracker Red DND-99 (1 mM, Molecular Probes, Life Technologies, Cat#L7528) is pre-prepared as a 1 mM cell culture ready stock solution, and stored in small aliquots at -20°C . Use at 100 nM to stain lysosomes for both SH-SY5Y cells and primary neurons.
5. FluoView 1000 (Olympus America) or a Zeiss LSM 510 Meta laser-scanning confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) is used for imaging
6. Mitochondria-targeted GFP (Mountain View, CA) or mtDsRed2 (CloneTech, MA, USA, Cat# 632421) is used to visualize mitochondria. GFP-LC3 (Addgene #11546, author: Dr. Tomatsu Yoshimori, Research Institute of Microbial Diseases, Osaka, Japan) and RFP-LC3 (AddGene #21075, author: Dr. Tomatsu

Yoshimori) plasmids for assessing initiation and maturation of mitophagy respectively.

7. Lipofectamine® 2000 (Life Technologies, Carlsbad, CA, Cat# 11688). Antibodies specific for mitochondrial markers: Anti- human mitochondrial antigen of 60kDa antibody (Biogenex, San Ramon, CA, 1:1,000), anti-human translocase of the outer mitochondrial membrane 20kDa antibody (TOM20, Santa Cruz Biotechnologies, 1:1,000, FL-145), anti-human cytochrome c oxidase antibody (Sigma, 1:100), and anti-human pyruvate dehydrogenase antibody (PDH, Molecular Probes, 1:1,000).
8. Small interfering RNA (siRNA) reagents: To determine whether decreased mitochondrial levels in response to a stimulus is caused by mitophagy, Atg7 and Atg8 can be knocked down in SH-SY5Y cells using the following validated siRNAs: human Atg7 (human, 5'-GCCAGUGGGUUUGGAUCAA- 3' or Atg8, (LC3B, human, 5' -GAAGGCGCUUACAGCUCAA-3') and non-targeting/ scrambled siRNA can be purchased from LifeTechnologies (40 nM stocks, Carlsbad, CA), transfect cells with 20 pmoles of siRNA per well for cells grown in 4 well chambered slides for 72 hours.

2.5. Western Blot Analysis of Mitophagy

1. Lysis buffer: 25 mM HEPES, pH 7.5; 150 mM NaCl; 1% Triton X-100; 10% glycerol containing freshly added proteinase and phosphatase inhibitors, including 100 μ M E64, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate and 2 mM PMSF.
2. 5 – 15% polyacrylamide gradient gels are used for the optimal separation of LC3-I and LC3-II.
3. Immobilon-PDVF membranes (Millipore, Bedford, MA, USA, Cat# IPVH00010).
4. Blocking solution: 5% nonfat dry milk in 20 mM potassium phosphate, 150 mM potassium chloride, pH 7.4, containing 0.3% (w/v) Tween-20 (PBST). Alternatively, 2% BSA (Fraction V) can be used to block PDVF membranes when immunodetecting for phosphorylated proteins in the same blot.
5. Antibodies: mouse-anti-LC3 (1:200, LC3-5F10: Nanotools, Cat# 0231-100), rabbit-anti-human LC3 antibody (1:1,000, Pierce Biotechnologies), rabbit anti- outer mitochondrial membrane protein TOM20 Antibody (1:10,000, Santa Cruz Biotechnologies, Santa Cruz, CA), ATP Synthase subunit β antibody (1:1000, Complex V, MitoSciences, distributed by Fisher Scientific), anti- human mitochondrial antigen of 60kDa antibody (1:1000, Biogenex, San Ramon, CA).
6. Small interfering RNA (siRNA) reagents: To measure mitophagic flux in SH-SY5Y cells using biochemical methods, siRNA targeting human ATG7 (5' -GCCAGUGGGUUUGGAUCAA- 3' or ATG8, (LC3B, human, 5' -GAAGGCGCUUACAGCUCAA-3') and non-targeting/scrambled siRNA can be

purchased from Life Technologies (prepared as 40 nM stocks). Transfect cells with 120 pmoles of siRNA per well of a 6-well plate for 72 hours.

3. Methods

3.1 Electron microscopy

To date, electron microscopy (EM) remains the gold standard for assessing mitophagy in eukaryotic cells. Although EM is not considered a robust technique, this technique can be used to qualitatively assess mitophagy. At the ultrastructural level, mitophagy is characterized by the presence of early AVs (AVi), which contain mitochondria with other electron dense material. Given that neuronal cells and primary neurons have a high level of autophagic flux under basal conditions, it is worth noting that AVi are rarely detected by EM in neurons under basal conditions. Therefore, it is important to assess for mitophagy by exposing cells with pharmacological inhibitors of lysosomal fusion such as bafilomycin, an inhibitor of vacuolar-type H(+)-ATPase which inhibits the fusion of AVs with lysosomes [40], to increase the number of AVs containing mitochondria.

The following protocol has been optimized for assessing mitophagy in neuronal cells, particularly in SH-SY5Y cells:

1. SH-SY5Y cells are maintained in antibiotic-free, high-glucose (4.4g/L) DMEM media containing 10% FBS and L-pyruvate. In their undifferentiated state, it is preferable to use early passages of SH-SY5Y cells (passages 15–25) which retain their characteristic fusiform bodies and neuronal-like processes. On the other hand, late passages of SH-SY5Y cells tend to lose their neuronal-like morphology and other non-neuronal like subpopulations –which contain a fibroblast-like morphology – tend to predominate.
2. For ultrastructural analyses of mitophagy, SH-SY5Y cells are plated at a density of 300,000 cells per well on a 6-well plate on either plastic or on glass coverslips kept in six well plates for three days. It is preferable to seed SH-SY5Y cells at this high cell density in order to include a sufficient number of cells when embedding the cell monolayer with Epon resin.
3. Given that neuronal cells have a high basal flux, it is challenging to visualize AVs with mitochondria contained inside them. Hence, to analyze for mitophagy, it is highly recommended to pulse cells with 10 nM bafilomycin A1 for 4 hours, or 20 nM bafilomycin A1 for 2 hours prior to fixation. It is also advisable to check for cell morphology and appropriate density prior to fixing cells with glutaraldehyde as it is possible that co-treating cells with bafilomycin and a presumed mitophagy-inducing stimulus may enhance toxicity.
4. Following pharmacological treatments, cells are rinsed with 0.1 M PBS for 10 minutes per wash and fixed for at least 30 minutes in 2.5% glutaraldehyde at RT, or overnight at 4°C.

5. After fixation, cell monolayers are washed three times in PBS (10 minutes per wash), followed by exposure to aqueous 1% OsO₄ and 1% K₃Fe(CN)₆ for 1 hour at 4°C.
6. After three washes with PBS for 10 minutes per wash, the cultures are dehydrated through a graded series of 30 – 100% ethanol solutions (30%, 50%, 70% and 90% ethanol for 10 minutes each wash and 100% ethanol for 10 minutes), and embedded in Polybed 812 epoxy resin contained in small capsules. The epoxy capsules containing cell monolayers are then transferred to a 37°C incubator overnight to allow for the efficient preservation of samples followed by incubation at 60 °C for 48 hours.
7. The preserved cell monolayers are cut as ultrathin sections and verified for cell morphology using a light microscope prior to mounting the samples on copper grids. The copper grids are sequentially stained with 2% uranyl acetate in 50% methanol for 10 minutes followed by a brief incubation in 1% lead citrate (7 minutes).
8. Cell monolayers are photographed, preferably at a magnification of 18,000 X, to visualize autophagosomes using a Phillips CM10 transmission *electron microscope or an equivalent electron microscope*. As a general rule, AVs should have a size range of 1–2 µm under physiological conditions.

3.2 Fluorescence imaging-based methods

1. SH-SY5Y cells are seeded on uncoated chambered LabTek II #1.5 (Nunc) coverglasses (137,000 cells per well) in complete medium (high glucose DMEM with 10% FBS, L-glutamine and sodium pyruvate). Primary cortical neurons are seeded on poly-L-lysine pre-coated LabTek II (Nunc) coverglasses in complete medium (Neurobasal medium with 1X B27, 0.5% FBS and L-glutamine). To study the effects of a stimulus on mitophagy in differentiated neuronal cells, SH-SY5Y cells can be differentiated by exposing cells with a 3 day treatment of an acute dose of retinoic acid (10 µM) or with db-cAMP treatment (250 µM). RA-differentiated SH-SY5Y cells should extend neurites that are greater than one cell body length by 24 hours of treatment whereas cAMP-differentiated SH-SY5Y tend to develop highly extended and complex neurite arbors. Primary cortical neurons should extend neurites immediately one day *in vitro* (DIV). Neurites of developing primary cortical neurons acquire polarity (axons or dendrites) by 3 DIV in culture and fully develop synapses by 7 DIV [41].
2. To monitor and quantify the initiation of mitophagy by confocal microscopy, 5 DIV mouse primary neurons or SH-SY5Y cells are transfected with 1 µg of GFP-LC3 (AddGene #11546, prepared as 100ng/µl) in 0.07% and 0.10 % Lipofectamine 2000 respectively. To monitor and quantify the progression of mitophagy induced by a presumed mitophagy-inducing stimulus, cells are transiently transfected with 1 µg of RFP-LC3 (AddGene #21075) in 0.10% for SH-SY5Y or in 0.07% Lipofectamine 2000 for primary neurons respectively. Four to six hours following transfection, it is critical to add one complete volume

of media per well onto the transfection mix in order to reduce any toxicity associated with any leftover Lipofectamine reagent present in the medium. It is important to add the media on top of the transfection mix without removing to avoid cell loss. Approximately two-thirds of the media is replaced 24 hours after transfection. Transfected cells are then incubated for an additional 48 hours in a 5% CO₂-supported tissue culture incubator at 37°C.

3. Three days post-transfection, the colocalization of AVs with mitochondria can be assessed in GFP-LC3 or RFP-LC3 expressing neurons by treating cells with 100 nM Mitotracker (MTR) Red CMXRos or with 250 nM MTR Green FM respectively for 45 minutes followed by one wash with warm media. The percentage of GFP-LC3 or RFP-LC3 puncta that colocalize with MTR-Red or MTR Green-stained mitochondria respectively gives an overall assessment of the steady-state levels of mitophagy in neurons (see Experimental variables section below).
4. Conditions that increase mitophagy, based on the colocalization of GFP-LC3 puncta with mitochondria, can be attributed to either increased or impaired mitophagic flux as both conditions can yield a high number and colocalization of GFP-LC3 puncta with mitochondria. Therefore, to analyze for flux, GFP-LC3 expressing cells can be treated with a single dose of bafilomycin A1 (10 nM for 4 hours), a concentration that elicits a maximal accumulation of GFP-LC3 puncta without inducing significant cell death (<5%). To further corroborate the involvement of autophagy for delivering mitochondria to lysosomes, cells can be co-transfected with ATG7/ATG8 siRNA for three days to suppress autophagy. It is important to note that this procedure requires transfecting the cell cultures twice within a span of two days. For instance, GFP-LC3 or RFP-LC3 plasmids are transfected in SH-SY5Y cells 48 hour after plating. The following day after the initial transfection, cells are re-transfected with ATG7/ATG8 siRNA at a 0.10% final concentration and incubated for an additional 48 hours (72 hours total transfection time). Likewise, 5 DIV primary neurons can be sequentially transfected with LC3 plasmids and ATG7/ATG8 siRNA using the same transfection protocol as described above for SH-SY5Y cells. We have found that the sequential transfection of plasmids and siRNA do not significantly injure cells based on intact cell morphology.
5. Alternatively, cells can be co-transfected with mitochondrially-targeted RFP (mito-RFP or mito-mCherry) or with mitochondrially targeted GFP (mito-GFP) and analyzed for the colocalization with GFP-LC3 and Lysotracker (LTR) Red respectively, an event defined as mitophagy maturation. It is highly recommended to allow neuronal cells/primary neurons to express mito-RFP or mito-mCherry for at least two days prior to mitophagy analyses in order to allow for sufficient import of mitochondrially- targeted fluorophores through the TOM complex.
6. Alternatively, the colocalization of lysosomes with mitochondria in SH-SY5Y cells or mouse primary cortical neurons can be analyzed by co-staining neurons

with 250 nM of MitoTracker Green and with 100 nM of LTR Red DND-99 in the same medium. Fluorescently-labeled cells are incubated for at least 45 minutes in a 37°C, 5% CO₂-maintained cell culture incubator. The colocalization of lysosomes, GFP-LC3 or RFP-LC3 with mitochondria can be analyzed by capturing high-resolution confocal slices (1024 × 1024 pixels) using a confocal microscope (Zeiss LSM510 or FluoView 1000) equipped with the appropriate set of filters (FITC and Texas Red-like filters) and a 60X objective. Based on experience, we typically analyze the colocalization of mitochondria with lysosomes, GFP-LC3 or with RFP-LC3 in at least 50 cells per condition in order to achieve statistically significant effects.

3.3. Biochemical methods

Unlike the plethora of complementary imaging-based techniques available for assessing mitophagy, there is currently only one biochemical method used to corroborate mitophagy in response to a presumed mitophagy-inducing stimulus. This method, immunoblotting for LC3-II is described below:

1. SH-SY5Y cells (5×10^5 cells per well) or mouse primary neurons (1.2×10^6 cells per well) are seeded in 6-well tissue culture dishes. Cells are treated with a presumed inducer of mitophagy inducer (e.g., 2.5 mM MPP+, 75 μ M 6-OHDA or 1.0 μ M rotenone for 4–6 h in SH-SY5Y cells) in the presence or absence of lysosomal fusion inhibitors (e.g. bafilomycin or chloroquine), or the cell permeable lysosomal protease inhibitors E64-D (40 μ M for SH-SY5Y cells) and pepstatin (25 μ M for SH-SY5Y cells) or transfected with siRNA targeting autophagy-related genes (ATG7/8, 120 pmols per well, 6-well plate × 3 days) to assess for basal levels of mitophagy and mitophagic flux. Following treatments, cell lysates are collected in cell lysis buffers containing 1% Triton X-100. Approximately 30 μ g of proteins are electrophoresed on 5–15% gradient gels and immuno-probed for the total levels of several OMM-, IMM- and matrix-localized mitochondrial proteins (e.g. OMM-localized proteins: TOM20, mitofusin 2 and porin; IMM-localized proteins: complexes I-V, and matrix-localized proteins: human pyruvate dehydrogenase).
2. The lipidated form of LC3, termed LC3-II, exhibits a faster electrophoretic migration on a gradient gel (5–15%) compared to the cytosolic-localized LC3-I (17 kDa for LC3-I vs. 15 kDa for LC3-II). The quantitation of LC3-II/ β -actin ratio is a valid index for assessing steady state levels of autophagy [36]. After blots have been probed for mitochondrial proteins, it is strongly recommended to verify the ability of ATG7/8 siRNA to suppress autophagy or of pharmacological and lysosomal fusion inhibitors (bafilomycin) for blocking the fusion of AVs with lysosomes by stripping and re-blotting the western blot membrane for LC3 and β -actin. Hence, a strong effect of bafilomycin on autophagy should increase the LC3-II/ β -actin ratios compared to untreated cells when assessing for flux. Conversely, RNAi-mediated knockdown of ATG7/8 should decrease the LC3-II/ β -actin ratio and reverse the loss of mitochondrial proteins in the case that a specific stimulus elicits mitophagy.

4. Data analysis

4.1 Qualitative assessment of mitophagy using GFP-LC3 and RFP-LC3

Generally, cells transiently transfected with GFP-LC3 show either diffuse cytosolic fluorescence or contain fluorescent puncta. It is worth noting that different morphological patterns of mitophagy can be identified when assessing for mitophagy using the GFP-LC3 construct as further described below. AVs, as monitored by GFP-LC3, tend to show high mobility and have a propensity to coalesce near mitochondria during the initiation of mitophagy. AVs that target mitochondria for sequestration exhibit decreased mobility upon associating with mitochondria, followed by the eventual entrapment and delivery of mitochondria to lysosomes [42]. While it is not considered colocalization – in the strict sense of two perfectly overlapping fluorescently labeled organelles – the clustering of GFP-LC3 near mitochondria is interpreted as mitochondrial association, the first step of the recognition phase of mitophagy. Secondly, the colocalization or overlap of GFP-LC3 or RFP-LC3 puncta with red and green fluorescently-labeled mitochondria respectively is interpreted as direct association of an AV with mitochondrial membranes. Alternatively, fragmented mitochondria can be completely engulfed by an AV. This morphological pattern is typically characterized by the presence of large GFP-LC3 “rings” that can sequester several pieces of mitochondria or the whole organelle (Fig. 1b, inset). These large AVs that contain mitochondria have been previously termed “mitophagosomes” [43]. As with EM analyses, it is critical to categorize and classify these three morphological patterns of mitophagy in order to gain a comprehensive understanding on the mechanisms by which a specific stimulus or protein promotes the initiation of mitophagy.

4.2 Quantitative analysis of autophagy and mitophagy using GFP-LC3, RFP-LC3 and fluorescent lysosome-specific dyes

To quantify mitophagy using fluorescence-based imaging protocols, the percentage of LC3 puncta (endogenous, GFP-LC3 or RFP-LC3) that colocalize with mitochondria in the soma is a valid method for assessing steady-state levels of mitophagy in primary neurons. The percentage of LC3 puncta that colocalize with mitochondria for a certain segment of proximal or distal neurites (axons or dendrites, 50 – 100 μ m) is a valid index for assessing mitophagy in neurites. Alternatively, the number of mitochondrial colocalizing LC3 puncta per soma or neurite segment can also be quantified in conjunction with the percentage of LC3 puncta that colocalize with mitochondria to assess for mitophagy. Given that mitochondria are highly dynamic in neurites, it is necessary to rule out transient colocalization of LC3 with mitochondria. To overcome this issue, it is highly recommended to monitor mitophagy at least two different time points in live or fixed cells. Data on autophagy/mitophagy can be represented as bar graphs that display the number of GFP-LC3 or RFP-LC3 puncta per neuron as well as the percentage of GFP-LC3 or RFP-LC3 puncta that colocalize with mitochondria in response to a presumed mitophagic inducer (Fig. 1–3). Likewise, to study lysosomal sequestration of mitochondria in response to a specific stimulus, the percentage of lysosomes that colocalize with mitochondria can be quantified to analyze late-stage mitophagy (Fig. 2).

Thus far, we have presented several methods and guidelines to measure mitophagy using LC3 as a marker of AVs. However, measuring the content of autophagosomes such as mitochondria in different neuronal compartments is a direct assessment of mitophagy that can further corroborate analyses involving mitochondrial colocalization of LC3. As numbers of mitochondria in neurites are governed by different factors including mitochondrial biogenesis, trafficking, and mitophagy, it is imperative to assess the contribution of mitophagy to mitochondrial content in the soma or neurites. Therefore, the percentage of cytosolic area occupied by mitochondria in the presence or absence of lysosomal fusion inhibitors or in cells transfected with ATG7/8 siRNA is a valid method for assessing mitophagy. As with western blots, it is imperative to immunostain for at least two different mitochondrial markers when assessing mitophagy using image-based methods. Secondly, to facilitate the concomitant analyses of mitochondrial content and mitophagy, cells can be co-transfected with mito-RFP and with GFP-LC3 to demarcate the cell boundaries given the mixed diffused and punctate staining pattern observed when cells express GFP-LC3.

Quantifying mitophagic flux—To facilitate the analyses of flux, treating neuronal cells with bafilomycin can be used to trap mitochondria in AVs. **Mitophagic flux**= (AVmbaf)/ (AVm)/Tbaf [43]. AVmbaf: # of AVs per cell containing mitochondria in the presence of bafilomycin; AVm: # of AVs per cell containing mitochondria in the absence of bafilomycin; Tbaf: time in bafilomycin treatment. Therefore, a high mitophagic flux ratio suggests that a specific stimulus or protein of interest enhances mitophagic flux whereas a low ratio (~1.0) indicates impaired flux.

4.3 Guidelines for measuring mitophagy in neurites

Mitochondria are highly dynamic organelles that show bidirectional movement in neurites [44]. When assessing mitophagy in presynaptic and postsynaptic compartments, live imaging can be performed in cells co-expressing mito-GFP and RFP-LC3. Mitophagy in neurites is assessed as the percentage of AVs that contain mitochondria at least two different time points. Hence, the colocalization of two dynamic organelles labeled with two different fluorophores can be analyzed by generating two five-minute kymographs for mitochondria (e.g. mito-RFP) and AVs (e.g. GFP-LC3). Individual kymographs for each organelle can then be assembled and overlaid to analyze for colocalized objects using Image J and the Multiple Kymograph” plug-in (J. Rietdorf, A. Seitz, EMBL, Heidelberg, http://www.embl.de/eamnet/html/body_kymograph.html).

4.4 Western blot quantification of mitophagy

Unlike imaged-based analysis, one major caveat is that western blots do not measure mitophagy in distinct neuronal compartments but represent an average of heterogeneous autophagic/mitophagic responses for a population of cells. We provide the following guidelines to measure mitophagy using western blot assays.

1. To corroborate mitophagy, it is important to perform densitometric quantification of at least two different mitochondrial proteins as measured by the integrated density of immunoreactive bands. Moreover, it is important to analyze the levels

of mitochondrial proteins in cells treated in the presence or absence of bafilomycin or in cells transfected with ATG7/8 siRNA to assess for mitophagy.

2. An increase in the LC3-II/ β -actin ratios (not the LC3- I/LC3-II levels) along with a concomitant decrease in the level of mitochondrial proteins suggests enhanced mitophagy. SQSTM1/P62 is a *bona fide* autophagy substrate that is sequestered by AVs and links the ubiquitin proteasome pathway with the autophagic machinery [18]. Induction of autophagy leads to decreased steady-state levels of SQSTM1/P62. Hence, the western blot membrane can be stripped and re-blotted for SQSTM1/P62 levels to further confirm for induction of autophagy. It is important to bear in mind that an induction of mitophagy transiently increases LC3-II/ β -actin ratios while a block in flux will show persistent increased levels of SQSTM1/P62 over time. On the other hand, a decrease in mitochondrial markers, increased LC3-II/ β -actin ratios along with decreased SQSTM1/P62 levels for at least two time points is a strong indication that mitophagy is induced by a specific stimulus or protein of interest.
3. If possible, it is helpful to include a positive control for all biochemical-based assessments of mitophagy such as treating cells with rapamycin (an inducer of mild, physiological mitophagy) or with staurosporine (a pathological inducer of mitophagy).

5. Results

In this section, we present several experimental examples that show how autophagy and mitophagy are properly analyzed in neuronal cell lines and primary neurons using image-based methods.

Also, as mitophagy can occur in the context of both physiological and pathological conditions, several caveats and potential pitfalls that can lead to a misinterpretation of the results will be indicated appropriately for each example.

5.1 Image-based analysis of mitophagy

Figure 4 shows an example of how mitophagy is properly analyzed at the ultrastructural level in SH-SY5Y cells treated with rotenone, a pathological inducer of mitophagy [30]. While untreated cells show elongated mitochondria and occasional AVs, a 4 hour treatment of rotenone (1 μ M) robustly swells/fragments mitochondria and increases the number of AVi in SH-SY5Y cells (Fig. 4a–b). Co-treating cells with a short pulse of bafilomycin (10 nM, 4 hours), leads to a further increase in the number of AVs per cell as well as the accumulation of swollen/fragmented mitochondria engulfed in AVi compared to cells treated with rotenone alone (Fig. 4c–e). These results suggest that rotenone alters mitochondrial structure and promotes mitophagy in SH-SY5Y cells. We calculated the mitophagic flux induced by rotenone in the same experiment using the formula described in section 4.2. Although rotenone increases the mean number of mitophagosomes per cell (0.22 vs. 0.62 mitophagosomes per cell in untreated or rotenone treated cells respectively), mitophagic flux of SH-SY5Y cells exposed to rotenone (0.15 mitophagosomes/hr) is similar to mitophagic

flux in the absence of rotenone (0.15 mitophagosomes/hr). These results suggest that rotenone elevates autophagic sequestration of mitochondria (Fig. 4e) without inducing flux.

The next three experimental examples demonstrate how studying the effects of overexpressing one protein on mitophagy can be assessed using multiple but complementary fluorescence-based techniques. The neuronal regulatory subunit of protein phosphatase 2A (PP2A) termed B β 2 targets the PP2A holoenzyme to the OMM to promote mitochondrial fission (fragmentation) and accelerate apoptosis in oxidatively-stressed neurons [45,46]. As mitochondrial fragmentation precedes mitophagy, we sought to determine whether PP2A/B β 2 modulates autophagy and mitophagy in SH-SY5Y neuronal cells. Overexpression of PP2A/B β 2 in SH-SY5Y neuronal cells significantly increased the number of GFP-LC3 puncta per cell as well as the percentage of GFP-LC3 that colocalize with mitochondria suggesting that overexpression of PP2A/B β 2 concomitantly upregulates autophagy and mitophagy (Rice M, Strack S, and Dagda RK, unpublished observations). As a specificity control for autophagy, cells transiently expressing a C-terminal lipid deficient mutant of GFP-LC3 (G120A) yields a significantly low number of GFP-LC3 puncta per cell. The presence of large GFP-LC3 rings engulfing mitochondria were also observed in some B β 2-expressing cells (Fig. 1b).

Alternatively, mitochondrial content in response to mitophagic stimuli can be measured at the single cell level as a valid index of mitophagy. Following treatment with a presumed mitophagy-inducing stimulus, in the presence or absence of flux inhibitors, live or fixed cells can be analyzed for the percentage of cytosol occupied by mitochondrial-specific fluorescent pixels by using a pre-validated Image J macro ("Mitophagy" macro, http://imagejdocu.tudor.lu/doku.php?id=macro:mitophagy_mitochondrial_morphology_content_lc3_colocalization_macro) [46,39]. Therefore, enhanced mitophagy in response to a specific stimulus is expected to decrease the percentage of the cytosolic area occupied by mitochondria but reversed by lysosomal fusion inhibitors (e.g. bafilomycin) or by ATG7/8 siRNA. Conversely, an increase in mitochondrial content in response to a stimulus, which is not further increased by lysosomal fusion inhibitors or by ATG7/8 siRNA, suggest an impairment of mitophagy [31].

It is important to determine whether a specific stimulus not only increases the initiation of mitophagy but also leads to the eventual sequestration and degradation of mitochondria by lysosomes. In conjunction with mitophagy, lysosomal expansion – defined as an increase in the number and size of acidic lysosomes per cell – is an indication of enhanced autophagy [47,31]. In addition to stimulating mitophagy initiation, to ascertain whether forced overexpression of PP2A/B β 2 promotes the lysosomal-mediated sequestration of mitochondria, SH-SY5Y cells were transiently transfected with an empty vector or Flag-tagged B β 2 for three days and analyzed for the number and percentage of lysosomes that colocalize with mitochondria per cell. Compared to empty vector expressing cells, transient expression of PP2A/B β 2 increases both the number and percentage of lysosomes that colocalize with mitochondria in SH-SY5Y cells (Fig. 2). Overall, these results suggest that PP2A/B β 2 not only promotes the initiation of mitophagy, but elicits lysosomal expansion and the active lysosomal-mediated degradation of mitochondria (Rice M, Strack S, and Dagda RK, unpublished observations).

5.2 Imaged-based analysis of mitophagy in neurites

The quantification of the number of GFP-LC3 or RFP-LC3 puncta (granules) per segment per neurite segment (50–100 μm) in either paraformaldehyde fixed or live neurons is a valid method to measure autophagy in neurites as previously published [17]. Mitochondria in neurites play critical physiological roles such as maintaining the structural stability of neurites and providing the necessary energy required for remodeling dendritic spines, synaptogenesis, and for enabling the release of synaptic vesicles [48,39,49–51]. Therefore, quantitating mitophagy in neurites in response to a specific stimulus can yield valuable insight into the physiological role that mitophagy plays in neuronal development and neurodegeneration. To quantitate mitophagy in neurons, the percentage of the area per neurite occupied by mitochondria (also known as mitochondrial content) in the presence or absence of flux inhibitors or ATG siRNA, can be used to assess mitophagy in neurites [52].

An experimental example of how mitophagy is measured in neurites is presented in figure 3. To study the effects of overexpressing PP2A/B β 2 on autophagy and mitophagy in neurites, 5 DIV primary cortical neurons were co-transfected with RFP-LC3 and with Flag-tagged B β 2 for three days prior to analyzing for both macroautophagy and mitophagy by confocal microscopy. While PP2A/B β 2 non-significantly increased the number of RFP-LC3 puncta per neurite (RFP-LC3 puncta/50 μm of neurite segment), transient expression of PP2A/B β 2 significantly elevated the percentage of RFP-LC3 puncta that colocalize with mitochondria per neurite (% of RFP-LC3 that colocalize with mitochondria/50 μm of neurite segment) and at the soma as well. Overall, these results suggest that PP2A/B β 2 upregulates the steady-state levels of mitophagy in neurites and soma of primary neurons (Rice M., Strack S., and Dagda RK, unpublished observations).

5.3 Biochemical quantification of mitophagy

The lipidated form of LC3, termed LC3-II, exhibits a much faster electrophoretic migration on a gradient gel (5–15 %) compared to LC3-I [36]. As with image-based analysis of LC3 puncta, macroautophagy and mitophagy can be analyzed in the same western blot. A specific effect of a stimulus on macroautophagy should transiently increase the LC3-II/ β -actin ratios and return to baseline levels, an indication of autophagic flux. On the other hand, a block on autophagic flux should lead to a persistent increase in the LC3-II/ β -actin ratio which is not further increased with bafilomycin treatment. In conjunction to enhanced autophagy, a specific effect of a stimulus on mitophagy should promote a decrease in the levels of at least two mitochondrial proteins (e.g. ATP synthase V, Mitochondrial antigen of 60kDa, and cytochrome c oxidase) and this effect should be blocked, or partially suppressed by co-treating cells with bafilomycin or by ATG7/8 siRNA.

6. Troubleshooting

In this section, we present the most common pitfalls that neurobiologists encounter when assessing mitophagy in neurons and provide solutions to overcome the following issues:

1. Pathological stimuli that elicit mitophagy, especially mitochondria-directed toxins, tend to damage and depolarize mitochondria. Hence, mitophagy assessments may be underestimated under these circumstances as oxidatively-

damaged and fragmented mitochondria do not stain well with mitochondrial membrane potential-dependent dyes (e.g. Mitotracker Red dye series) and may appear not to colocalize with AVs. Therefore, it is highly advisable to analyze mitophagy using live fluorescent reagents that do not depend on the membrane potential (e.g. MitoTracker green) or transfect with mitochondria- targeted fluorescent proteins.

2. There are several caveats to consider when analyzing mitophagy by transient vs. stable expression of fluorescent LC3 fusion proteins (e.g. GFP-LC3). For instance, a major caveat of transiently co-expressing a protein of interest and fluorescent LC3 fusion proteins is that only short-term mitophagy responses can be monitored. On the other hand, using stable cell lines affords the ability to analyze mitophagy for prolonged periods of time. Secondly, use of stable cell lines eliminates potential toxicity of liposome-mediated transient transfection. Third, it facilitates biochemical analysis of mitophagy in response to stably overexpressing or downregulating a protein of interest. However, a major caveat of stable cell lines is that GFP-LC3 may be incorporated into aggregates (not AVs), or the GFP moiety may aggregate itself, which can confound analyses of autophagy and mitophagy [53].
3. It is important to check the quality of cells prior to starting an experiment as a high baseline level of autophagy and mitophagy in primary neurons likely indicates an unhealthy, stressed or contaminated culture.
4. Primary neurons transfected with GFP-LC3 or cultured from transgenic GFP-LC3 mouse embryos [42] predominantly show a diffuse staining pattern and a low number of AVs which precludes analyzing steady-state basal levels of mitophagy using this construct. On the other hand, primary neurons tend to display a higher number of RFP-LC3 puncta compared to GFP-LC3 puncta. Given that the RFP moiety is fluorescently stable in the acidic environment of the lysosome compared to GFP [38], a high number of RFP-LC3 puncta relative to the number of GFP-LC3 puncta per cell is indicative of high autophagic flux. Neurons display a high autophagic flux under basal conditions as indicated by the high number of RFP-LC3 puncta per cell relative to the GFP-LC3 puncta. To this end, mitophagy in the soma and neurites of primary neurons is better assessed by analyzing for the colocalization of RFP-LC3 puncta with MitoTracker Green-labeled mitochondria (Fig. 4).
5. Although analyses of mitophagy in presynaptic or postsynaptic compartments is technically challenging in primary neurons, it is highly advisable to use a spinning disc confocal microscope equipped with high performance camera (e.g., EMCCD Hamamatsu) to perform time-lapse imaging in order to prevent phototoxicity-induced mitophagy[54] and to have the ability to analyze the dynamic movement and colocalization of dynamic organelles (AVs and mitochondria) at a high frame rate (40 – 62 frames per second). Alternatively, the mitochondrial content in the neurites in response to a specific stimulus can be assessed to further confirm mitophagy as described in section 3.4.

6. Unlike IMM-localized proteins, OMM-localized proteins can undergo fast turnover through the ubiquitin-proteasome pathway. Therefore, a major pitfall when assessing mitophagy by western blot is that the protein levels of OMM-localized proteins may not change in parallel with IMM- or matrix-localized proteins [36]. To overcome this issue, it is important to strip and re-blot a western blot membrane for multiple mitochondrial markers, preferably IMM- and matrix-localized proteins. Secondly, a decrease in IMM- and matrix-localized proteins in response to a specific stimulus can be attributed to proteolytic processing, impaired mitochondrial import or impaired biogenesis. Therefore, it is critical to determine whether specific decreases in mitochondrial proteins can be reversed by RNAi-mediated knockdown of ATG7/8 or by short-term treatment of cells with bafilomycin. In addition, biochemical assessments of mitophagy should be qualitatively confirmed by EM as well.
7. Another possibility is that treatments that promote high mitophagic flux may not change the total levels of mitochondrial proteins due to increased compensatory mitochondrial biogenesis. To overcome this issue, it is important to analyze for potential compensatory mitochondrial biogenesis by re-blotting the western blot membrane for the total protein levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- α) and mitochondrial transcription factor A (TFAM), two regulators of mitochondrial biogenesis [55,56].
8. When performing LC3 shift assays, LC3-II may not be very visible in treatments that modestly induce autophagy. In addition, unlike other tissue types, brain tissue contains a high amount of LC3-I. Hence, high levels of LC3-I may block the detection of LC3-II in a western blot [51]. Hence, to ascertain whether a specific treatment induces macroautophagy, it may be necessary to probe for LC3-II in membrane fractions obtained by centrifugation steps as previously described [51]. Finally, immunodetection of endogenous LC3 by immunofluorescence or by western blot can be difficult at times. Some good commercial antibodies available include mouse anti-human LC3 monoclonal antibodies (Nanotools, clone 5F1 or clone 2G6 for western blot). In addition, good antibodies that can be used to detect the GFP moiety to resolve GFP-LC3-II from GFP-LC3-I by western blot have been used as well (Rabbit anti-GFP serum, Life Technologies, Cat# A-6455) [17].

7. Conclusions

Although a vast amount of knowledge has been garnered over the last decade regarding the role of mitophagy in the soma of neurons, studies that address the physiological roles of mitophagy in other neuronal compartments have only recently taken center stage. Indeed, the seminal discovery that LC3 is a *bona fide* marker of AVs and the development of sophisticated, yet affordable live cell imaging equipment have allowed neuroscientists to assess the role of mitophagy in the context of neuronal development and neurodegeneration. It is important to keep in mind that the image-based techniques presented in this chapter may allow for the simultaneous analyses of autophagy, mitophagy, mitochondrial morphology,

bioenergetics and mitochondrial dynamics in neurons when multiplexed with other techniques described in this book. Secondly, it is important to keep in mind that a single technique is not sufficient to establish mitophagy induced by a specific physiological/pathological stimulus. Therefore, we encourage the use of complementary techniques, including electron microscopy, fluorescence- and biochemistry-based techniques to analyze mitophagy in neurons as described in this chapter. Finally, the techniques outlined in this chapter are meant to encourage the development of new methodology and reagents to assess mitophagy in *ex vivo* and in *in vivo* models in the near future. However, it should be noted that regardless of the experimental paradigm(s) used to analyze mitophagy in neurons, the concepts and guidelines for assessing mitophagy should remain the same.

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Figure 1. Example of autophagy/mitophagy analyses using the GFP-LC3 construct in live SH-SY5Y cells

The effects of overexpressing B β 2 on autophagy/mitophagy were analyzed in SH-SY5Y cells. Representative epifluorescence images of SH-SY5Y cells transiently co-expressing GFP-LC3 and either an empty vector (**a**) or Flag-tagged B β 2 (**b**) and stained with Mitotracker Red to visualize mitochondria. Note that forced overexpression of PP2A/B β 2 increases the number and mitochondrial colocalization of GFP-LC3 puncta (arrows). Some B β 2-expressing neuronal cells showed the presence of large GFP-LC3 ring structures containing mitochondria (asterisk, inset in **b**). Scale bars: 2 μ m. **c-d**: Representative quantifications of GFP-LC3 puncta per cell (**c**) and the percentage of GFP-LC3 puncta that colocalize with mitochondria per cell (**d**). Note that forced overexpression of PP2A/B β 2 increases the number and mitochondrial colocalization of GFP-LC3 puncta per cell. As a negative control, the number and mitochondrial colocalization of GFP-LC3 puncta were quantified in cells transiently expressing the lipidation deficient mutant construct of GFP-LC3 (GFP-LC3 G120A). (*: $p < 0.05$ vs. empty vector, $\pm$ S.E.M., $n = 22 - 30$ cells per condition, Student's t-test).

Figure 2. Example of autophagy/mitophagy analyses using lysosomal and mitochondrial specific fluorescent dyes in live SH-SY5Y cells

The effects of forced overexpression of B β 2 on lysosomal-mediated sequestration of mitochondria were analyzed in SH-SY5Y cells. Representative epifluorescence images of SH-SY5Y cells transiently expressing empty vector (**a**) or Flag-tagged B β 2 (**b**) were co-stained with Lysotracker Red and Mitotracker Green FM to visualize lysosomes and mitochondria respectively. Note that forced overexpression of B β 2 increases the number and the colocalization of lysosomes with mitochondria (white arrows). Scale bars: 2 μ m. **c–d**: Quantifications of the number Lysotracker (LTR) stained structures per cell and the average percentage of LTR puncta that colocalize with mitochondria (mitophagy). (*:p<0.05 vs. empty vector, $\pm$ S.E.M., n=22 – 30 cells per condition, Student's t-test).

Figure 3. Example of autophagy/mitophagy analyses in the soma and neurites of primary cortical neurons expressing the RFP-LC3 construct

The effects of forced overexpression of PP2A/B β 2 on autophagy/mitophagy were further confirmed in 8 DIV primary cortical neurons. Representative epifluorescence images of primary cortical neurons transiently co-expressing RFP-LC3 and either an empty vector (a) or Flag-tagged B β 2 (b) and stained with Mitotracker Green (MTRG) to visualize mitochondria. Scale bars: 2 μ m. Note that forced overexpression of B β 2 increases the mitochondrial colocalization of RFP-LC3 puncta (white arrows) or its association with mitochondria (arrow heads). For clarity, a dendrite was traced with white hatched lines in the representative neuron shown in panel a. c–d: Quantification of RFP-LC3 puncta per cell and the average percentage of RFP-LC3 that colocalize with mitochondria (mitophagy) in the soma or neurites. While overexpression of B β 2 induces a non-significant increase in RFP-LC3 puncta per neurite (50 μ m), forced overexpression of B β 2 significantly increases the mitochondrial colocalization of RFP-LC3 puncta in the soma and neurites of neurons (*:p<0.05 vs. empty vector, $\pm$ S.E.M., n=22 – 30 cells analyzed per condition as shown in panel c or n=20 – 45 neurites analyzed per condition as shown in panel d, Student's t-test).

Figure 4. Example of ultrastructural analyses of autophagy/mitophagy in SH-SY5Y cells
a. SH-SY5Y cells were treated with rotenone (**b**) or DMSO (**a**) as a vehicle control in the absence (**b**) or presence of bafilomycin (**c–d**) for 4 hours prior to processing for ultrastructural analyses of mitophagy. **e.** The number of AVs (arrows) per cell were quantified for the indicated conditions. An asterisk in panel **d** shows an example of an AV containing mitochondria (electron dense structure with noticeable cristae). Legend: m= mitochondria, n= nuclei, arrows= AVs, asterisk= mitophagosome. Scale bar: 500 nm. (*:p<0.05 vs. DMSO, **:P<0.05 vs. rotenone, ±S.E.M., n=15–22 cells per condition, Student's t-test).