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Monitoring Mitophagy During Aging in *Caenorhabditis elegans*

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Abstract

Mitochondria constitute the main energy-producing centers of eukaryotic cells. In addition, they are involved in several crucial cellular processes, such as lipid metabolism, calcium buffering, and apoptosis. As such, their malfunction can be detrimental for proper cellular physiology and homeostasis. Mitophagy is a mechanism that protects and maintains cellular function by sequestering harmful or dysfunctional mitochondria to lysosomes for degradation. In this report, we present experimental procedures for quantitative, in vivo monitoring of mitophagy events in the nematode *Caenorhabditis elegans*.

Keywords: Aging, Autophagy, *Caenorhabditis elegans*, Mitochondria, Mitophagy, Ratiometric imaging, Rosella biosensor

1 Introduction

The nematode *C. elegans* is widely appreciated as an invaluable model for the study of fundamental cellular processes. Its versatility can be attributed to a unique combination of beneficial properties the organism possesses. First and foremost, its small and transparent body renders it ideal for in vivo, noninvasive, and real-time monitoring through simple optical and fluorescent microscopy. Furthermore, the organism can be manipulated genetically with relative ease, thus facilitating the dissection of molecular pathways through forward and reverse genetics. This endeavor is further supported by the invariant number and lineage of the organism's cells and the complete knowledge of its neuronal connectome. Finally, its short lifespan and its abundant reproduction make it ideal for the study of processes related to aging [1].

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Organelle quality and quantity can be crucial for proper cellular function. Cells need to tightly regulate the amount of each organelle so that they can meet their current needs without excess, while damaged organelles need to be promptly removed and replaced. Autophagy is a cellular mechanism which contributes greatly to both these goals by deconstructing unwanted organelles. There is evidence for the existence of specific autophagic pathways for most cellular components, including peroxisomes [2, 3], lipid droplets [4], lysosomes [5], the nucleus [6], ER [7], ribosomes [8], and mitochondria.

Mitophagy, the specific macroautophagic mechanism targeting mitochondria, plays a critical role in cellular survival. Respiration causes the formation of reactive oxygen species (ROS) that can damage vital macromolecules and especially the vulnerable mitochondrial DNA. This damage can cause anomalies in mitochondrial function and render the malfunctioning organelles dangerous for the whole cell. Mitophagy removes such aberrant organelles, protecting cells and maintaining normal organismal function and lifespan [9–11]. Impaired mitochondrial function has been implicated in the onset of aging [12] and severe disorders, such as neurodegeneration [13] and cardiomyopathies [14]. Dysfunctions in mitophagy have been also associated with Parkinson's disease [15, 16].

The mechanisms which initiate mitophagy exhibit some variation among organisms, since not all species use the same signaling to recruit mitochondria into autophagosomes [17]. Yeast possesses a unique pathway involving Atg-32 and Atg-11, while other eukaryotes generally use the NIX (NIP3-like protein X), FUNDC1, and PINK1/PARKIN pathways [17, 18]. The Bcl-2 family protein NIX is a receptor that has been associated with mitophagy during cell maturation events [19, 20]. It has also been shown to facilitate regular mitochondrial turnover based on organelle energetic status in collaboration with the small GTPase Rheb [21]. FUNDC1 is an outer mitochondrial membrane protein that is necessary for hypoxia-induced mitophagy in mammalian systems [22]. PINK1 is a kinase that normally enters the mitochondria and is deactivated by PARL (presenilin-associated rhomboid-like protease) but becomes stabilized in the outer mitochondrial membrane of dysfunctional organelles, thus recruiting the ubiquitin ligase Parkin. The combination of ubiquitination and phosphorylation by this newly formed complex leads to the labeling of proteins on the outer mitochondrial membrane and the recruitment of the defunct mitochondria into autophagosomes (Fig. 1a). Regardless of how mitophagy is initiated, all pathways converge into a similar sequence of events. Mitochondria are enclosed by the isolation membrane of the autophagosomes, which binds Atg-8/LC3 (LGG-1 in *C. elegans*). The autophagosomes are eventually sequestered to lysosomes, where mitochondria are degraded [17, 23].

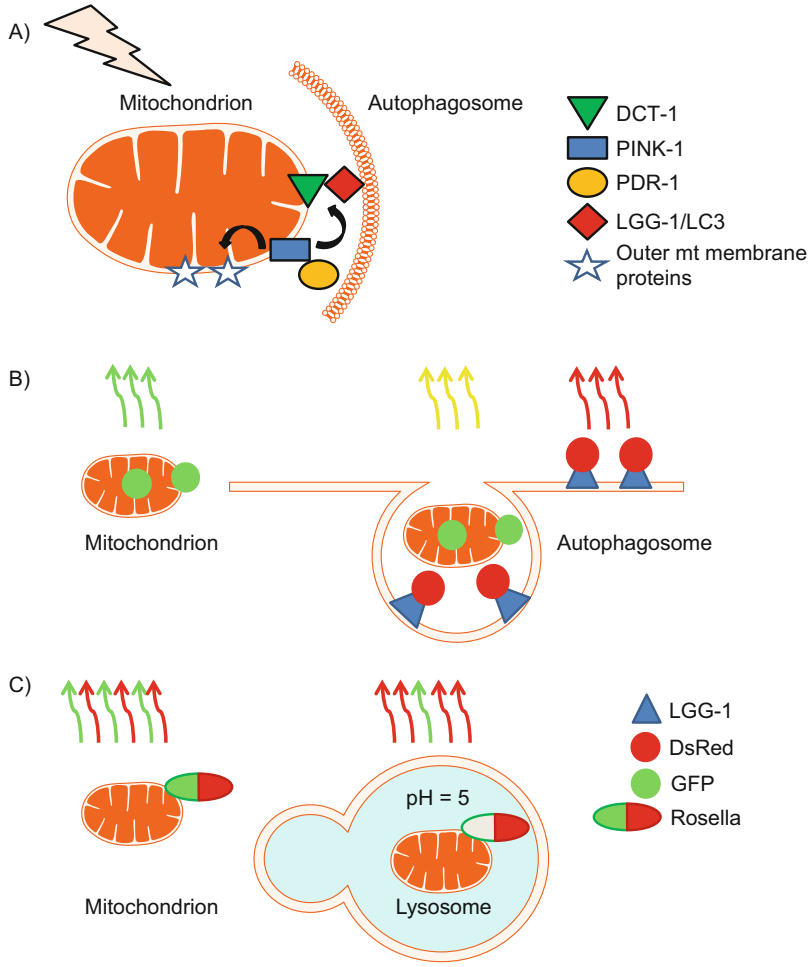

Fig. 1 A *C. elegans* platform for monitoring mitophagy in vivo. (**a**) Mitophagy components in *C. elegans*. When the mitochondrial environment is challenged by exogenous or endogenous insults, mitochondria-specific macroautophagy (mitophagy) is activated to prevent accumulation of dysfunctional organelles. In *C. elegans*, mitophagy is mediated by DCT-1, a receptor which localizes on the outer mitochondrial membrane, as well as the PINK-1 kinase and PDR-1 E3 ubiquitin ligase. Combined ubiquitination and phosphorylation of several outer mitochondrial membrane proteins, among them DCT-1 itself, by the PINK-1/PDR-1 complex signals for the sequestration of mitochondria to autophagosomes and finally to lysosomes for degradation. (**b**) Mitophagy can be monitored by observing the co-localization of GFP-tagged mitochondria with DsRed-tagged autophagosomes. (**c**) Alternatively, mitophagy events can be quantified by observing the reduction of green channel emission of the hybrid fluorescent reporter Rosella upon entry to the acidic lysosomes

Initial evidence for the existence of active mitophagy in nematodes arose from reports supporting that paternal sperm mitochondria are eliminated upon fertilization through macroautophagy, thus preventing aberrant heteroplasmy of the mitochondrial genome [24, 25]. Recently, our lab identified DCT-1 as the putative worm homologue of the mammalian mitophagy receptor NIX/BNIP-3 [26]. DCT-1 acts cooperatively with PINK-1 kinase

and Parkin E3 ubiquitin ligase to orchestrate mitophagy induction under stressful conditions (Fig. 1a). Intriguingly, our work also highlighted that mitophagy accounts for the majority of autophagic influx in response to stimuli which are reported to trigger autophagy. Furthermore, intact mitophagy is indispensable for the extended lifespan of various long-lived mutants, such as animals with reduced insulin/IGFR signaling. Mitophagy is also essential for the extended longevity of worms which exhibit perturbed electron transport chain activity or bear a depletion of frataxin, a mitochondrial protein involved in iron-sulfur cluster biogenesis. Frataxin inhibition causes mitochondrial stress due to iron starvation, an insult which strongly induces mitophagy [27]. Collectively, mitophagy serves as a core cellular mechanism, which preserves homeostasis and determines lifespan in cases where the mitochondrial environment is challenged. In this article, we describe the experimental methodology primarily used in our lab to track mitophagy events in *C. elegans* in vivo.

2 Materials

2.1 C. elegans Manipulation

2.1.1 Equipment

1. Worm pick and eyelash hair: We use a platinum pick for routinely transferring worms in plates and an eyelash hair to transfer them on agarose pads just before observation to avoid stressing the animals.
2. 2% agarose pads: 1 g agarose powder in 50 mL distilled water and heat until it is completely dissolved. While the solution is still hot, leave a droplet (~1 cm in diameter) on top of a glass slide. Put a second slide on top and press until a large gel pad is formed.
3. Dissecting microscope.

2.1.2 Solutions and Chemicals

1. M9 buffer: Dissolve 3 g KH_2PO_4, 6 g Na_2HPO_4, and 5 g NaCl in 1 L distilled water. Autoclave and add 1 mL 1 M $MgSO_4$. M9 buffer can be stored at room temperature or 4 °C.
2. Tetramisole: Tetramisole is used for the anesthetization of the animals. Prepare a 100 mM tetramisole solution by dissolving 1.2 g tetramisole powder in 50 mL M9 solution, and store at 4 °C. We normally dilute this further and prepare a 20 mM working solution for everyday use.

2.2 Nematode Food

1. OP50 bacterial food: Inoculate 50 mL of liquid, sterile LB broth with a single OP50 colony, and let it grow for approximately 8 h at 37 °C with shaking. Plate 180–200 μL of the OP50 culture in the center of freshly prepared NGM plates, and let the bacterial lawn grow overnight (for approximately

16 h). In the next morning, the plates are ready to be used for animal transferring. This procedure requires sterile conditions.

2. Petri dishes containing nematode growth medium (NGM): For preparing 1 L of NGM, dissolve 3 g NaCl, 2.5 g bacto-peptone, 17 g agar, and 0.2 g streptomycin sulfate powders in a flask containing 900 mL distilled H_2O. Stir the mix for 5–10 min and then autoclave. Let the flask cool down to 55 °C, and add 1 mL $CaCl_2$ (stock solution 1 M), 1 mL $MgSO_4$ (stock solution 1 M), 1 mL cholesterol (stock solution 5 mg/mL), and 1 mL nystatin (stock solution 10 mg/mL). Finally, add 25 mL 1 M phosphate KPO_4 buffer (pH = 6.0) and distilled water to the final volume of 1 L. Stir the mix for 5–10 min and dispense the medium into the plates in the desired volume.
3. RNAi Petri dishes: The preparation of those plates is similar to the one described above for NGM plates. However, instead of adding streptomycin sulfate powder before autoclaving, in this case add ampicillin after autoclaving, at a final concentration of 100 μg/mL.
4. Petri dishes containing RNA inhibition medium: To perform gene silencing experiments, streak the desired HT115 bacterial strain containing the RNAi expressing vector of choice (a pL4440 vector backbone containing a fragment of your gene of interest) on LB agar plates with ampicillin at a final concentration of 100 μg/mL and tetracycline at a final concentration of 10 μg/mL. Incubate the plates at 37° for 16–18 h. Inoculate 5 mL of liquid LB with ampicillin and tetracycline (at similar concentrations with those of solid plates) with a single colony from the plate. Incubate at 37° for 16–18 h with shaking. Inoculate the desired amount of LB containing ampicillin with the bacterial culture, at a ratio of 50 μL overnight culture per 1 mL of LB with ampicillin. Incubate for 2–4 h at 37° with shaking. Disperse 200 μL of a mix of the over-day culture plus IPTG at a final concentration of 2 mM at the center of freshly prepared RNAi Petri dishes. Incubate overnight at room temperature to allow bacterial lawn grow. This procedure requires sterile conditions. In the next morning, animals can be transferred on these plates.

2.3 Chemical Compounds

1. Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride): Prepare 0.5 M stock solution, and dilute it in distilled water, to a final concentration of 8 mM per plate. Paraquat stock solution can be stored at 4 °C. *Note*: Paraquat is extremely toxic for human beings; thus, any contact should be avoided!
2. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP): Dissolve 100 mg CCCP in 10 mL dimethyl sulfoxide (DMSO), aliquot, and store at −20 °C. Add to the plates at a final concentration of 10 mM.

2.4 Nematode Strains

1. IR1284: WT;*Is*[p_{myo-3}mtGFP];*Ex*[p_{lgg-1}DsRed::LGG-1].
2. IR1511: WT;*Ex*[p_{myo-3}DsRed::LGG-1;p_{dct-1}DCT-1::GFP].
3. IR1631: WT;*Ex*[p_{myo-3}TOMM-20::Rosella].

3 Methods

3.1 CCCP and Paraquat Treatment

1. Add both paraquat and CCCP on NGM plates, at a final concentration of 8 and 10 mM per plate, respectively, on the top of UV-treated OP50 bacteria (*see* **Notes 1** and **2**). We normally kill bacteria through exposure to UV light for 15 min in a UV cross-linker.
2. Spread the drug-containing solution throughout the entire surface of the plate, and let it dry at room temperature.
3. Place at least 20 nematodes on each of the drug-containing plates as well as on plates with pure solvent (distilled water or DMSO) for negative control (*see* **Note 3**).
4. Monitor induction of mitophagy 2 days later using the methods described below.

3.2 Sample Preparation for Imaging (All Strains)

1. Place a 10–20 μL droplet of 20 mM tetramisole on a 2% agarose pad.
2. Use an eyelash hair to transfer the worms onto the droplet. Exposure of the worms to tetramisole will render them immobile within a few seconds and allow efficient imaging (*see* **Note 4**).
3. Cover gently with a coverslip.

Worms should not dry out during the imaging process. Ignore any worms that appear to have been damaged due to mishandling (worms with vulva rupture).

3.3 Monitoring Mitophagy by Simultaneous Labeling of Mitochondria and Autophagosomes

The first method for quantifying mitophagy relies on observing co-localization between the mitochondrial outer membrane, identified by GFP either fused to the DCT-1 mitophagy receptor or marking the mitochondrial matrix and the autophagosomal isolation membrane, identified by DsRed fused with LGG-1 (Fig. 1b) [26]. This method follows a similar principle to older techniques that utilized fluorescent antibodies and MitoTracker staining (*see* **Note 5**) [24]:

1. Photograph body wall muscle cells using a confocal fluorescent microscope (equivalent to a Zeiss AxioObserver Z1 confocal microscope) and performing *z*-stack imaging.
2. Use image analysis software of choice to count mitochondria engulfed by autophagosomes per body wall muscle cell. These can be observed as puncta where mitochondrially localized GFP (mtGFP) and the autophagosomal marker (LGG-1::DsRed) co-localize (*see* **Notes 6** and **7**).

3. Perform statistical analysis with appropriate software (Microsoft Excel/Graphpad Prism) to examine possible changes in mitophagy between samples. Use the student's t-test for comparison between samples with a significance cutoff level of $p < 0.05$. Use ANOVA corrected by the post hoc Bonferroni test for multiple comparisons. We recommend examining at least 50 individual cells from different animals per experiment, and repeating each experiment thrice.

3.4 Monitoring Mitophagy Using the Rosella Biosensor

As an alternative method to monitor mitophagy events, we have utilized the Rosella biosensor (Fig. 1c). Rosella is a dual fluorescent reporter which originated from the combination of a pH-sensitive GFP variant and a pH-insensitive DsRed variant [28]. We have fused Rosella with an amino terminal fragment of TOMM-20, which localizes in the outer mitochondrial membrane and mediates import of nucleus-encoded proteins into mitochondria [29]. Via this approach, we can quantify mitophagy events by merely assessing the fraction of green fluorescence which has quenched as a consequence of fusion of mitochondria-tagged Rosella with lysosomes (*see* **Note 8**). Mitochondria-tagged Rosella was specifically overexpressed in *C. elegans* body wall muscle cells, using the minor isoform of myosin heavy chain promoter (*myo-3* gene):

1. Observe the animals under a fluorescent microscope (equivalent to Zeiss Axio Imager Z2 Epifluorescence/DIC Microscope), and acquire images of the head region of 20 or more animals using a 10X lens and a digital camera.
2. Using the ImageJ/FIJI image processing package, split the channels to separately quantify the green and red fluorescence intensity in a representative head region, just anterior to the animal's intestine to avoid gut autofluorescence (*see* **Notes 6**, **7**, and **9**).
3. Divide the individual values of green versus red fluorescence from single animals using Microsoft Excel or GraphPad Prism. Comparisons between samples can be performed using the student's t-test with a significance cutoff level of $p < 0.05$. Use ANOVA corrected by the post hoc Bonferroni test for multiple comparisons between different experimental conditions.

Under mitophagy-inducing conditions, the ratio of green versus red fluorescence is expected to drop significantly, as more mitochondria will be sequestered to acidic lysosomes for degradation. We recommend examining at least 50 individual animals per experiment and repeating each experiment thrice.

4 Notes

1. An important parameter which should be taken under consideration is that the measurements should be conducted in animals of the same age under the desired experimental conditions. Macroautophagy is inseparably linked with aging, and it can be influenced by interventions which accelerate or delay its onset [30]. For mitophagy measurements described here, animal synchronization based on the crescent-like vulval morphology of L4-staged worms is preferable than synchronization in the egg stage via hypochlorite treatment or egg laying. This is because treatment with CCCP or paraquat is restrictive for normal development and only few of the treated animals can reach adulthood.
2. Unless your study is explicitly related to starvation/caloric restriction, you should always only use non-starved animals in your experiments. We recommend ensuring your strains have been well fed for at least three generations to avoid any transgenerational effects that might be inherited from starved parents [31, 32].
3. We recommend preparing fresh solutions for both the mitophagy-inducing drugs (paraquat and CCCP) prior to each experiment. Freeze-thaw cycles may reduce drug efficiency and diminish the robust induction of mitophagy that makes them ideal positive controls.
4. Sodium azide, widely used for worm anesthesia before imaging, is a strong inhibitor of oxidative phosphorylation [33]. Since it is detrimental for mitochondrial function, it is also a potential activator of mitophagy. Hence, we suggest the exclusive use of tetramisole to anesthetize the animals prior to observation.
5. In the case of the autophagosome co-localization method, an increase in observed LGG-1::GFP puncta might not necessarily result from increased autophagy, but instead represent a side effect of a dysfunctional pathway. For instance, defunct autolysosomes can lead to autophagosome accumulation and increased LGG-1::GFP puncta [34]. Hence, it is advisable to also rely on an alternative approach for drawing safe conclusions regarding mitophagy induction.
6. Thoroughly document the method and specific parameters of the analysis process, and ensure consistency across experiments. This is particularly important if you use an automated method to count the amount of LGG-1::DsRed and mitochondrial GFP co-localization.

7. It is important to distinguish actual DsRed fluorescence from bleed-through emission of GFP into "red wavelengths." This can happen if you overexpose your samples during imaging. In the case of the LGG-1::DsRed and mitochondrial GFP co-localization method, observing perfect alignment between all green and red spots may be an indication of bleed-through.
8. As an alternative to the Rosella biosensor, we suggest the use of pHluorin [35]. This is a pH-sensitive version of GFP whose fluorescence is quenched in acidic compartments, such as the lysosomes. Tagging of an outer membrane mitochondrial protein with pHluorin would allow monitoring of mitophagy events based on a single-color method.
9. Always document the parameters of your imaging as thoroughly as possible, and ensure they remain consistent between experiments. This is particularly important for the Rosella method, as changes in the imaging process might not affect both color channels equally, thus leading to skewed fluorescence ratios.

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