Towards an understanding of the side effects of anti-HIV drugs using Caenorhabditis elegans

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Appendix

*In vivo* visualization and quantification of mitochondrial morphology in *C. elegans*

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In vivo visualization and quantification of mitochondrial morphology in C. elegans

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Summary

Caenorhabditis elegans is a highly malleable model system, intensively used for functional, genetic, cytometric and integrative studies. Due to its simplicity and large muscle cell number, C. elegans has frequently been used to study mitochondrial deficiencies caused by disease or drug toxicity. Here we describe a robust and efficient method to visualize and quantify mitochondrial morphology in vivo. This method has many practical and technical advantages above traditional (manual) methods and provides a comprehensive analysis of mitochondrial morphology.

1. Introduction

Mitochondria are highly dynamic tubular organelles that continuously remodel by fusion and fission in a regulated manner. The balance between these opposing events determines the morphology of the mitochondrial network, with excessive fission resulting in mitochondrial fragmentation and increased fusion leading to extended and highly interconnected mitochondria. Mitochondrial morphology plays an essential role in cell division, cell survival, cellular redox status, mtDNA rescue and mitochondrial protein quality control and specific morphological changes in mitochondrial structure and organisation are considered to reflect cellular responses to stress and pathological conditions in worms, mice and humans. For example, changes in mitochondrial structure and function are known to occur in age-associated disorders such as Parkinson’s disease, sarcopenia and in metabolic diseases, including heart-disease and diabetes mellitus.

The nematode Caenorhabditis elegans has proven to be one of the most versatile model organisms for the elucidation of molecular pathways implicated in many human diseases and ageing. Wild type C. elegans has a relatively short lifespan of two weeks, enabling researchers to rapidly assess the effects of different mutations or treatments on mitochondrial function or longevity. Mitochondrial research in C. elegans has yielded insights in the genetic regulation of ageing and mitochondrial function, and it has provided a vast array of mutants to study these effects. C. elegans has many technological advantages. It is self-
fertilizing, transparent, has a fully described anatomy and development, and a completely sequenced genome. Moreover, many tools exist to manipulate *C. elegans*, for example RNAi. Although limited to less than 1000 cells, *C. elegans* has highly differentiated tissues such as skin, nerves, muscles, reproductive organs and gut tissue. The preferred tissue to study mitochondria is muscle as it has well organized and extensive mitochondrial networks to provide ATP for their high energy demands.

Specific cell-permeable dyes, such as Mito-Tracker, are available for visualizing mitochondria in *C. elegans*. However, the uptake and retention of Mito-Tracker by the mitochondria is dependent on the mitochondrial membrane potential and the specific staining is lost upon disruption of the membrane potential. To avoid this, one can make use of fluorescent proteins that can be targeted to the mitochondria. Constitutive expression guarantees mitochondrial staining under all conditions and during all nematode developmental stages. Additionally, fluorescent proteins are highly stable, remain localized and allow in vivo imaging. Using a strain expressing mito::GFP in muscle cells, we established a protocol for reliable and robust quantification of the mitochondrial network. This assay is easily amenable to up-scaling and implementation in high-throughput assays.

## 2. Materials

Ensure that all solutions are sterile before use. Typically, the nematode growth medium does not contain any antibiotics and is therefore prone to contamination. Work in a sterile environment when handling the worms. Transgenic strains and chemical waste must be disposed of correctly. Special care is to be taken when handling sodium azide (NaN₃) as it is toxic. *C. elegans* strains can be obtained from the Caenorhabditis Genetics Centre (https://www.cbs.umn.edu/cgc/strains).

- Transgenic *C. elegans* strain JI1271 injected with expression construct mito::GFP expressed from the myo-3 promoter, which is mitochondrial matrix specific, and transformation marker rol-6, which distinguishes transgenic animals by a rolling phenotype [22](see Note 1).
- Temperature controlled stove (20°C).
- Nematode growth medium (NGM): NaCl (3g), Bactopeptone (2.5 g), Agar (20g), dd H₂O (1L), 1M CaCl₂ (1ml), 1M MgSO₄ (1ml), Potassium phosphate pH 6.0 (KPO₄) (25ml). Autoclave. Add 5mg/mL Cholesterol in 95% EtOH (1ml).
- OP50 *Escherichia coli* (OD~2.0) in LB: Bacto Trypton (10g), Bacto Yeast (5g), NaCl (5g), dd H₂O (1L). Autoclave.
- M9: NaCl (5g), Na₂HPO₄ (6g), KH₂PO₄ (3g), dd H₂O (1L). Autoclave and add 1M MgSO₄ (1mL).
- Worm picker and platinum wire tip (Genesee Scientific, San Diego, California, USA)
- MatTek Glass bottomed petri-dishes: P35G-1.0-14C (Corning 35mm dish with 14mm glass diameter and coverslip No. 1 (0.13-0.16mm thick)) Depth of well = 0.70 – 0.75mm (see Note 2).
- 10mM Sodium azide (NaN₃) in M9. Use ice cold.
- Microscopy coverslips: Menzel-Gläser, 18 x 18mm, No. 1 (see Note 3)
- Confocal microscope with camera (see 3.3). Argon laser; 488nm excitation and 525-550nm emission.
- GFP reference (green chroma fluorescent slide: 2273-G. Ted Pella, Inc.)
3. Methods

3.1 Culture of *C. elegans*

Worms are cultured on nematode growth medium (NGM) plates seeded with OP50 *Escherichia coli*. Culture at 20°C is preferred as other temperatures induce stress and adaptation, which can alter mitochondrial morphology. For detailed descriptions of nematode culture and physiology see www.wormbook.org or www.wormbase.org/species/c_elegans.

1. Pour NGM plates (~20mL per 9cm Ø petri dish) and let harden at room temperature. Once solid, seed the plates with 500μL fresh OP50 culture by pipetting and spreading the bacteria evenly over the plate surface area with a sterile dri-galski spatula (see Note 4).
2. Synchronous populations of worms are obtained by alkaline hypochlorite treatment of gravid adults. Synchronize ~100 gravid adults.
3. Place the eggs overnight in 10mL M9 in an Erlenmeyer whilst gently shaking at 20°C, so as to let all the eggs hatch to L1 larvae (see Note 5).
4. Collect the larvae the following morning in a 15mL conical tube and centrifuge at 1.4 x g for 2 minutes. Aspirate the M9 without disturbing the pellet.
5. Wash the worm pellet in 10mL M9 to rid the culture of debris, and centrifuge at 1.4 x g for 2 minutes before aspirating most of the M9.
6. Plate the worms sparsely on fresh OP50 seeded plates with a glass pipette; approximately 200 worms per 9cm Ø petri dish. Incubate for 48h at 20°C to the L4 stage.
7. When having reached the L4 stage, transfer the worms to a 15mL conical tube by pouring 10mL M9 onto the plates and gently swirling to dislodge the worms.
8. Centrifuge the worms at 1.4 x g for 2 minutes. Aspirate most of the M9 and then using a glass pipette transfer the worms to fresh OP50 seeded plates (In this case containing the compound of interest). For RNAi seed the NGM plates with the *E. coli* transformant strain carrying the desired gene target construct.
9. Incubate further at 20°C for the desired exposure time (In this case 24h) (see Note 6).

3.2 Microscopy slide preparation

1. Keeping the MatTek slides clean, pipette 120μL of ice cold 10mM NaN₃ into the glass bottomed well.
2. Pick approximately 50 worms and place them inside the NaN₃ droplet.
3. Cover the well gently with a glass coverslip (Figure 1). Wait for approximately 10 minutes for the worms to become paralyzed.
4. Your samples are ready for immediate imaging (see Note 7).

![Figure 1](image_url)
3.3 Imaging of mitochondria

It is important to have optimized your confocal microscope settings beforehand, and once set, to keep them constant at all times. Settings mentioned here were used for a Nikon A1 confocal microscope and are indicative. Because the sample is suspended in a water-based solution, the preferred objective is a water immersion objective. Additionally, a water immersion objective provides superior focus in depth because it minimalizes aberration under these circumstances. We use a Plan Apo 60x WI objective with a numerical aperture of 1.27. Keep the acquisition settings such as laser power (3 / 5.8kW/cm²), pinhole size (2 A.U. / 55μm) and HV value (85), pixel dwell time (1.9μsec) and pixel size (0.2μm/pixel) identical between images.

1. Place the glass-bottom dish on the viewing platform with the glass bottom facing the microscope objective.
2. Using widefield microscopy, locate a single worm and align the focal center just behind the clearly distinguishable area of the posterior bulb (Figure 2) (see Note 8).
3. Using the scanning function (to avoid bleaching) on the confocal microscope, locate the bottom of the well and set this point as the z-stack’s lower limit. Now locate the topside of the muscle quadrant using the fast scanning function and set this point as the z-stack’s upper limit. (see Note 9)
4. Set the z-stack slices at 1μm intervals (see Note 10) and acquire the images at the optimal acquisitions settings.
5. Collect a minimal of 10 z-stacks, i.e. 10 worms, per condition, to ensure statistically relevant sampling.
6. After every imaging session, acquire an image stack from a reference fluorescence slide, which can be used for correcting spatial and temporal fluctuations in illumination intensity (see 3.4).

Figure 2. Region of interest (red). Align the focal center just behind the clearly distinguishable area of the posterior bulb (yellow).

3.4 Automated image analysis (see Note 11)

Images of mitochondria are segmented by means of a dedicated image-processing pipeline. Before proceeding, inspect images visually to only retain those of consistent quality. The following image processing steps are performed before feature extraction:

1. Uninformative slices or slices with reflections are removed from the image stacks. This can be done automatically using an image quality criterion that only retains slices with an intensity covariance (stdev / mean) > 1.
Quantification of mitochondrial morphology in *C. elegans*

2. A flat-field correction is performed to buffer for spatial intensity variations (illumination heterogeneity). To this end the image is divided by the corresponding image from the reference slide.

3. The flat-field corrected stack is then projected according to the maximum pixel intensity. This allows capturing the majority of the mitochondria in one image. A potential disadvantage of this procedure is superposition of mitochondria from different levels in the worm, although we found this effect to be limited due to the coarse axial sampling.

4. A duplicate image of the maximum projection image is pre-processed, by background subtraction (rolling ball radius = 15) and local contrast enhancement (block size = 15, slope = 3), so as to buffer intensity variations between the different objects of interest (mitochondria).

5. Mitochondria are then specifically enhanced by means of a multi-scale Laplacian operator \(^{232}\) (see Note 12).

6. The enhanced image is binarized according to an autothresholding procedure (Yen or Isodata), yielding a mask that can be used for analysing the mitochondria in the original image. Before doing so, the mask should be filtered to only retain objects of a predefined size (> 7 pixels), this to avoid noise or debris from skewing the results.

Using the mask, shape and intensity metrics, individual mitochondria are extracted from the original image as well as the total number of mitochondria. In addition, general texture metrics can be calculated from a gray-level co-occurrence matrix analysis. We specifically calculate the average texture parameters over a horizontal and a vertical GLCM matrix with a 1 pixel offset (a reference pixel and its immediate neighbor) (see Note 13).

3.5 Mitochondrial quantification

After feature extraction, results are summarized per worm or per condition, by averaging individual mitochondrial metrics. Dedicated statistical analyses can be performed in Matlab 2010a® or in R freeware. In a first approach, individual parameters can be statistically compared between conditions, by means of pairwise students T-tests or, in case of non-normal distributions, Wilcoxon rank sum tests. Subsequently, a more holistic cluster analysis can be performed, integrating all relevant features. To this end, the data set is first standardized (values are converted to Z-scores) so as to avoid differences in magnitude or range from pulling the weight too much towards one particular variable. Using the standardized data set, the different conditions (e.g. chemical treatments or RNAi) are then clustered using Euclidean distance as distance metric and the average value as linkage value for establishing the dendrogram. The same is done for the features and the final output is displayed in a two-dimensional clustergram, color-coded by the z-value (Figure 3). This representation allows for quickly resolving conditions with similar effects on mitochondrial morphology.
Figure 3. Mitochondrial morphological changes caused by 10mM NaN₃. Mitochondrial networks slowly deteriorate over time and become more fragmented. A = 10min, B = 30min, C = 60min, D = 120min, E = 150min, F = 180min, G = 240min, H = Typical mitochondrial morphology after vulvar gonad protrusion.

4. Notes

1. Other strains such as SJ4103⁴⁷⁴ also service and are easier to maintain, yet the strain mentioned is preferred due to the fact that it lacks autofluorescent and birefringent gut granules and the mitochondrial network in control animals is neatly organized and clearly distinguishable, making image analysis straightforward.

2. Glass bottomed petri-dishes have the advantage over traditional agar-pad microscopy slides as they do not need to be made beforehand, are sterile, do not dry out the worms, and importantly minimize light refraction and background during image acquisition.

3. The type is irrelevant for imaging; they are merely needed to seal off the well.

4. Add the chemical of interest before the medium is poured and mix well with a magnetic bead stirrer, making sure that the chemical can withstand temperatures above ~50°C beforehand. Pour the NGM plates in a sterile environment when the medium has cooled to ~50°C, or you can comfortably hold the medium flask. The agarose in the NGM plates solidifies during cooling, which takes approximately 30 minutes. NGM plates without chemical additions can be stored at 4°C for approximately 2 months in a sealed container or petri dish sleeve. Plates including drugs or other chemicals should be used as quickly as possible, depending on the compound’s stability. Seeded plates can be kept for several days in a cool, dark and dry environment. Ensure that the OP50 can grow normally when exposed to your chemical of interest. If this is not the case, OP50 stocks can be 5x concentrated, spread on the NGM plates and ‘inactivated’ by UV exposure, before drying at room temperature and placing the worms on the plates.

5. Because there is no food present the larvae halt growth at the L1 stage. L1 worms can be kept for approximately 48h in these conditions before use.

6. During this period worms become adults and lay eggs. Therefore the plates are prone to become overcrowded, rapidly reducing the *E. coli* food source. In this case worms can be sterilized at the L4 stage by adding 5-fluoro-2'-deoxyuridine (FUdR) to the agar ²², or if FUdR may interfere with the experiments, meshed using a Sefar Nitex μM filter (Sefar AG Filtration Solutions, Heiden, Switzerland) every 24h to rid the culture of progeny.

7. Immediate analysis is essential as NaN₃ inhibits the respiratory enzyme cytochrome oxidase and therefore affects mitochondria in the long run. After approximately 1 hour of exposure to 10mM NaN₃, worm mitochondrial networks become disengaged and they fragment (Figure 4). This is particularly evident in muscles adjacent to the vulva, and prolonged NaN₃ exposure can cause gonad protrusion through the vulva (Figure 3). NaN₃ is, however, still preferred above other immobilizing agents as its effects are rapid. Frequently used anesthetizing agents for *in vivo* analysis include Levamisole (tetramisole hydrochloride) or
Aldicarb, but these compounds show full paralyzing effects only after hours [475]. Fixating agents, such as formaldehyde, can also be used [476]. However, the effects of these chemicals and their relatively long-term incubation time to obtain full immobilization may affect mitochondrial morphology.

8. This area contains somatic muscle cells nr. 7, 8 & 9 from the D-lineage, which are present at hatching and have the highest exposure to the compound through ingestion.

9. *C. elegans* has distinct muscle segments which are divided into two dorsal and two ventral quadrants, with two rows of muscle cells per quadrant (http://www.wormatlas.org/hermaphrodite/muscleintro/MusIntroframeset.html). Each quadrant in an adult hermaphrodite is approximately 8μm thick at the area just behind the posterior bulb. Typically, ~25 slices at 1μm intervals will sufficiently cover the quadrant closest to the bottom coverslip. Because of the orientation of the muscle quadrants (see Note 10) it can be quicker to scan for the top of the worm and set this as the z-stack’s upper limit (~50 slices at 1μm intervals). This limit no longer needs to be adjusted for each worm, thus speeding up image acquisition, although it will provide you with many useless images.

10. This should provide you with approximately 15-25 images per z-stack, of which, on average, 6 images provide sufficient muscle cross-sectional surface area so they can be used for image analysis. Because the nematode strain described here has the roller phenotype, the nematode’s rows of muscle are likely slightly corkscrewed when paralyzed. Depending on the position of the nematode in the well, the z-stack segmentation may not be optimal to obtain large surface area images of the muscle quadrants. In this case, select a different worm.

11. Traditional mitochondrial morphology analysis methods rely on blind-scoring by the researcher and, although adequate, have limited descriptive power due to the amount of variables that can be taken into account [476]. In addition, these scoring methods are time consuming, labor intensive and due to their lack of sensitivity, a considerable amount of worms or muscle cells needs to be imaged before sufficient statistical power can be attained.

12. This requires the FeatureJ plugin by Erik Meijering (http://www.imagescience.org/meijering/software/featurej/), which is part of the FIJI package but should be downloaded and installed when using ImageJ.

13. This requires the GLCM_Texture plugin by Julio Cabrera (http://rsbweb.nih.gov/ij/plugins/texture.html).

![Figure 4](image_url) **Figure 4.** Heatmap obtained after analysis of a set of images from *C. elegans* worms treated with different chemical compounds or RNAi. The columns represent different features of mitochondrial shape and intensity as well as image texture and the rows represent the different treatments. On the right, representative images are shown for the most dominant phenotypical patterns of mitochondrial networks (normal, fragmented and complex).