Chapter 43

Mitophagy Dynamics in *Caenorhabditis elegans*

Konstantinos Palikaras, Eirini Lionaki, and Nektarios Tavernarakis

Abstract

Mitochondrial selective autophagy (mitophagy) is a critical cellular process for mitochondrial homeostasis and survival both under basal and stress conditions. Distinct cell types display different requirements for mitochondrial turnover depending on their metabolic status, differentiation state, and environmental cues. This points to the necessity of developing novel tools for real-time, tissue-specific assessment of mitophagy. *Caenorhabditis elegans* is an invaluable model organism for this kind of analysis providing a platform for simultaneous monitoring of mitophagy in vivo in different tissues and cell types, during development, stress conditions, and/or throughout life span. In this chapter we describe three versatile, noninvasive methods, developed for monitoring in vivo early and late mitophagic events in body wall muscles and neuronal cells of *C. elegans*. These procedures can be readily used and/or provide insights into the generation of novel imaging methods to investigate further the role of mitophagy at the organismal level under normal and pathological conditions.

Key words Aging, Autophagosome, Autophagy, *Caenorhabditis elegans*, DsRed, Green fluorescent protein (GFP), Lysosomes, Fluorescent microscopy, Mitochondria, Mitophagy, mtRosella

1 Introduction

Mitochondria represent central hubs of cellular metabolism and play pivotal roles in a variety of cellular processes, including ATP generation, iron metabolism, production of several metabolites and cofactors, Ca^{+2} buffering and signaling, among others. Their importance for cellular physiology predicts the adverse effects of mitochondrial perturbations for cellular and organismal homeostasis. Damaged mitochondria can negatively impact cellular survival through the generation of toxic levels of reactive oxygen species and pro-apoptotic factors. Mitochondrial dysfunction has been associated with multiple pathologies in humans including cancer, type II diabetes, cardiovascular disorders, myopathies, and neurodegenerative diseases [1, 2].

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Given their important role for cellular homeostasis, mitochondrial welfare is monitored and controlled at several levels. Mitochondria fuse to dilute toxic agents and refresh their components. An arsenal of local chaperones and proteases, whose coordinated expression consist the mitochondrial unfolded protein response, tackles mild proteotoxic stress. When mitochondrial damage exceeds the threshold of protein quality control mechanisms, dysfunctional organelles are removed by mitophagy, a selective type of autophagy [3]. Mitochondrial fission primes mitochondria for autophagic degradation, while the fission/fusion machinery is closely implicated in mitophagy initiation [4, 5]. As distinct cell types experience different metabolic needs, the mitochondrial network varies significantly between cell types or among different developmental states of a cell lineage [6]. Therefore, mitochondrial quality control mechanisms may have different impacts depending on the variable cellular context.

Mitophagy has been at the spotlight of research for more than a decade. Mitochondrial selective autophagy is also induced under nonpathogenic conditions, when the cell needs to adapt its metabolic status to environmental changes, such as hypoxia [7], or to developmental changes, like in the maturing reticulocytes, the precursors of red blood cells [8, 9]. These findings highlight the importance of assessing mitophagy in specific cell types, developmental stages, and/or under defined stress conditions.

Methods to monitor mitophagy include applications of biochemical and imaging techniques on distinct model organisms, in vitro and in vivo. Biochemically, mitophagy can be assessed by the lysosomal-dependent loss of mitochondrial proteins or mitochondrial-associated enzymatic functions (citrate synthase activity). These approaches cannot distinguish mitophagic activity between different cell types within tissues and usually cannot assess subtle differences in the rate of mitophagy. Moreover, electron microscopy has yielded images of mitochondria surrounded by autophagosomal/lysosomal membranes. Its limitations with regard to sample preparation and quantification of the results have restricted the broad use of this method. Fluorescence microscopy has gained increasing attention when it comes to monitoring of dynamic processes. Mitophagy is monitored by colocalization of mitochondria-targeted probes with autophagosomal/lysosomal markers or by mitochondria-targeted ratiometric and dual pH-sensitive probes [10–12]. The latter have revolutionized our understanding of mitophagy process in vivo.

C. elegans is a genetically modifiable small nematode with a transparent body, which comprises different tissues and organs, with high functional similarities to the mammalian counterparts. These nematodes can be monitored alive under the fluorescent

microscope for real-time in vivo assessment of dynamic cellular processes, without dissection or other intrusive methods prior to microscopy. Therefore, although amenable to all the aforementioned approaches, *C. elegans* was used for the establishment of tools for monitoring in vivo real-time mitophagy, both systemically and in a tissue-specific manner. In this chapter, we describe the development of complementary systems for monitoring mitophagy in vivo, which are used to uncover several conditions that either promotes or inhibits mitophagy in *C. elegans* [11, 13]. In the first approach, we generated transgenic animals expressing the pH-sensitive *Rosella* biosensor in mitochondria of either body wall muscles or neurons. mtRosella is a mitochondria-targeted dual fluorescent probe, which comprises a pH-sensitive GFP and a pH-insensitive DsRed moiety. Thus, mitophagy is signified by a reduction in the ratio of GFP/DsRed fluorescent signal. We examined transgenic animals expressing mtRosella either in body wall muscle cells or in neurons under normal and mitophagy-inducing conditions, such as mitochondrial and oxidative stress. Mitochondrial stress, induced by RNAi against *isp-1* and *frh-1* genes, results in decreased GFP/DsRed ratio of mtRosella, highlighting mitophagy stimulation (Fig. 1a). Furthermore, oxidative stress triggers elimination of impaired mitochondria in both soma and axons of neurons (Fig. 2). Neuronal mitophagy is of particular importance as neurons rely heavily on their mitochondrial network, and even mild changes may affect their functionality and survival [14]. In addition to mtRosella, we developed a method for monitoring earlier mitophagic events by generating transgenic animals that express the mitophagy receptor protein DCT-1, the homologue of the mammalian BNIP3/NIX, fused with GFP together with the autophagosomal marker LGG-1, the homologue of the mammalian cytosolic microtubule-associated protein 1 light chain 3 (MAP 1LC3/LC3), fused to DsRed in body wall muscle cells [11, 13, 15, 16]. We examined animals carrying both the mitophagy receptor and autophagosomal markers, under normal and mitophagy-inducing conditions. Mitophagy stimulation induces the formation of autophagosomes that extensively colocalize with DCT-1 (Fig. 2).

The investigation of the molecular mechanisms and physiological role of mitochondrial elimination in cellular and organismal homeostasis demands the development of reliable, non-invasive, quantitative methods for mitophagy assessment in vivo. In the following sections, we describe detailed protocols for in vivo imaging of mitochondrial selective autophagy in *C. elegans*, using three versatile imaging tools.

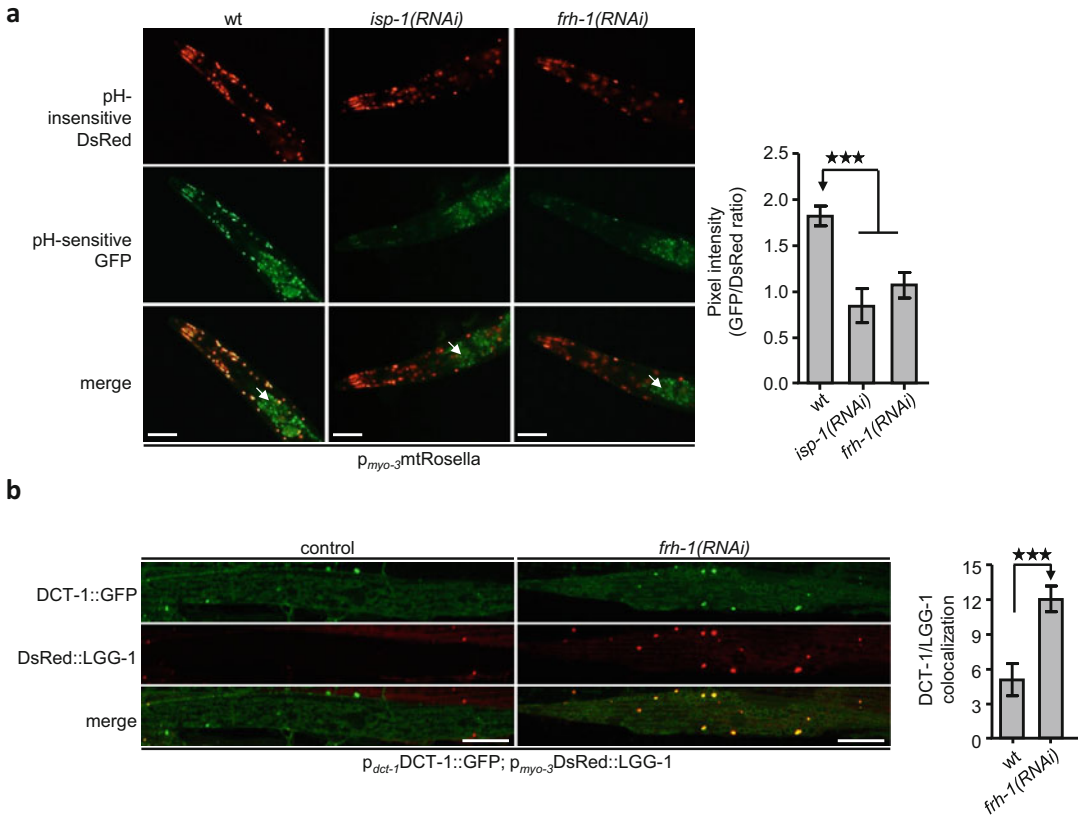

Fig. 1 Mitophagy activation in response to mitochondrial dysfunction. (**a**) Transgenic animals expressing mtRosella in body wall muscles were subjected to *isp-1(RNAi)* and *frh-1(RNAi)*. The decreased ratio between pH-sensitive GFP to pH-insensitive DsRed signifies mitophagy stimulation ($n = 40$, $^{***}P < 0.001$; one-way ANOVA). Arrows point out intestinal autofluorescence. Acquisition information: exposure time, 200 ms; contrast, medium. Images were acquired using 10× objective lens. (**b**) Transgenic nematodes expressing the mitophagy receptor DCT-1 fused with GFP together with the autophagosomal marker LGG-1 fused with DsRed were subjected to RNAi against *frh-1* gene. Mitophagy stimulation is indicated by the increased colocalization events between DCT-1::GFP and DsRed::LGG-1 ($n = 50$, $^{***}P < 0.001$; unpaired *t*-test). Acquisition information: resolution, 1024 × 1024; master gain, Track1, 562, and Track2, 804; emission filters, Track1 Channel1, 575–703, and Track2 Channel2, 493–545; laser intensity, Track1 (543 nm), 12.9%, and Track2 (488 nm), 25%. Images were acquired using 63× objective lens. Scale bars, 20 μm. Error bars, SEM values

2 Materials

2.1 Reagents

1. Wormpick[a] or eyebrow/eyelash hair[b] (*see* **Note 1**).
2. Cholesterol stock solution, 5 mg/mL: dissolve cholesterol in absolute ethanol by stirring. Store at 4 °C. Do not flame or autoclave.
3. Nystatin stock solution, 10 mg/mL: dissolve nystatin in 70% (V/V) ethanol. Store at 4 °C and shake prior to use as this is a suspension. Do not autoclave.

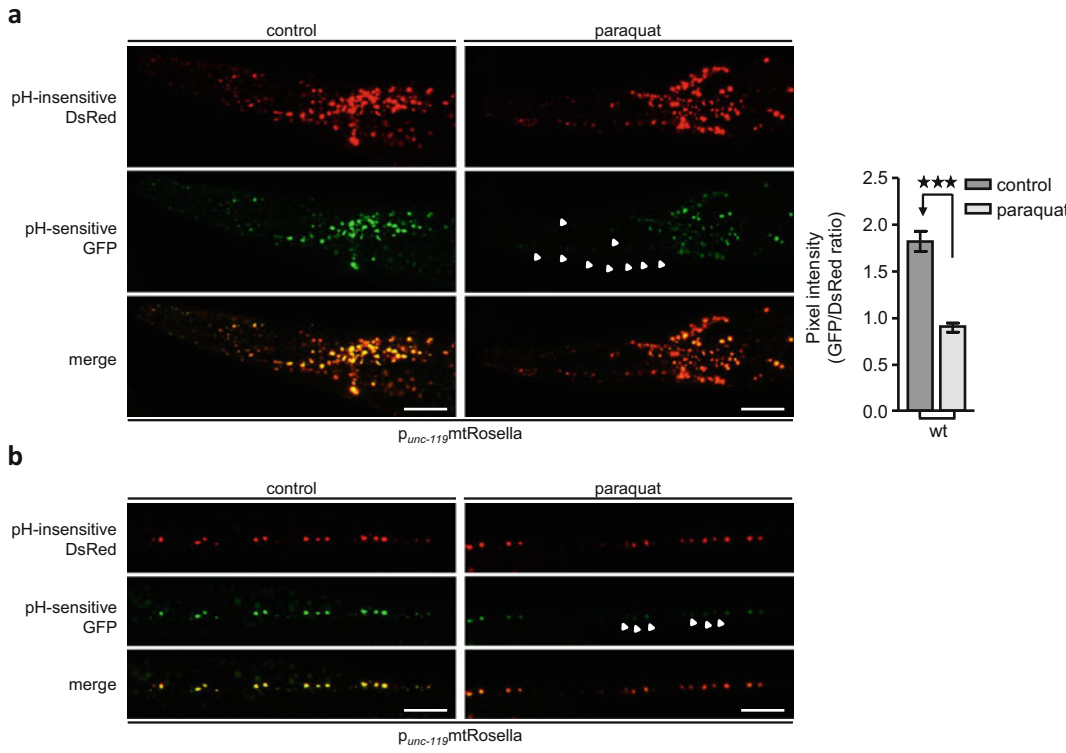

Fig. 2 Assessment of mitophagy in *C. elegans* neurons. Transgenic nematodes expressing mtRosella in neuronal cells were exposed to paraquat. (**a**) Induction of neuronal mitophagy is underlined by the reduced ratio between pH-sensitive GFP to pH-insensitive DsRed ($n = 30$, $^{***}P < 0.001$; unpaired *t*-test). Error bars, SEM values. (**b**) Local elimination of impaired mitochondria in neuronal axons. Acquisition information: resolution, 1024 × 1024; master gain, Track1, 658, and Track2, 714; emission filters, Track1 Channel1, 548–703, and Track2 Channel2, 493–550; laser intensity, Track1 (543 nm), 7.9%, and Track2 (488 nm), 6.2%. Images were acquired using 40× objective lens. Scale bars, 20 μm

4. 1 M phosphate buffer, pH 6: 102.2 g KH_2PO_4, 57.06 g K_2HPO_4 in 1 L distilled water. Autoclave and keep at room temperature.
5. 1 M $MgSO_4$.
6. 1 M $CaCl_2$.
7. Levamisole.
8. Petri dishes (60 mm × 15 mm).
9. LB medium.
10. LB plates: 100 μg/mL ampicillin, 10 μg/mL tetracycline.
11. Nematode growth medium (NGM) agar plates: 3 g NaCl, 2.5 g Bacto Peptone, 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, 1 mL 1 M $CaCl_2$, 1 mL 1 M $MgSO_4$, 1 mL nystatin stock solution, 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 allow for solidification. Keep the plates at 4 °C until used.

12. RNAi agar plates: 3 g NaCl, 2.5 g Bacto Peptone, 17 g agar, and add 900 mL distilled water. Autoclave. Let cool to 55–60 °C. Add 1 mL cholesterol stock solution, 1 mL 1 M $CaCl_2$, 1 mL 1 M $MgSO_4$, 1 mL nystatin stock solution, 25 mL 1 M phosphate buffer, pH 6.0, 100 μg/mL ampicillin, 4 mL 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG), 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**).
13. NGM plates seeded with *Escherichia coli* (OP50 strain): 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) culture every 4 days.
14. Ampicillin.
15. Tetracycline.
16. M9 buffer: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, in 1 L distilled water. Autoclave and add 1 mL 1 M $MgSO_4$. Store at 4 °C.
17. 0.5 M paraquat: 1 g paraquat in 8 mL distilled water. Prepare aliquots of 400 μL to avoid contamination, and store them at 4 °C (*see* **Note 3**).
18. 0.5 M levamisole: 1.2 g levamisole in 10 mL distilled water. Store levamisole solution at 4 °C (*see* **Note 4**).
19. 2% agarose pads: 0.5 g agarose into a glass beaker. Add 25 mL M9 buffer. Heat the mixture in a microwave until the agarose is completely dissolved. Stir the mixture periodically, and keep it warm on a heating plate. Add 2–3 drops of 2% agarose on a glass slide, and cover it quickly with a second glass slide, so as to form a thin agarose film (pad). Wait for 1 min until the agarose pad solidifies, and remove the top slide (*see* **Note 5**).
20. Nematode strain expressing mtRosella biosensor in body wall muscle cells and neurons; IR1631: N2;*Ex003* [$p_{myo\text{-}3}$TOMM-20::Rosella; *rol-6(su1006)*] and IR1864: N2;*Ex001* [$p_{unc\text{-}119}$TOMM-20::Rosella; *rol-6(su1006)*] (*see* **Notes 6–8**).
21. Nematode strain co-expressing the mitophagy receptor DCT-1 fused with GFP together with the autophagosomal marker

LGG-1 fused with DsRed; R1511: N2; *Ex*[p_{dct-1}DCT-1::GFP; *rol-6(su1006)*]; *Ex011*[p_{myo-3}DsRed::LGG-1; p_{myo-2}GFP] (*see* **Notes 6**, **7**, **9**).

2.2 Equipment

1. Incubators for stable temperature (20 and 37 °C).
2. Dissecting stereomicroscope.
3. UV-dissecting stereomicroscope.
4. UV cross-linker.
5. Microwave.
6. Zeiss AxioImager Z2 epifluorescence microscope (Zeiss, model: Zeiss AxioImager Z2).
7. Zeiss AxioObserver Z1 confocal microscope (Zeiss, model: Zeiss AxioObserver Z1).

2.3 Software

1. ImageJ image processing software: freely available at http://rsb.info.nih.gov/ij/-[17].
2. Zeiss ZEN 2012 software.
3. Microsoft Office 2011 Excel (Microsoft Corporation, Redmond, USA).
4. GraphPad Prism software package (GraphPad Software Inc., San Diego, USA).

3 Methods

3.1 Preparation of RNAi Agar Plates Seeded with dsRNA-Expressing Bacteria

1. Streak *E. coli* (HT115) bacteria expressing dsRNA *isp-1* and *frh-1* genes or bearing an empty vector (EV), onto LB agar plates containing 100 μg/mL ampicillin, 10 μg/mL tetracycline.
2. Incubate the plates at 37 °C overnight.
3. Use a sterilized toothpick to isolate few bacterial colonies of each condition (EV, *isp-1* and *frh-1*) and place them in separate bacteriological culture tubes containing 5 mL LB medium, 5 μL of 100 mg/mL ampicillin, and 5 μL of 10 mg/mL tetracycline.
4. Incubate cultures at 37 °C overnight.
5. Prepare three different tubes, and add 5 mL LB medium and 5 μL of 100 mg/mL ampicillin.
6. Add 300 μL (50 μL per 1 mL LB/amp) of each overnight culture (EV, *frh-1* and *isp-1*) into separate bacteriological culture tubes containing 5 mL LB medium, 5 μL of 100 mg/mL ampicillin, and 5 μL of 10 mg/mL tetracycline.

7. Incubate cultures at 37 °C until OD_{600} is between 0.5 and 0.8 (approximately 4 h).
8. Place 200 μL of each culture (EV, *frh-1* and *isp-1*) described in Subheading 3.1, **step 7**, on RNAi agar plates.
9. Let the plates dry at room temperature overnight.

3.2 Maintenance, Expansion, and Synchronization of Transgenic Nematodes Population

Use the nematode strains expressing mtRosella, in body wall muscles (IR1631) and neurons (IR1864), and DCT-1::GFP together with autophagosomal marker LGG-1 fused with DsRed (IR1511) to monitor mitophagy.

1. Use a regular or a UV-dissecting stereomicroscope to select transgenic L4 larvae based on the selection marker (*see* **Notes 8** and **9**).
2. Use a wormpick to select and transfer 10 L4 larvae of transgenic nematodes on a freshly *E. coli* (OP50)-seeded NGM agar plate. Use at least five plates for each strain to expand transgenic population (*see* **Note 1**).
3. Incubate the nematodes at 20 °C.
4. Four days later, the plates (described in Subheading 3.2, **step 2**) contain mixed larval populations.
5. Synchronize worm population by selecting transgenic L4 larvae from the NGM agar plates to start the experiments.

3.3 Mitophagy-Inducing Conditions

3.3.1 Mitophagy Stimulation by RNAi Against isp-1 and frh-1 Genes

1. Pick and transfer 15–20 L4 larvae of transgenic animals expressing either mtRosella in body wall muscles (IR1631) or DCT-1::GFP together with DsRed::LGG-1 (IR1511) onto separate RNAi agar plates seeded with dsRNA-expressing bacteria. For each experimental condition, use at least three plates containing transgenic nematodes.
2. Keep and grow the animals at 20 °C.
3. After 2 days, either use 2-day-old adult worms directly or transfer them to freshly seeded RNAi plates to avoid progeny and starvation (*see* **Note 10**). After 2 days, the latter would represent 4-day-old adult worms.
4. Nematodes are ready for microscopic examination. Censored or dead animals are eliminated from the imaging process (*see* **Notes 11** and **12**).

3.3.2 Mitophagy Stimulation in Response to Oxidative Stress

1. Place six NGM *E. coli* (OP50) bacteria-seeded plates in a UV irradiation chamber.
2. Irradiate with UV light (254 nm) the NGM bacteria-seeded plates for 15 min (*see* **Note 13**).
3. Use paraquat as a chemical inducer of oxidative stress. Add 100 μL 0.5 M paraquat on top of OP50-seeded NGM plates

(8 mM final concentration/total agar volume). Add equivalent volume of water, which is the solvent of paraquat, to the three remaining plates that will serve as control.

4. Spread the drug over the entire surface by swirling the plates.
5. Cover the plates with foil or place them in a dark space and let them dry at room temperature.
6. Select and transfer 10–15 2- or 4-day-old adult transgenic animals expressing mtRosella in neuronal cells (IR1864) on plates containing paraquat or control plates using a wormpick (*see* **Note 14**).
7. Incubate the animals at 20 °C for 2 days.
8. Nematodes are ready for microscopic examination. Censored or dead animals are eliminated from imaging process (*see* **Notes 11** and **12**).

3.4 Prepare and Mount the Samples for Imaging

1. Prepare 2% agarose pads by adding 25 mL M9 buffer to 0.5 g agarose into a glass beaker. Heat the mixture in a microwave until the agarose is completely dissolved. Stir the mixture periodically, and keep it warm on a heating plate. Add 2–3 drops of 2% agarose on a glass slide, and cover it quickly with a second glass slide, so as to form a thin agarose film (pad). Wait for 1 min until the agarose pad solidifies and remove the top slide. Only use freshly prepared 2% agarose pads (*see* **Note 5**).
2. Add 10 μL of 20 mM M9-levamisole buffer on the agarose film (*see* **Note 15**).
3. Use an eyebrow/eyelash hair to transfer the transgenic nematodes in M9-levamisole drop (*see* **Note 1)**. Place 15–30 worms per drop.
4. Gently place a coverslip on top of nematodes.
5. Seal the agarose pads with nail polish to maintain humidity during imaging.
6. Proceed to microscopic examination of the samples.

3.5 Acquisition Process

3.5.1 Acquisition Process Using Nematode Strains (IR1631 and IR1864) Expressing mtRosella in Body Wall Muscle and Neuronal Cells

1. Detect single transgenic animals expressing mtRosella either in body wall muscles or neurons using an epifluorescence microscope.
2. Use 10x objective lens and capture images of entire transgenic nematodes by using a microscope-attached camera (*see* **Note 16**).
3. Use the same imaging settings (lens and magnifier used, filters exposure time, resolution, laser intensity, gain, etc.) throughout the imaging process.
4. Save and collect the acquired images.

3.5.2 Imaging and Data Analysis Using Nematode Strain (IR1631) Expressing mtRosella in Body Wall Muscle and Neuronal Cells

1. Download and install ImageJ software (https://imagej.nih.gov/ij/).
2. Open images obtained in Subheading 3.5.1, **step 4** with ImageJ program.
3. Convert images to grayscale images with a pixel depth of 8 bit by selecting the "split channel" command via the "image" and "color" drop-down menu.
4. Use the "freehand selection" too to manually set the fluorescent area of interest.
5. Measure emission intensity by selecting the "measurement" command via the "analyze" drop-down menu to perform pixel intensity analysis.
6. Copy the displayed data from the separate "results" window.
7. Paste and import the data by using any software package, such as the Microsoft Office 2011 Excel software package (Microsoft Corporation, Redmond, USA).
8. Normalize pixel intensity values to the selected area.
9. Measure GFP to DsRed ratio. The levels of GFP/DsRed ratio underline mitophagy upregulation since GFP fluorescent signal is quenched upon the fusion with the acidic environment of lysosomes, whereas DsRed fluorescent signal remains stable.

3.5.3 Acquisition Process Using Nematode Strain (IR1511) Co-expressing Mitophagy Receptor and Autophagosomal Marker in Body Wall Muscle Cells

1. Detect single body wall muscle cells of transgenic animals co-expressing the mitophagy receptor DCT-1::GFP together with autophagosomal marker DsRed::LGG-1 using a confocal microscope.
2. Use 63× objective lens.
3. Image an entire single body wall muscle cell by performing z-stack scanning method (*see* **Note 16**). Keep the same imaging and acquisition settings (lens and magnifier used, filters exposure time, resolution, laser intensity, gain, etc.) during imaging process.
4. Save and collect the acquired images.

3.5.4 Data Analysis Using Nematode Strain (IR1511) Co-expressing Mitophagy Receptor and Autophagosomal Marker in Body Wall Muscle Cells

1. Open and process images acquired in Subheading 3.5.3, **step 4** with any confocal software.
2. Analyze mitophagy levels by manually counting the colocalization events between mitophagy receptor (DCT-1::GFP) and autophagosomal marker (DsRed::LGG-1) in each stack of body wall muscle cell (*see* **Note 17**).
3. Document the obtained data by using the Microsoft Office 2011 Excel (Microsoft Corporation, Redmond, USA).

3.5.5 Statistical Analysis

Report the significance of each experiment by using any statistical analysis software.

1. Open the desired statistical analysis software.
2. Create a new "table and graph."
3. Select a specific type of graph to display the data (e.g., scatterplot, column graph bar, etc.) and import data.
4. Decrease the variability of mitophagy levels between animals and cells by increasing the sample size. For each experimental procedure, examine at least 30 transgenic animals or 40 body wall muscle cells for each strain and treatment. Each assay should be repeated at least three (3) times.
5. Suggested statistical analysis tests: (a) Student's t-test with a significance cutoff level of $p < 0.05$ for comparisons between two groups and (b) one-factor (ANOVA) variance analysis corrected 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. Flatten the wire tip using pincers or a light hammer. Before using the wormpick, always sterilize the tip over flame. (b) Take a toothpick, and glue an eyebrow/eyelash hair to the tip of it. Let it dry at room temperature. Before using the eyebrow/eyelash hair, always sterilize it in 70% of EtOH.
2. Prepare fresh IPTG-containing RNAi plates every 2 weeks. IPTG efficiency diminishes over time.
3. Paraquat is a photosensitive chemical. Protect stock solutions, aliquots, and plates from light by enwrapping them with foil or placing them in a dark space.
4. Prepare fresh levamisole stock solution every 6 months.
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 by Professor Tavernarakis N. (tavernarakis@imbb.forth.gr).

8. Maintain the following transgenic nematode strains by selecting rollers under a regular dissecting stereomicroscope, IR1631: N2;*Ex003* [p_{myo-3}TOMM-20::Rosella; *rol-6 (su1006)*] and IR1864: N2;*Ex001* [$p_{unc-119}$TOMM-20::Rosella; *rol-6(su1006)*].
9. Maintain the following transgenic nematode strain by selecting rollers with GFP-positive pharynx under a UV-dissecting stereomicroscope, R1511: N2; *Ex*[p_{dct-1}DCT-1::GFP; *rol-6 (su1006)*]; *Ex011*[p_{myo-3}DsRed::LGG-1; p_{myo-2}GFP].
10. Caloric restriction and starvation promotes autophagy and mitophagy elevation [11]. Thus, well-fed and non-starved nematodes should be assessed for mitophagy induction under desired conditions.
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.
    (b) Decrease the concentration of paraquat.
    (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. Bacteria may metabolize chemical compounds diminishing their efficacy. Therefore bacteria should be killed by UV irradiation prior to drug application. *Caution*: UV-killed dsRNA-expressing bacteria will display also decreased gene silencing efficiency.
14. The appropriate developmental stage, age, drug concentration, and duration of oxidative stress should be experimentally determined each time, when animals of different genetic backgrounds, that might be sensitive to stress, are used. L1–L4 larvae are hypersensitive to paraquat leading to severe lethality.

15. M9-levamisole buffer (final levamisole 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.
16. In *C. elegans*, intestinal autofluorescence increases with age. Therefore, body wall muscles or neurons close to the intestine should be avoided during the imaging process. Focus on body wall muscles and neurons in the pharyngeal area to avoid intestine-derived autofluorescence.
17. Mitophagy events are defined by the colocalization of GFP and DsRed signals, which correspond to the mitophagy receptor (DCT-1::GFP) and autophagosomes (DsRed::LGG-1), respectively.

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