Chapter 21

Assessing Mitochondrial Selective Autophagy in the Nematode *Caenorhabditis elegans*

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Abstract

Eukaryotic cells heavily depend on ATP generated by oxidative phosphorylation (OXPHOS) within mitochondria. Besides being the main suppliers of cell's energy, mitochondria also provide an additional compartment for a wide range of cellular processes and metabolic pathways. Mitochondria constantly undergo fusion/fission events and form a mitochondrial network, which is a highly dynamic, tubular structure allowing for rapid and continuous exchange of genetic material, as well as, targeting dysfunctional mitochondria for degradation through mitochondrial selective autophagy (mitophagy). Mitophagy mediates the elimination of damaged and/or superfluous organelles, maintaining mitochondrial and cellular homeostasis. In this chapter, we present two versatile, noninvasive methods, developed for monitoring in vivo mitophagy in *C. elegans*. These procedures enable the assessment of mitophagy in several cell types during development or under stress conditions. Investigating the role of mitophagy at the organismal level is essential for the development of therapeutic interventions against age-related diseases.

Key words Ageing, Autophagosome, Autophagy, *Caenorhabditis elegans*, DsRed, Green Fluorescent Protein (GFP), Lysosomes, Fluorescent microscopy, Mitochondria, Mitophagy, mtRosella

Abbreviations

CCCP	Carbonyl cyanide m-chlorophenylhydrazone
DMSO	Dimethyl sulfoxide
DsRed	Red fluorescent protein
FUdR	Fluorodeoxyuridine
GFP	Green fluorescent protein
MAP1LC3/LC3	Microtubule-associated protein 1 light chain 3
mtGFP	Mitochondria-targeted green fluorescent protein
mtRosella	Mitochondria-targeted Rosella
NGM	Nematode growth medium

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1 Introduction

Mitochondria are a distinguishing feature of eukaryotic cells and are often described as cellular powerhouses, since they are the major energy producers through oxidative phosphorylation (OXPHOS) and ATP production. In addition, these organelles play an essential role in several fundamental cellular processes including calcium homeostasis, metabolite synthesis, and apoptosis, among others [1].

Impairment of mitochondrial function is a major hallmark of many pathological conditions and ageing, highlighting the importance of proper mitochondrial activity [2]. Maintenance of cellular and organismal homeostasis necessitates a tight regulation of mitochondrial biogenesis, as well as the elimination of damaged or superfluous mitochondria [3–6]. Therefore, eukaryotic organisms have developed complex and highly specialized molecular and cellular pathways to preserve mitochondrial homeostasis [7]. Mitophagy is a selective type of autophagy mediating removal of dysfunctional and/or aged mitochondria, and the major degradation pathway by which cells regulate mitochondrial population in response to metabolic state [8].

The cytosolic E3 ubiquitin ligase Parkin and the mitochondrial phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1), which are associated with the autosomal recessive form of parkinsonism [9, 10], have been implicated in the regulation of mitophagy. The PINK1/Parkin pathway is the best studied molecular pathway mediating selective autophagy of damaged and/or aged mitochondria. In healthy mitochondria, PINK1 translocates to the inner mitochondrial membrane and is cleaved by mitochondrial proteases [11]. Subsequently, N-terminally truncated form of PINK1 is released into the cytosol and degraded there by the proteasome [12]. Impairment of mitochondrial function inhibits import of PINK1 to the inner mitochondrial membrane and PINK1 is consequently stabilized on outer mitochondrial membrane. Accumulation of PINK1 on the mitochondrial surface stimulates mitophagy through recruitment of Parkin [8]. Following its translocation to defective mitochondria, Parkin ubiquitylates several outer mitochondrial membrane proteins, resulting in the flagging of dysfunctional organelles within the mitochondrial population [13–15]. Damaged mitochondria are so recognized and degraded by the autophagic machinery. The highly conservation of PINK1 and Parkin among diverse species underlines their vital role in the maintenance of energy homeostasis [8].

Despite advances in the delineation of the molecular mechanisms that regulate mitophagy, no reliable and quantitative, in vivo approach for monitoring mitochondrial elimination in multicellular organisms is currently available. Aiming to fill this gap, we developed two complementary systems for monitoring mitophagy

in vivo and identified conditions that either induce or suppress mitophagy in the nematode *Caenorhabditis elegans* [4, 6]. In the first approach, we generated transgenic animals expressing the Rosella biosensor in mitochondria. Rosella is a purpose-built reporter comprising a fast-maturing pH-insensitive DsRed fused to a pH-sensitive GFP variant. The Rosella biosensor mode of function is based on the pH differences between lysosomes and other cellular compartments. GFP fluorescence becomes quenched in acidic lysosomes, whereas DsRED fluorescence remains unaffected. This biosensor has been used successfully in previous studies to monitor mitophagy in the unicellular organism *Saccharomyces cerevisiae* [16]. We adapted this versatile fluorescent microscopy assay and examined *C. elegans* animals carrying mtRosella under normal and mitophagy-inducing conditions such as oxidative stress, mitochondrial stress, and heat stress. All treatments reduce the GFP/DsRed ratio of Rosella fluorescence, indicating stimulation of mitophagy (Fig. 1). In addition to mtRosella, we generated

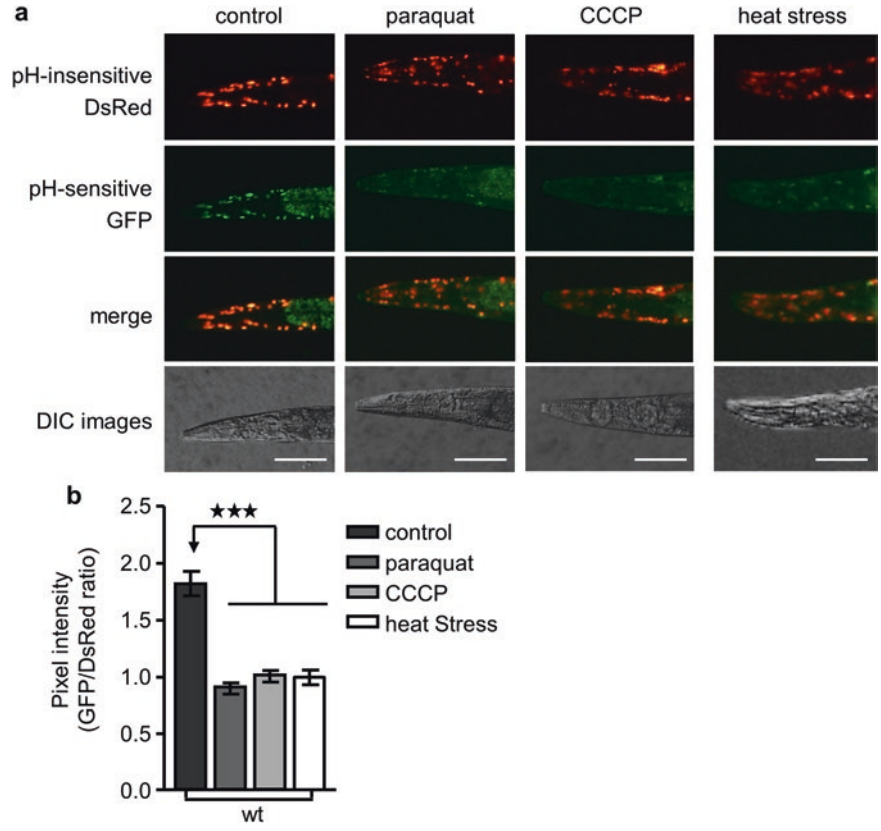

Fig. 1 Mitophagy is induced under stress conditions. (**a**) Transgenic animals expressing the mtRosella biosensor in body wall muscle cells were treated with either paraquat or CCCP or exposed to heat stress (37 °C). (**b**) Mitophagy stimulation is described as the reduction of the ratio between pH-sensitive GFP to pH-insensitive DsRed ($n = 120$; ***$P < 0.001$; one-way ANOVA). Acquisition information: Exposure time, 100 ms; Contrast, medium. Images were acquired using a 10× objective lens. Size bars denote 20 μm. Error bars denote S.E.M. values

transgenic animals expressing a mitochondria-targeted GFP (mtGFP), together with the autophagosomal marker LGG-1, the homologue of the mammalian cytosolic microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3), fused with DsRed in body wall muscle cells. We examined animals carrying both the mitochondrial and autophagosomal markers, under normal and mitophagy-inducing conditions. Mitophagy stimulation induces the formation of autophagosomes that extensively co-localize with mitochondria (Fig. 2). The availability of reliable and quantitative methods for monitoring mitochondrial elimination is a prerequisite for the elucidation of the molecular mechanisms and pathophysiological implications of mitophagy in living cells and organisms and therefore for the development of therapeutic interventions to treat several human disorders including mitochondrial diseases and neurodegenerative conditions.

In the following sections, we describe detailed protocols for in vivo imaging of mitochondrial-selective autophagy in *C. elegans*, using two versatile imaging tools.

2 Materials

1. Wormpick or eyebrow/eyelash hair (*see* **Note 1a, b**).
2. Cholesterol stock solution: Prepare 5 mg/ml solution of cholesterol in absolute ethanol. Dissolve by stirring. Store at 4 °C. Do not flame or autoclave.
3. Nystatin stock solution: Prepare 10 mg/ml solution of nystatin in 70% (V/V) ethanol. Store at 4 °C and shake prior to use as this is a suspension. Do not autoclave.
4. Phosphate buffer: For 1 l, dissolve 102.2 g KH_2PO_4 and 57.06 g K_2HPO_4 in distilled water and fill up to 1 l. This is a 1 M solution, pH 6.0. Autoclave and keep at room temperature.
5. Prepare and autoclave 1 M $MgSO_4$ (Sigma cat. no. M-7506) stock solution.
6. Petri dishes (60 mm × 15 mm).
7. Nematode growth medium (NGM) agar plates: Mix 3 g NaCl, 2.5 g Bactopeptone, 0.2 g Streptomycin, 17 g Agar and add 900 ml distilled water. Autoclave. Let cool to 55–60 °C. Add 1 ml cholesterol stock solution (SERVA Electrophoresis GmbH, Heidelberg, Germany, cat. no. 1701), 1 ml 1 M $CaCl_2$ (Sigma cat. no. C-5080), 1 ml 1 M $MgSO_4$ (Sigma cat. no. M-7506), 1 ml Nystatin stock solution (Sigma cat. no. N-3503), 25 ml 1 M phosphate buffer, pH 6.0, and distilled sterile water up to 1 l. Pour about 8 ml medium per Petri dish and leave to solidify. Keep the plates at 4 °C until used (*see* **Note 2**).

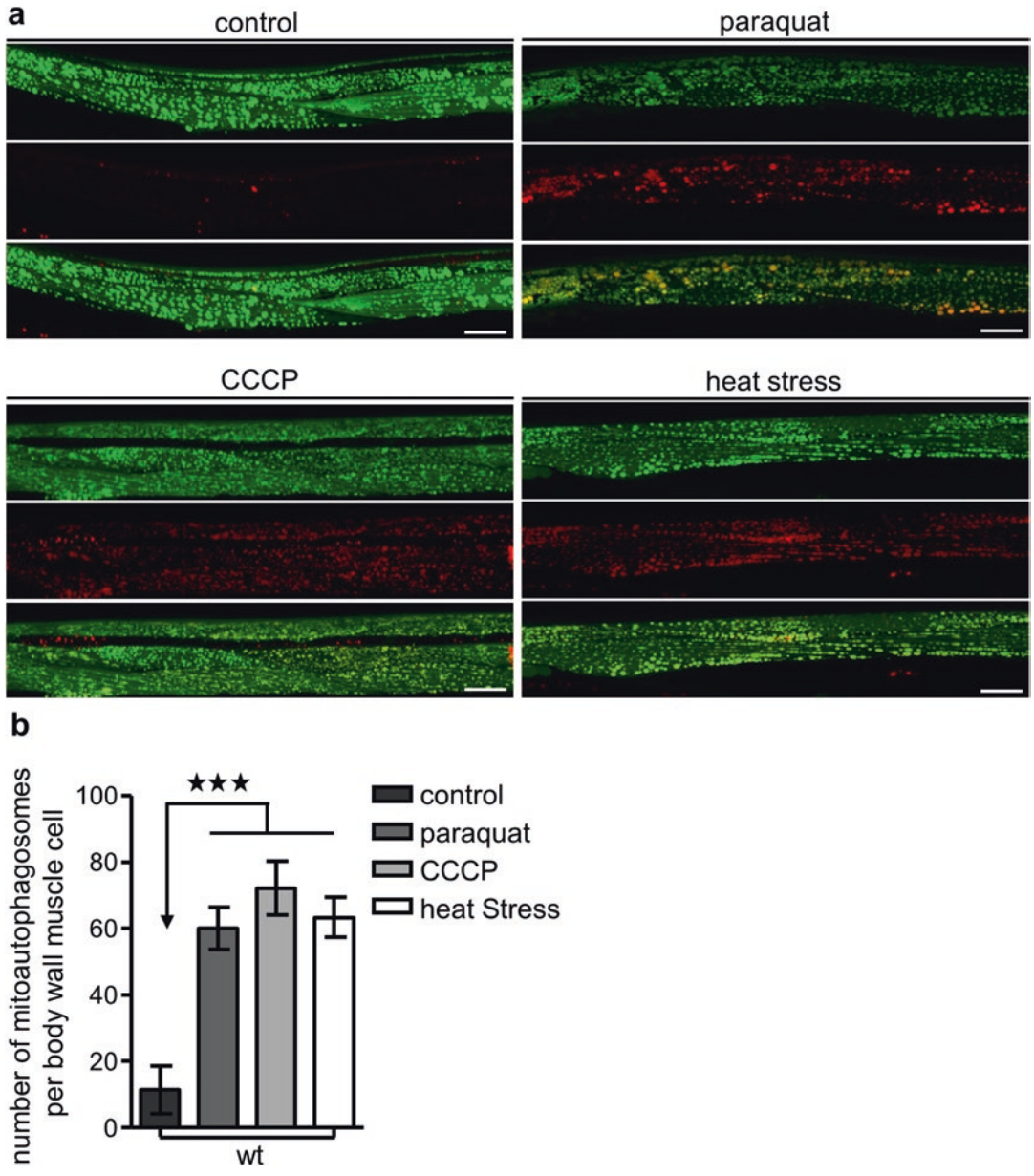

Fig. 2 Monitoring mitophagy in vivo. (**a**) Transgenic animals co-expressing in body wall muscle cells a mitochondrially targeted GFP (mtGFP) and the autophagosomal protein LGG-1 fused with DsRed were treated with paraquat, CCCP or exposed to heat stress (37 °C) and subsequently analyzed by confocal microscopy. Z-projection images of GFP signals depicting mitochondria are shown in the *upper panels*, DsRed signals depicting autophagosomes are shown in the *middle panels*, and the merged images in the *lower panels*. Mitophagy induction is described as co-localization of GFP and DsRed signals. (**b**) Increased number of mitoautophagosomes under mitophagy-inducing conditions ($n = 60$; $^{***}P < 0.001$; one-way ANOVA). Acquisition information: Resolution, 1024 × 1024; Master gain, Track1: 775 and Track2: 510; Emission filters, Track1 Channel1: 612–671 and Track2 Channel1: 493–562; Laser intensity, Track1 (543 nm): 12% and Track2 (488 nm) 2%. Images were acquired using a 40× objective lens. Size bars denote 20 μm. Error bars denote S.E.M. values

8. NGM plates seeded with *Escherichia coli* (OP50 strain): OP50 strain is available at the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, USA; https://www.cbs.umn.edu/research/resources/cgc). Use a single colony of *E. coli* (OP50) and inoculate a 25 ml culture using Luria Bertani (LB) liquid medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl and distilled water up to 1 l and sterilize by autoclaving). Allow inoculated culture to grow for 8–10 h at 37 °C with shaking. Seed NGM plates with 150 μl *E. coli* (OP50) solution and incubate the plates at room temperature overnight to allow the growth of the bacterial lawn. Store the *E. coli* (OP50) solution at 4 °C. Prepare freshly new *E. coli* (OP50) solution every 4 days.
9. M9 buffer: Dissolve 3 g KH_2PO_4 (Merck cat. no. 1.04873.1000), 6 g Na_2HPO_4 (Merck cat. no. 1.06586.0500), 5 g NaCl (Merck cat. no. 1.06404.1000) in 1 l distilled water. Autoclave and add 1 ml 1 M $MgSO_4$ (sterile; Sigma cat. no. M-7506). Store M9 buffer at 4 °C.
10. Paraquat: Dissolve 1 g paraquat (Sigma-Aldrich, St. Louis, USA cat. no. 856,177) in 8 ml distilled water. This is a 0.5 M solution. Prepare aliquots of 400 μl to avoid contamination and store them at 4 °C (*see* **Note 3**).
11. Dimethyl sulfoxide cell culture grade BC (DMSO; Applichem, USA cat. no. A3672,0250; *see* **Note 4**).
12. CCCP: Dissolve 100 mg Carbonyl cyanide m-chlorophenylhydrazone (Sigma-Aldrich, St. Louis, USA cat. no. 857,815) in 10 ml of DMSO to make a 49 mM stock solution. Prepare aliquots of 1 ml and store them at −20 °C (*see* **Note 3**).
13. Levamisole: To make a 0.5 M solution, dissolve 1.2 g levamisole (Sigma-Aldrich, St. Louis, USA cat. no. 196,142) in 10 ml distilled water. Store levamisole solution at 4 °C.
14. Incubators for stable temperature (20 and 37 °C).
15. Agarose pads: Prepare 2% agarose in distilled water and keep it warm on a heating plate. Add 2–3 drops of boiled 2% agarose on a blank slide glass and cover it quickly with a second blank slide glass at 90° to the first one. Wait for 1–3 min until the agarose pads dry and remove the second blank slide (*see* **Note 5**).
16. Nematode strain expressing mtRosella biosensor; IR1631: N2; *Ex003* [$p_{myo\text{-}3}$TOMM-20::Rosella] (*see* **Notes 6** and 7).
17. Nematode strain expressing mtGFP together with the autophagosomal marker LGG-1 fused with DsRed; R1284: N2; *Is* [p*myo-3*mtGFP]; *Ex011* [p*lgg-1*DsRed::LGG-1] (*see* **Notes 6** and 7).
18. Dissecting stereomicroscope.
19. UV irradiation chamber (356 nm).

20. Epifluorescence microscope.
21. Confocal microscope.
22. ImageJ image processing software: freely available at: http://rsb.info.nih.gov/ij/ [17].
23. Microsoft Office 2011 Excel (Microsoft Corporation, Redmond, USA).
24. GraphPad Prism software package (GraphPad Software Inc., San Diego, USA).

3 Methods

3.1 Growth and Synchronization of Nematodes Population

Use both nematode strains expressing either mtRosella (IR1631) or mtGFP together with autophagosomal marker LGG-1 fused with DsRed (IR1284) to monitor mitophagy.

1. Use a dissecting stereomicroscope to select L4 larvae. Then, add 8–10 L4 larvae of transgenic animals on a freshly *E. coli* (OP50) seeded NGM plate. Use at least ten plates containing transgenic worms.
2. Incubate the worms at the standard temperature of 20 °C.
3. Four days later the plates contain mixed nematode population.
4. Synchronize worm population by picking L4 larvae of transgenic animals under a dissecting stereomicroscope. Select with a wormpick and transfer them onto separate plates to start the experiments (*see* **Note 1a**).
5. Add 15–20 L4 larvae of transgenic animals per plate. For each experimental condition, use at least three plates containing transgenic worms.
6. After 2 days, either use 2-days old adult worms directly or transfer them to freshly seeded NGM plates to avoid progeny and prevent starvation due to lack of food (*see* **Note 8**). After 2 days, the latter would represent 4-days old adult worms.

3.2 Induction of Mitophagy

3.2.1 Induction of Mitophagy by Heat Stress

1. Incubate three freshly *E. coli* (OP50) seeded NGM plates for 30 min at 37 °C and keep three plates at 20 °C, as a control.
2. Transfer 10–15 2- or 4-days old adult transgenic animals on pre-warmed plates for each condition (*see* **Note 9**).
3. Incubate the animals for 2 h at 37 °C. Keep the control set at 20 °C.
4. Remove the plates from 37 °C and let worms recover for 30 min at 20 °C (*see* **Note 10**). Censored or dead animals are removed from the study (*see* **Notes 11** and **12**).
5. Animals are ready for microscopic examination.

3.2.2 Induction of Mitophagy by Oxidative Stress and Mitochondrial Stress

1. Kill *E. coli* (OP50) bacteria seeded on six NGM plates by exposing them to UV light (365 nm) for 15 min in an UV irradiation chamber (*see* **Note 13**).
2. Use paraquat to induce oxidative stress. Add paraquat to the top of three seeded NGM plates at 8 mM final concentration in the total agar volume. Add the same volume of water to the three remaining plates that will serve as controls. To specifically induce mitochondrial stress, add CCCP, instead of paraquat, to the top of three seeded NGM plated at 15 μM final concentration in the total agar volume. Use DMSO for control plates, since CCCP is not dissolved in water (*see* **Notes 4** and **14**).
3. Gently swirl the plates and allow the liquid to spread over the entire surface.
4. Let the plates to dry at room temperature.
5. Transfer 10–15 2- or 4-days-old adult transgenic animals on paraquat- or CCCP-containing plates and on control plates using a wormpick (*see* **Note 15**).
6. Incubate the animals at 20 °C.
7. After 2 days of exposure to the drug, worms are ready for microscopic examination. Censored or dead animals are removed from the analysis (*see* **Notes 11** and **12**).

3.3 Sample Preparation for Imaging

1. Collect transgenic animals with an eyebrow/eyelash hair (*see* **Note 1b**).
2. Place and let the animals to crawl into an unseeded NGM plate to remove bacteria for 5 min.
3. Add a droplet of 10 μl 20 mM M9-levamisole buffer on 2% agarose pad.
4. Collect transgenic animals with an eyebrow/eyelash hair and place them in M9-levamisole droplet to immobilize transgenic animals for imaging. Place 15–30 animals per droplet (*see* **Note 16**).
5. Gently place a coverslip on the top to press the animals in agarose.
6. Samples are ready for imaging.

3.4 Imaging, Data, and Statistic Analysis

3.4.1 Imaging Process Using Nematode Strain (IR1631) Expressing mtRosella in Body Wall Muscles

1. Capture images of single transgenic animals expressing mtRosella in body wall muscle cells using a camera attached to epifluorescence microscope. Acquire fluorescent images of whole transgenic animals by using 10× objective lens (*see* **Note 17**).
2. Document and keep the same microscope and camera settings (lens and magnifier used, filters exposure time, resolution, laser intensity, gain, etc.) during imaging process.
3. Collect and save images from each method.

3.4.2 Imaging and Data Analysis Using Nematode Strain (IR1631) Expressing mtRosella in Body Wall Muscles

1. Open and process images with ImageJ software.
2. Select the "split channel" command via the "image" and "color" drop-down menu to convert images to grayscale images with a pixel depth of 8 bit (256 shades of gray).
3. Measure emission intensity. To analyze the area of interest manually (anterior, middle, and posterior body wall muscles), use the "freehand selection" tool to enclose the fluorescent area (*see* **Note 17**).
4. Select the "measurement" command via the "analyze" drop-down menu to perform pixel intensity analysis.
5. Copy the data from "results" window.
6. Paste and import the data by using any software package, such as the Microsoft Office 2011 Excel software package (Microsoft Corporation, Redmond, USA).
7. Normalize pixel intensity values to the selected area.
8. Calculate GFP to DsRed ratio. GFP/DsRed ratio is decreased upon mitophagy stimulation since GFP signal is quenched due to acidic environment of lysosomes, whereas DsRed signal remains stable.

3.4.3 Imaging Process Using Nematode Strain (IR1284) Co-expressing Mitochondrial and Autophagosomal Marker in Body Wall Muscles

1. Capture single body wall muscle cells of transgenic animals co-expressing mtGFP together with autophagosomal marker LGG-1 fused with DsRed using a confocal microscope.
2. Perform z-stack method, of an entire single body wall muscle cell by using 40× or 63× objective lens (*see* **Note 17**).
3. Document and keep the same microscope and camera settings (lens and magnifier used, filters exposure time, resolution, laser intensity, gain, etc.) during imaging process.
4. Collect and save images from each method.

3.4.4 Imaging and Data Analysis Using Nematode Strain (IR1284) Co-expressing Mitochondrial and Autophagosomal Marker in Body Wall Muscles

1. Open and process images acquired in Subheading 3.4.3 with the confocal software.
2. Analyze the number of mitoautophagosomes, autophagosomes containing dysfunctional or superfluous mitochondria, by manually counting the mitophagy/co-localization events between mitochondrial (mtGFP) and autophagosomal marker (DsRed::LGG-1) in each stack of body wall muscle cell (*see* **Note 18**).
3. Document and import the data by using the Microsoft Office 2011 Excel (Microsoft Corporation, Redmond, USA).

3.4.5 Statistic Analysis

1. Use any statistic analysis software, such as GraphPad Prism software package (GraphPad Software Inc., San Diego, USA), to report the significance of the study.
2. Open the desired statistic analysis software.

3. Create a new "table and graph."
4. Select a graph (e.g., Scatter plot, column graph bar, etc.).
5. Import and analyze data obtained in Subheading 3.4.2 or 3.4.4.
6. Increase sample size to obtain more accurate results. For each experiment, at least 50–60 transgenic animals or 50 body wall muscle cells should be examined for each strain and condition. Each assay should be repeated at least three times.
7. Use the Student's t test with a significance cut-off level of $P < 0.05$ for comparisons between two groups. Use the one-factor (ANOVA) variance analysis and correct by the post hoc Bonferroni test for multiple comparisons.

   Examples of such experiments are given in Figs. 1 and 2.

4 Notes

1. (**a**) Cut 2–3 cm of platinum wire (90% platinum, 10% iridium wire, 0.010 in. diameter, e.g., Tritech Research, Los Angeles, CA). Break off the thin part of a glass Pasteur pipette and melt the glass at the site of breakage on a Bunsen burner. Attach the end of the platinum wire. Make flat one end using pincers or a light hammer. Before using the wormpick always sterilize the tip over a flame.
   (**b**) Take a toothpick and glue an eyebrow/eyelash hair to the tip of it. Let it dry at room temperature. Then, use this tool to pick and place transgenic animals on agarose pads for imaging. Before using the eyebrow/eyelash hair always sterilize it by using 70% of EtOH.
2. 1 l of NGM agar medium is sufficient for 125 plates (60 mm × 15 mm). At least 30 NGM plates are needed for the described method.
3. Paraquat and CCCP are photosensitive chemicals. Cover the stock solutions, the aliquots, and plates with foil or keep them in a dark place.
4. The universal solvent DMSO could influence animal's physiology. Use less than 1% of DMSO to avoid possible side effects and/or toxicity [18, 19].
5. Agarose pads have to be freshly made every time.
6. For basic *C. elegans* culture, maintenance, and manipulation techniques, see WormBook, http://www.wormbook.org/). Follow standard procedures for *C. elegans* strain maintenance. Nematode rearing temperature was kept at 20 °C, unless noted otherwise.
7. The nematode strains are available upon request to Professor Tavernarakis N (tavernarakis@imbb.forth.gr).

8. It is reported that autophagy and mitophagy are stimulated upon caloric restriction and starvation [4, 20]. Therefore, well-fed and non-starved animals should be used.
9. It is well known that autophagy efficiency diminished over ageing [21]. Therefore, young adult individuals should be used to monitor mitophagy.
10. The appropriate duration of heat stress and the recovery time should be experimentally determined each time, especially when animals of different genetic backgrounds that are likely to be sensitive to high temperatures are used.
11. Animals are characterized as censored when they display defects that interfere with normal physiology or have been compromised by experimental mishandling. Censored and dead animals are excluded from analysis. To avoid increased censoring and lethality due to excessive internal egg hatching (bag-of-worms phenotype or worm bagging), under mitophagy inducing conditions:
    (a) Incubate specimens for shorter period in the presence of each drug or at 37 °C.
    (b) Decrease the concentration of paraquat or CCCP.
    (c) Use NGM plates containing fluorodeoxyuridine (FUdR), an inhibitor of DNA synthesis that blocks egg hatching.
    (d) Use older adult hermaphrodites (e.g., 4-day-old worms) that display reduced egg production.
12. Contaminations may appear during the experimental procedure. Contamination of NGM plates with bacteria not indented for feeding or with fungi may have a detrimental impact on animal survival and mitophagy stimulation. Contaminated plates and animals should be removed from the study.
13. Drug efficiency could be affected by bacterial metabolism and therefore bacteria are killed by UV irradiation in this step.
14. Use drug-free plates containing identical solutions of distilled water or DMSO for control conditions.
15. The appropriate developmental stage, age, drug concentration, and duration of oxidative and mitochondrial stress should be experimentally determine each time, when animals of different genetic backgrounds that might be sensitive to stress are used. L1–L4 larvae are hypersensitive to paraquat and CCCP leading to severe lethality.
16. M9-levamisole buffer (final concentration 20 mM). Use M9 buffer instead of water to ensure a favorable osmotic environment. Protect the animals from drying out during the imaging process.
17. In *C. elegans*, intestinal autofluorescence increases with age. Therefore, body wall muscle cells close to the intestine should be

avoided during the imaging process. Focus on body wall muscle cells in the pharynx to avoid intestinal autofluorescence.

18. Mitophagy/co-localization event is defined by the overlap between GFP and DsRed signals, which correspond to mitochondria (mtGFP) and autophagosomes (DsRed::LGG-1) respectively.

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