Video Article

Assessing Lysosomal Alkalinization in the Intestine of Live *Caenorhabditis elegans*

Kunal Baxi[1], Carlos E. de Carvalho[1]

[1]Department of Biology, University of Saskatchewan

Correspondence to: Carlos E. de Carvalho at carlos.carvalho@usask.ca

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Abstract

The nematode *Caenorhabditis elegans* (*C. elegans*) is a model system that is widely used to study longevity and developmental pathways. Such studies are facilitated by the transparency of the animal, the ability to do forward and reverse genetic assays, the relative ease of generating fluorescently labeled proteins, and the use of fluorescent dyes that can either be microinjected into the early embryo or incorporated into its food (*E. coli* strain OP50) to label cellular organelles (*e.g.* 9-diethylamino-5H-benzo(a)phenoxazine-5-one and (3-{2-[(1H,1'H-2,2'-bipyrrol-5-yl-kappaN(1))methylidene]-2H-pyrrol-5-yl-kappaN}-N-[2-(dimethylamino)ethyl]propanamidato)(difluoro)boron). Here, we present the use of a fluorescent pH-sensitive dye that stains intestinal lysosomes, providing a visual readout of dynamic, physiological changes in lysosomal acidity in live worms. This protocol does not measure lysosomal pH, but rather aims to establish a reliable method of assessing physiological relevant variations in lysosomal acidity. cDCFDA is a cell-permeant compound that is converted to the fluorescent fluorophore 5-(and-6)-carboxy-2',7'-dichlorofluorescein (cDCF) upon hydrolysis by intracellular esterases. Protonation inside lysosomes traps cDCF in these organelles, where it accumulates. Due to its low pKa of 4.8, this dye has been used as a pH sensor in yeast. Here we describe the use of cDCFDA as a food supplement to assess the acidity of intestinal lysosomes in *C. elegans.* This technique allows for the detection of alkalinizing lysosomes in live animals, and has a broad range of experimental applications including studies on aging, autophagy, and lysosomal biogenesis.

Video Link

The video component of this article can be found at https://www.jove.com/video/57414/

Introduction

The appearance of protein aggregates is widely accepted to be a hallmark of aging in eukaryotic cells[1,2,3], and the formation of which is thought to be among the principle drivers of cellular senescence[4,5,6,7]. There is growing evidence that as cells age, protein catabolism is impaired, leading to an increase in protein aggregation. The collapse of proteolysis in aging cells involves an impairment of autophagy[8] as well as proteasome-mediated protein degradation[9]. Finally, irreversible protein oxidation is increased in old cells, further impairing protein catabolism[10].

Autophagy was initially thought to be a non-selective process for bulk degradation of damaged proteins, but recent studies have indicated that autophagy is highly selective to the catabolism of protein aggregates and dysfunctional organelles that are not amenable to degradation via other protein clearance mechanisms[11]. During the process of autophagy, damaged and aggregated proteins are sequestered into a double-membrane vesicle called the autophagosome. This autophagosome then fuses with the acidic organelles called lysosomes, which leads to degradation of the autophagosome cargo[12]. Lysosomes represent the end-point of the autophagic pathway and participate in different cellular processes such as membrane repair, transcriptional control and nutrient sensing; highlighting their centralized role in cellular homeostasis (reviewed in ref. 13). Several studies have shown an association between an age-dependent decrease in lysosomal function and various neurodegenerative disorders[13]. Consistently, restoring lysosomal function in older cells can delay the onset of aging-related phenotypes[14,15]. Studies of the composition of the intralumen milieu suggest that the collapse of lysosomal function in older cells is not due to a reduction in the production of lysosomal proteases[16]. Alternatively, it has been proposed that loss of intralysosomal acidity, a critical requirement of its enzymatic activity, may underlie the drop in lysosome-mediated proteolysis[17]. To be able to explore this hypothesis, it is essential to develop reagents and protocols to probe dynamic changes in lysosomal pH in live cells in a replicable and consistent manner.

The intestine of *C. elegans* is the major metabolic tissue in worms and it is a critical regulator of systemic homeostasis and lifespan. We have developed assays to evaluate changes in the acidity of the lumen of intestinal lysosomes of worms to determine how lysosome-mediated proteolysis contributes to aging. Though pH-sensitive fluorophores have been used previously in *C. elegans* to mark intestinal lysosomes, there hasn't yet been an effort to establish a successful protocol that can detect small increases in lysosomal pH *in vivo*[18]. Here, we provide a protocol that can be used to detect loss of lysosomal acidity in the intestinal cells of *C. elegans* using a simple and convenient feeding protocol that incorporates a pH-sensitive fluorophore (cDCFDA) into OP50 food.

Protocol

1. Stain and Image intestinal lysosomes

1. **Seed nematode growth media (NGM) plates with OP50**
   1. Prepare NGM plates as per the recommended protocol[19] and allow the closed plates to dry for 2 days at room temperature.
   2. Inoculate OP50 bacteria into sterile Luria Broth (LB) broth and grow in a shaking incubator or water bath at 37 °C for 36 h or until the OD is between 0.2 and 0.4. Avoid using a bacterial culture with OD >1 or OD <0.2.
   3. Once NGM plates are dry, place a drop (~30 µL) of OP50 inoculum onto the center of the plate, and then spread the drop into a patch roughly that is 2 cm x 2 cm in size.
      NOTE: Any remaining OP50 inoculum can be stored at 4 °C for up to a month.
   4. Invert the OP50 plates once the inoculum has dried (roughly 10 or 15 min), and then incubate the plates at 37 °C for 36 h.
   5. After 36 h, remove the plates from the incubator and store at 4 °C until later use.
      NOTE: The plates should be good for up to 1 month at 4 °C.

2. **Supplementing cDCFDA to OP50**
   1. To supplement cDCFDA onto OP50 plates, prepare a working stock of 10 mM cDCFDA in dimethyl sulfoxide (DMSO). Keep the cDCFDA solution protected from light and store at -20 °C for later use.
   2. Gently place 100 µL of 10 mM cDCFDA solution onto the surface of the 2 cm x 2-cm OP50 patch, such that the entire patch is uniformly covered with cDCFDA.
      NOTE: It is very important that the entire patch of OP50 is covered with cDCFDA. If required, use up to 150 µL of cDCFDA for each plate. It is also imperative that the cDCFDA solution does not extend beyond the borders of the OP50 patch, since any cDCFDA that flows out of the OP50 patch will not be incorporated into the food, and will result in reduced staining intensity.
   3. Place the OP50 plates in a dark box on the bench top until all of the cDCFDA solution is absorbed into the bacterial patch (usually about 25 to 30 min).

3. **Staining worms using cDCFDA**
   1. Once the cDCFDA has completely permeated the OP50 patch, place no more than 20 worms per plate and incubate the plates inverted at 20 °C for a minimum of 14 h.
      NOTE: It is recommended to place the cDCFDA plates in an opaque box to minimize exposure to light.
   2. To control for intake differences between experiments and to normalize cDCFDA signals, (recommended) co-stain with 3-{2-[(1H,1'H-2,2'-bipyrrol-5-yl-kappaN(1))methylidene]-2H-pyrrol-5-yl-kappaN}-N-[2-(dimethylamino)ethyl]propanamidato)(difluoro)boron (2.5 µL of a freshly made 1 mM solution), an acidotropic weak amine dye whose fluorescence is largely non-variant over the acidic pH spectrum (see **Table of Materials** for common names of reagents).
      NOTE: Do not stain worms for less than 14 h since this may provide inadequate cDCFDA staining intensity and give a false interpretation of lysosomal pH.

4. **Preparing slides for microscopy**
   1. Place two parallel strips of labeling tape about 3 inches apart on a smooth flat surface, such as a mirror (**Figure 1**).
   2. Coat the surface of the mirror with water repellent spray, and wipe surplus liquid off.
   3. Melt 2% agarose (dissolved in distilled H_2O) in a microwave until completely liquefied, then place a 50 µL drop of agarose between the two strips of tape and quickly cover the drop with a microscope slide, such that the slide is perpendicular to the strips of tape. After the agarose has solidified, forming a circular pad under the slide, flip the slide over and add 10 to 15 µL of 5mM sodium azide (NaN_3) onto the agarose pad.
      NOTE: NaN_3 is a cytochrome C inhibitor that when used in low concentrations, reversibly anesthetizes worms so that they will not move during imaging.
   4. Pick worms from the OP50 plate supplemented with cDCFDA, and transfer them to a clean NGM plate without any OP50. Allow the worms to move about for a few seconds to allow most of the OP50 to be removed from the surface of the worms, and then transfer the worms to the agarose pad containing 5 mM sodium azide.
   5. Immediately cover the agarose pad with a cover slip. Be sure to gently place the cover slip without applying too much pressure, since this can result in worms bursting.
      NOTE: OP50 bacteria supplemented with cDCFDA fluoresce when visualized by microscopy, hence it is important to clean the worms as best as possible before placing them on the agarose pad containing sodium azide. If prepping more than 6 strains of *C. elegans* (including N2, wild type control), it is advisable to prepare the samples in batches of 6, so as to ensure that the worms do not dry out on the agarose pad. In some strains, the vulva starts to rupture after extended periods of time due to pressure from the cover slip, so it is important to plan accordingly.

5. **Confocal microscopy**
   NOTE: Some microscopes have a built-in feature that automatically increases fluorescence intensity to compensate for variable levels of fluorescence. Such microscopes might not work well for imaging worms stained with cDCFDA, since they will inherently increase the fluorescence intensity of samples.
   1. Perform imaging on a standard confocal microscope using excitation/emission wavelengths set to 488/530 nm for cDCFDA. Take single plane images (not Z-stacks) of intestinal lysosomes and use only lysosomes that are in focus (maximal signal intensity) for intensity quantification.
      NOTE: Perhaps because of differences in dye availability, lysosomes of the intestinal cells directly posterior to the pharynx maintain cDCFDA staining intensity irrespective of genotype or age, hence care should be taken to avoid imaging lysosomes in these cells.
   2. Image the slide containing 2 day old wild type N2 worms first. While doing so, adjust the confocal imaging parameters such as laser power, pinhole, and aperture to ensure that the cDCFDA staining intensity is not oversaturated.

NOTE: Once these settings are set, do not change them for the entire duration of the imaging for that day.

3. Capture 1024 x 1024 pixel images of a single plane of intestine (3 to 4 images per worm) using a 60X magnification lens.
   NOTE: The cDCFDA emission spectrum in worms will yield a single prominent peak at 520 nm coinciding with its reported fluorescence spectrum[20], providing a specific signal readout that is distinct from intestinal autofluorescence (**Figure 3**).
4. Export raw confocal image (.lsm files) as .tiff files for each channel.
5. Next, open ImageJ and click on **File| Open** to open the image file to be quantified. Once the image loads, use the oval shape selection tool in ImageJ to select a region of interest (ROI).
6. Thereafter, click on **Analyze| Measure** to quantify fluorescence intensity for that ROI. Select 4–5 different in-focus lysosomes per image and calculate the average relative fluorescence intensity of each region of interest (ROI).
7. For each image, measure the background fluorescence intensity in a region of the intestine between lysosomes. Subtract the background fluorescence intensity values from the lysosomal fluorescence intensity values.
8. In total, collect around 30 to 50 individual normalized values for cDCFDA intensity (6 to 10 animals) for each strain being tested. Use these values to plot a box and whisker plot using any appropriate statistical software.
   NOTE: Staining can vary considerably depending on factors like temperature, incubation time and the overall health/age of animals such that fluorescence intensity comparisons are only relevant within samples process in the same staining experiment. It is therefore important to process controls in every staining experiment. Calculate statistical differences (*t*-tests) using any appropriate statistical software.
9. Image all strains from the same experiment (stained on the same day) in sequence, taking care not to change any of the imaging parameters.

Representative Results

cDCFDA stains lysosomes in a pH-dependent manner, and its low pKa and ready uptake into lysosomes makes it an ideal pH sensor[21]. cDCFDA staining intensity is inversely proportional to lysosomal pH (*i.e.* staining intensity increases as pH decreases)[18,22]. cDCFDA signals are consistently weak in lysosomes of animals treated with 20 mM of chloroquine, an inhibitor of lysosomal acidification, and in worms depleted of v-ATPase, the protein complex on the membrane of lysosomes that is required for proton import[22]. These are important controls to use when assessing the relative levels of cDCFDA staining in other genetic backgrounds or treatments.

We also find no overt effects of cDCFDA exposure on the fitness or fertility of treated worms (data not shown). Using this protocol, we identified an endogenous loss of acidity in lysosomes of post-reproductive wild type worms. As shown in figure 3, the cDCFDA fluorescence signal is robust in the lysosomes of young (2 day post L4) reproducing *C. elegans*, signifying acidified lysosomes. The staining intensity reduces considerably upon alkalinization of lysosomes, as observed in old (8-day post L4) worms as well as in worms where the proton influx into these organelles is reduced (via knockdown of *vha* genes encoding v-ATPase subunits). Using this method, we detected a physiological loss of lysosomal acidity in the intestine of post-reproductive animals (diagram in **Figure 2**, fluorescence images in **Figure 3**). The reduced cDCFDA signal in intestines of day 8 (post-L4) worms pointed to alkalinized lysosomes and a possible impairment in protein clearance. This is in fact the case, as these animals significantly accumulate protein aggregates inside lysosomes at this life stage[22]. To validate a role of lumen de-acidification in this process, we co-stained young, reproducing animals that had been depleted by RNAi of two core components of the v-ATPase pump machinery (VHA-2 and VHA-8) and therefore should not properly import protons into lysosomes. As expected, *vha-2* and *vha-8* RNAi animals, even at the reproductive stage, showed reduced cDCFDA fluorescence signals comparable to those of post-reproductive wild type worms and consistent with alkalinized lysosomes (**Figure 3**).

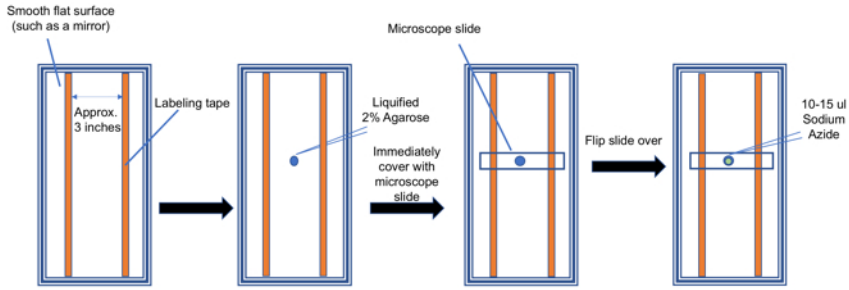

Figure 1: Preparing slides for microscopy. A simple step-by-step schematic showing the steps to be followed for preparing slides for microscopy.

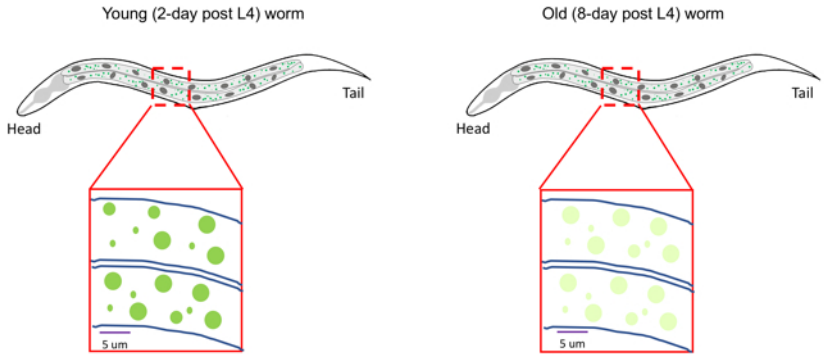

Figure 2: Use of pH-sensitive dyes to probe acidity in intestinal lysosomes of *C. elegans*. Diagram showing the patterns of intestinal cDCFDA fluorescence intensity in reproducing (day 2) and post-reproductive (day 8) worms. The top diagrams show whole worms. The boxes in the bottom represent zoomed in, projected diagrams of the midgut region, the intestinal lumen in the center. Lysosomes are depicted as vesicles of varying size in the cytoplasm of intestinal cells. Compare these schematic diagrams with **Figure 3** images.

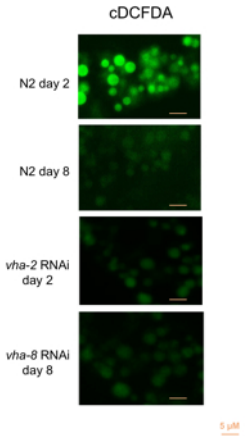

Figure 3: cDCFDA staining images. Representative images showing day 2 and day 8 (post L4) *C. elegans* intestinal lysosomes (N2 wild type) stained with cDCFDA as well as intestinal lysosomes of *vha-2* and *vha-8* RNAi worms stained with cDCFDA (positive control). *vha-2* and *vha-8* encode subunits of the vacuolar ATPase (vATPase), the proton pump lining the lysosomal membrane that is responsible for acidification of the lysosomal lumen.

Discussion

A variety of cellular and molecular events contribute to aging, influenced by life history traits and genetic factors. Our recent study[22] suggests that the reproductive cycle plays an important role in controlling the fitness of the soma through the regulation of lysosomal pH dynamics. We showed that lysosomal-mediated proteolysis is promoted while animals actively reproduce by upregulation of v-ATPase transcription, which in turn ensures acidic lysosomes. Upon the end of reproduction, v-ATPase expression drops, lysosomes alkalinize, and protein aggregates accumulate in these cells.

Disorders that impair lysosomal acidification have been shown to be caused by altered v-ATPase activity, and declining v-ATPase function in the brain has also been proposed to underlie various neurodegenerative diseases[23]. Our results further support the hypothesis that impaired v-ATPase function is among the causes of cellular senescence and aging[24].

One cytological hallmark of aging cells is the progressive formation of misfolded and aggregated proteins in old cells[1,25,26,27,28,29]. As in post-reproductive *C. elegans*, similar protein aggregates are found in the lysosomal lumen of late passage mammalian cultured cells[27,29]. Since lysosomes are the terminal endpoint of the autophagic pathway, it is plausible that the continuous influx of damaged proteins and organelles into alkalinized lysosomes results in the functional collapse of these lysosomes, which then leads to other downstream events that ultimately contribute to cellular senescence.

The protocol described here has been designed to generate a simple and reproducible assessment of the relative acidity in intestinal lysosomes of live *C. elegans*. Because a narrow pH range (4.5–5) is required in these organelles for proper proteolysis, subtle increases in pH have dramatic consequences for cellular and tissue homeostasis[17]. The fluorescence characteristics and low pka of cDCFDA allows this dye to capture changes in acidity in this physiologically relevant pH window[18]. The concentration of cDCFDA recommended for staining lysosomes has been optimized to provide the best possible readout of changes in lysosomal pH based on our imaging parameters and imaging system. Even so, the results of cDCFDA staining might vary for different end users based on their imaging setup such that lysosomal pH changes might not be accurately reflected in the cDCFDA staining pattern. If this happens, the best course of action would be to use varying the concentrations of cDCFDA (10 to 100 mM) to determine the optimum concentration for imaging variations in wild type N2 worms at day 2 and day 8 post L4. The

ideal cDCFDA concentration is one that stains lysosomes of young (day 2 post L4) worms efficiently without being oversaturated but also shows a decreased staining intensity for old (day 8 post L4) worms.

One of the limitations of the cDCFDA staining technique is its long incubation time. When worms are incubated at a particular time point, the results can be visualized only after ~24 h. It would be more efficient to develop a strain of *C. elegans* that provides a real-time readout of lysosomal pH, presumably using a pH-sensitive fluorophore tagged to a lysosomal membrane protein.

While there exist a few different techniques for assessing pH in the intestine of *C. elegans,* most of these techniques either assess the pH of the intestine as a whole[30], or require injection into the body cavity, which is time-consuming and cumbersome[31]. cDCFDA can easily be applied for large scale genetic screens using feeding RNAi libraries to search for *C. elegans* mutants that show altered lysosomal pH or physiology. The only critical aspects of this method are the concentration of cDCFDA used and being sure to include a young (day 2 post L4) N2 control strain as a benchmark for configuring confocal imaging during every session.

To better understand the contribution of lysosomal pH to aging, it will be important to dissect the regulatory mechanisms behind the dynamic changes in lysosomal acidity in response to different cellular and environmental inputs. cDCFDA staining may be used as one strategy to extend this research in *C. elegans*.

Disclosures

The authors declare no conflict of interest.

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