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***In vivo* Determination of Mitochondrial Function using Luciferase-Expressing *Caenorhabditis elegans*: Contribution of Oxidative Phosphorylation, Glycolysis, and Fatty Acid Oxidation to Toxicant-Induced dysfunction**

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Abstract

Mitochondria are a target of many drugs and environmental toxicants; however, how toxicant-induced mitochondrial dysfunction contributes to the progression of human disease remains poorly understood. To address this issue, *in vivo* assays capable of rapidly assessing mitochondrial function need to be developed. Here, using the model organism *Caenorhabditis elegans*, we describe how to rapidly assess the *in vivo* role of the electron transport chain, glycolysis or fatty acid oxidation, in energy metabolism following toxicant exposure, using a luciferase-expressing ATP-reporter strain. Alterations in mitochondrial function subsequent to toxicant exposure are detected by depleting steady-state ATP levels with inhibitors of the mitochondrial electron transport chain, glycolysis, or fatty acid oxidation. Differential changes in ATP following short-term inhibitor exposure indicate toxicant-induced alterations at the site of inhibition. Because a microplate reader is the only major piece of equipment required, this is a highly accessible protocol for studying toxicant-induced mitochondrial dysfunction *in vivo*.

Keywords

Caenorhabditis elegans; mitochondrial toxicity; PE255; PE327; glycolysis; oxidative phosphorylation; fatty acid oxidation

INTRODUCTION

Mitochondria are best known for the role they play in ATP production via oxidative phosphorylation; however, mitochondria also play crucial roles in apoptosis (Susin et al.,

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1999), calcium homeostasis (Duchen, 2000) and retrograde signaling (Liu and Butow, 2006), thus playing diverse roles in cellular and organismal health. Mitochondrial dysfunction is causative and/or associated with numerous human diseases, including cancer (Baysal et al., 2000; Wallace, 2012; Yan et al., 2009), metabolic syndrome (Bugger, 2008), and various neurological disorders (Beal, 2005; Lin and Beal, 2006). Furthermore, growing evidence has demonstrated that mitochondria are an important target of many drugs (e.g. antibiotics and nucleoside reverse transcriptase inhibitors) (Dyken and Will, 2007; Guan, 2011; Poirier et al., 2015) and environmental toxicants (e.g. polycyclic aromatic hydrocarbons and pesticides) (Backer and Weinstein, 1980; Meyer et al., 2013; Tanner et al., 2011), and toxicant-induced mitochondrial dysfunction has been implicated in many diseases, including cancer and neurodegeneration (Robey et al., 2015; Tanner et al., 2011; Zhao et al., 2014).

Because mitochondrial function is dependent upon cellular context and environmental cues (Chan, 2012; McBride et al., 2006), it is critical to develop assays capable of rapidly assessing mitochondrial function, *in vivo*, following toxicant exposure. A short lifecycle (2-3 weeks), high reproductive rate (~300 offspring per gravid adult), and highly conserved mitochondrial biology (Tsang and Lemire, 2003) and biochemical pathways (Braeckman et al., 2009) contribute to the utility of the model organism *Caenorhabditis elegans* for studying toxicant-induced mitochondrial dysfunction. Furthermore, significant overlap between the activities of Toxcast phase I and II libraries have recently been described between nematodes and zebrafish, further validating *C. elegans* as an important non-mammalian model (Boyd et al., 2015). Currently, mitochondrial respiration in *C. elegans* can be measured via low-throughput Clark type electrodes (Braeckman et al., 2002), or with the higher-throughput, but more expensive Seahorse XF[®] Bioanalyzer (Luz et al., 2015a; Luz et al., 2015b). Additionally, small metabolites, such as ATP, pyruvate, or NADH can be extracted from nematodes and used to assess mitochondrial health (Brys et al., 2010; Krijgsveld et al., 2003); however, this is a time consuming process. Alternatively, transgenic, firefly luciferase-expressing PE255 nematodes can be used to rapidly assess steady-state ATP levels *in vivo* (Lagido et al., 2009; Lagido et al., 2008). This transgenic model has proven valuable to environmental toxicologists, and has been used to study the effects of heavy metals and 3,5-dichlorophenol (Lagido et al., 2009; Lagido et al., 2001), 5-fluoro-2-deoxyuridine (Rooney et al., 2014), sewage sludge extract (McLaggan et al., 2012), the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Bodhicharla et al., 2014), and ultraviolet C radiation (Bess et al., 2012; Bess et al., 2013; Leung et al., 2013) on steady-state ATP-levels, and has more recently been used to track nematode development (Olmedo et al., 2015), and screen drug-libraries (Lagido et al., 2015).

Here, using PE255 luciferase-expressing nematodes and well-established inhibitors of the mitochondrial electron transport chain (ETC), glycolysis, and fatty acid oxidation (FAO) we describe a novel method that can be used to rapidly screen for alterations in mitochondrial energy metabolism following drug or toxicant exposure. Short-term incubation with these inhibitors depletes steady-state ATP levels. Thus, differential depletion of ATP in toxicant exposed nematodes in response to inhibitors indicates the relative contribution of the targeted cellular process to energy metabolism. Using this approach we recently demonstrated induction of a Warburg-like effect in arsenite exposed PE255 *gfp-4* nematodes

(Luz et al., Submitted), which was confirmed via Seahorse XF^e and small metabolite analysis, thus further demonstrating this protocol's utility in detecting toxicant-induced mitochondrial dysfunction.

BASIC PROTOCOL 1

Luciferase-based *in vivo* assessment of mitochondrial energy metabolism in

C. elegans—Here, using the PE255 ATP reporter strain (Lagido et al., 2015; Lagido et al., 2008), and the well-known pharmacological inhibitors rotenone (complex I), thenoyltrifluoroacetone (TTFA, complex II), antimycin A (complex III), sodium azide (complex IV), dicyclohexylcarbodiimide (DCCD, ATP synthase), carbonyl cyanide-p-trifluoromethoxyphenylhydrazon (FCCP, mitochondrial uncoupler), perhexiline (fatty acid oxidation (FAO)), and 2-deoxy-D-glucose (2-DG, glycolysis)) we outline how to rapidly assess mitochondrial energy metabolism following toxicant exposure. All of these inhibitors have previously been demonstrated to work in *C. elegans* (Luz et al., 2015b; Schulz et al., 2007; Taylor et al., 2013; Zubovych et al., 2010). Short-term (1.0 or 4.5 hour) incubation with inhibitors results in changes in steady-state ATP levels. Thus, altered function at the site of inhibition is detected through differential depletion of steady-state ATP levels. For example, the magnitude of ATP depletion following inhibition of ETC complex I with rotenone will be less than in toxicant exposed relative to unexposed nematodes if toxicant exposure has the effect of reducing complex I activity. This is because complex I is already contributing less to maintenance of steady-state ATP levels. Alternatively, the magnitude of ATP depletion will be greater in toxicant exposed nematodes if toxicant exposure is increasing activity of complex I. Figure 1 details the main principles of this assay.

Materials

REAGENTS: OP50 seeded K-agar plates (see Support Protocol 1)

Synchronous populations of L1 PE255 nematodes (see Support Protocol 2; Transgenic (PE255) N2 (wild type) and PE327 *glp-4* (*bn2*) nematodes available through the *Caenorhabditis* Genetics Center, University of Minnesota)

K-medium (see recipe)

Inhibitor stocks (Table 1 outlines all required inhibitors, as well as storage conditions)

Dimethylsulfoxide (DMSO)

Unbuffered EPA H₂O (see recipe)

0.1% (v/v) Triton X-100 (diluted in ddH₂O; store at room temperature indefinitely)

Glass microscope slides

Disposable reagent reservoirs

Multi-channel pipette (capable of pipetting 20-200µl)

White 96-well plates without lids

Luminescence buffer (see recipe)

EQUIPMENT: Incubator (capable of maintaining temperatures in the range of 15-25°C)

Centrifuge (e.g. Beckman Coulter equipped for 15mL tubes)

Dissecting light microscope

Horizontal vortexer (e.g. Eppendorf MixMate PCR 96)

Orbital shaker

Microplate reader (FLUOstar OPTIMA, BMG Labtech) equipped with luminescence optic, 502nm emissions filter, and 485 nm excitation filter

Nematode culturing—Nematodes are cultured on k-agar plates seeded with *E. coli* strain OP50 as previously described (Stiernagle, 1999).

1. Using a sterile Pasteur pipet transfer age-synchronous L1 PE255 nematodes, obtained from sodium hydroxide bleach treatment (see Support Protocol 2), to an OP50 seeded k-agar plate (see Support Protocol 1). Culture the nematodes until the appropriate life stage for toxicant or drug exposure is reached.

Both PE255 glp4 (strain PE327) and PE255 N2 (wild type) nematodes are available for purchase for a nominal fee, through the National Institutes of Health-supported Caenorhabditis Genetics Center (CGC, University of Minnesota).

This assay was originally developed using germline-deficient, PE255 *glp-4* (bn2) nematodes, which are maintained at the permissive temperature of 15°C. Shifting *glp-4* nematodes to the restrictive temperature, 25°C, results in sterile, germ cell free nematodes (Beanan and Strome, 1992). However, we have successfully used most of the concentrations of inhibitors outlined in this assay (see Table 1) with both L4 and 8 day old adult PE255 N2 nematodes, which are maintained at 20°C.

Toxicant or Drug Exposure—This assay can be used to assess mitochondrial function following toxicant or drug exposure. The precise length of exposure is at the discretion of the experimenter. However, if using PE255 N2 nematodes we recommend assaying prior to, or after the reproduction period, as reproduction will add variability to experiments. Nematodes can be exposed in liquid or on agar; however, liquid exposures can facilitate drug uptake in nematodes (Zheng et al., 2013). Finally, be sure to thoroughly rinse toxicant exposed nematodes 3-4 times with 15ml k-medium to remove excess toxicant prior performing this assay.

Preparation of Inhibitors

2. Prepare stocks of 2mM rotenone, 100mM TTFA, 15mM antimycin A, 2mM sodium azide, 2mM DCCD, 2.5mM FCCP, 10mM perhexiline, and 400mM 2-DG in either 100% unbuffered EPA water or DMSO as outlined in Table 1. To minimize freeze/thawing, stocks can be stored in 30µl aliquots at either 4°C or –20°C (see Table 1).

Titration of each drug was performed in sterile young adult (cultured on agar for 72h at 25°C) PE255 glp-4 nematodes (Supplementary Figures 1-8). Concentrations of each ETC inhibitor that result in a 40-60% depletion of ATP after a one hour exposure were then chosen.

A 4.5 hour exposure to 50mM 2-DG gave the most consistent reduction in luminescence in the context of arsenite exposure (Luz et al., Submitted), thus was chosen for all future experiments.

A 1 hour exposure to 100µM perhexiline increased nematode luminescence (~25%) in PE255 glp-4-deficient nematodes (Supplemental Figure 8), and thus was chosen for all future experiments. Our rationale for this result is detailed in the Anticipated Results, perhexiline section.

3. Dilute inhibitor stocks with unbuffered EPA H₂O to the appropriate 8X working concentrations as outlined in Table 1.

All inhibitors are dissolved in either DMSO or unbuffered EPA water. 8X Working stocks contain zero or 8% DMSO, such that when 12.5µl of the 8X working stock is pipetted into a well of a white 96-well plate (containing 50 nematodes in 87.5µl unbuffered EPA H₂O) the inhibitor is diluted to its final, 1X, working concentration in 1% DMSO. A one hour exposure to 1% DMSO does not significantly affect ATP levels in young adult PE255 glp-4, or L4 or 8 day old PE255 N2 nematodes (Supplemental Figure 1, 9).

Preparation of nematodes for inhibitor exposure

4. Remove PE255 nematodes from the incubator. If nematodes are being exposed to a drug or toxicant (either on agar or in liquid), be sure to rinse the nematodes thoroughly to remove excess toxicant.

Excess toxicant can be removed by transferring toxicant exposed nematodes to a new 15ml centrifuge tube, and resuspending them in 15ml unbuffered EPA H₂O. Nematodes can then be pelleted by centrifuging at 2200 RCF for 2 minutes at room temperature. The supernatant can then be discarded in accord with your university's guidelines. This process should then be repeated an additional 2-3 times to ensure toxicant is completely removed through dilution.

5. Resuspend the nematodes to a final concentration of 1.0 ± 0.2 nematodes per microliter in unbuffered EPA H₂O.

The minimal acceptable concentration is 0.6 nematodes per microliter, as this concentration results in approximately 50 nematodes per 87.5µl; however, we recommend diluting nematodes to a concentration of 0.8-1.2 nematodes per microliter for all samples to minimize variation.

To estimate the number of nematodes per microliter, trim the tip of a 200µl pipette tip, and pipette 20µl of 0.1% Triton X-100 up and down. The triton prevents worm loss due to sticking. Pipet four 20µl drops of nematodes onto a glass slide and count the number of nematodes per 20µl on a dissecting light microscope. Be sure to use a new, triton rinsed tip for each drop, and invert the centrifuge tube several times between counts to resuspend the nematodes.

6. Calculate the volume required to obtain 50 nematodes.

For example, if your concentration of nematodes is 1.0 nematode per microliter, you will pipette 50µl into each well of the 96-well plate to achieve 50 nematodes per well.

7. Pour 5ml 0.1% Triton X-100, 5ml unbuffered EPA H₂O, and the nematode suspension into three separate, new, 25ml disposable reagent reservoirs.

8. Using a 200µl multi-channel pipette, pipet 50 nematodes into the appropriate wells of a white 96-well plate.

Prior to pipetting nematodes into a white 96-well plate trim the pipet tips with scissors to increase each tips circumference, which allows large adult nematodes to be pipetted without injury. Then rinse the pipette tips with 0.1% Triton X-100 by pipetting up and down, which prevents nematode loss due to sticking. Nematodes can then be re-suspended in the reagent reservoir prior to their addition to the 96-well plate by pipetting up and down 3-4 times with the multi-channel pipette. Use new, trimmed, triton-rinsed tips each time you resuspend and transfer nematodes.

Figure 2 illustrates how a 96-well plate may be set up for an experiment containing two experimental groups. For example, each group (i.e. control and toxicant exposed) is pipetted into 4 wells of a 96-well plate for each inhibitor or control (i.e. EPA H₂O or 1% DMSO) used. We recommend setting up 2 plates, one for the one hour inhibitor exposure (rotenone, TTFA, antimycin A, azide, DCCD, FCCP, perhexiline), and one for the 4.5 hour inhibitor exposure (2-DG; not shown, but can be setup in a manner similar to Figure 2. Note that 2-DG does not require a 1% DMSO control, as it is dissolved in EPA H₂O).

9. Using a multi-channel pipette bring the volume in each well to 87.5µl with unbuffered EPA H₂O.

EPA H₂O controls (i.e. nematodes unexposed to inhibitors or DMSO) and blank wells can be brought to a final volume of 100µl with unbuffered EPA H₂O.
10. Using a 20µl pipette, add 12.5µl of each 8X inhibitor (prepared in steps 2-3 and outlined in Table 1) to the appropriate wells. Figure 2 outlines how nematodes can be loaded into a 96 well plate; however, this will vary depending upon the number of exposure groups, and inhibitors chosen for each experiment (outlined in Figure 2).

It should take no longer than 3-4 minutes to load all of the inhibitors for each plate. This is important, because the inhibitor incubation period is only 60 minutes (for ETC, and FAO inhibitors); thus, a longer loading period will introduce variability into the assay. If necessary, samples can be divided onto multiple 96-well plates to limit the amount of time it takes to load all of the inhibitors. However, be sure to include the appropriate EPA H₂O and DMSO controls, as well as blanks for each plate. The addition of inhibitors should be staggered 15-20 minutes for each plate to avoid overlap on the plate reader.

At minimum, two plates will be run. The first plate is designated for one hour inhibitor incubations (rotenone, TTFA, antimycin A, azide, DCCD, FCCP, perhexiline, EPA H₂O and 1% DMSO controls), while the second plate is for the 4.5 hour inhibitor incubations (i.e. 2-DG, EPA H₂O control).
11. Vortex the white 96-well plate for 10 seconds at 1000 rpm using a horizontal vortexer after the final inhibitor has been added.

Vortexing will help ensure that inhibitors are mixed and completely in solution.
12. Place the 96-well plate on an orbital shaker at 20°C for 60 minutes or 4.5 hours depending upon which inhibitors are being tested.

Measuring steady-state ATP levels—In the presence of ATP, firefly luciferase catalyzes the oxidation of luciferin to generate light. Thus, steady-state ATP levels can be determined *in vivo* by measuring light output, which is proportional to steady-state ATP levels in PE255 nematodes (Lagido et al., 2008). Nematode luminescence can be measured using a microplate reader equipped with a luminescence optic and filters capable of measuring GFP fluorescence (502nm emissions, 485nm excitation). Below we detail how to measure ATP in PE255 nematodes using a FLUOstar OPTIMA (BMG LABTECH) plate reader; however, precise instruction will vary depending upon the microplate reader model being used. A recent visual presentation of this assay is also available (Lagido et al., 2015).

- 13.** Prepare the luminescence buffer (see recipe) 15 minutes prior to the end of the incubation period.
- Luminescence buffer can be prepared in 15ml centrifuge tube covered in foil to protect light-sensitive luciferin.
- 14.** Turn on the plate reader and open the OPTIMA software 15 minutes before the incubation period has ended. Prepare the plate reader for measuring GFP fluorescence.
- PE255 nematodes express a firefly luciferase – GFP fusion protein. Thus by normalizing each wells luminescence reading to GFP, you can account for overall enzyme content, which will help to normalize each well for slight discrepancies in nematode size and overall nematode counts.
- 15.** Under reader configuration, select the fluorescence optic.
- 16.** Select test setup. Click on fluorescence intensity, and make a new program for measuring GFP. Name (i.e. PE255::GFP) and save the program for future use. Guidelines for preparing the program are outlined below.
- Plate type: (fill in with the appropriate plate brand)
- Optic used: Top
- Excitation filter: 485nm
- Emission filter: 502nm
- Position delay (s): 0.2
- Kinetic window: 1
- Number of cycles: 1
- Measurement start time (s): 0.0
- Number of flashes: 10
- 17.** Open the newly designed program. Under the Layout tab select the appropriate sample containing wells. Click Okay.
- 18.** Approximately 10 minutes before the incubation period has ended, insert the 96-well plate into the microplate reader. Click on measure and select the appropriate protocol for measuring GFP. Name the current run in the pop-up menu.
- 19.** Click on the gain tab and highlight the entire plate. Click gain adjust.
- Gain adjusting the entire plate will identify the well with the highest GFP fluorescence, which will be used to normalize the entire plate. The raw gain value should be somewhere around 58,000, although this may vary for other microplate reader models.

- 20.** Click start measurement. When the measurement has finished exit the OPTIMA software. Turn off the plate reader.
- GFP measurements are automatically saved in the OPTIMA software.
- 21.** Carefully remove the fluorescence optic from the microplate reader and replace it with the luminescence optic.
- For more details see the plate reader's user manual.
- 22.** Turn on the plate reader and open the OPTIMA software. Click on Reader Configuration and select luminescence optic.
- 23.** Prime the injector (if applicable) for luminescence buffer injection. First, insert the injector needle into a waste container (50ml centrifuge tube covered with foil) and the tubing into a 50ml centrifuge tube containing 70% ethanol. Next, select the priming function and flush the injector needle with 2ml 70% ethanol, following by 2ml ddH₂O, and finally prime the injector needle with 1.5ml of luminescence buffer.
- If your plate reader is not equipped with an injecting apparatus you can manually pipette the luminescence buffer into your 96-well plate using a multi-channel pipette and then read luminescence 3 minutes later.
- 24.** Remove the injector needle from the waste container and place it into the machine's injection port.
- 25.** Select test setup. Click on luminescence intensity, and make a new program for measuring luminescence. Name (i.e. PE255-Luminescence) and save the program for future use. Guidelines for measuring luminescence are outlined below.
- Plate type: (fill in with the appropriate plate brand)
- Optic used: Top
- Gain: 3600
- Emission filter: lens
- Position delay (s): 0.2
- Shaking width (mm): 7
- Shaking mode: orbital
- Number of cycles: 2
- Cycle time (s): 180
- Measurement start time (s): 0.0
- Measurement interval time (s): 1.0

Injector Setup

Volume (μ l): 50

Pump used: 1

Pump speed (μ l/s): 420

Pump syringe volume (ml): 0.5

Injection cycle: 1

Injection start time (s): 0.0

26. Open the newly designed program. Under the layout tab select the appropriate sample containing wells.

27. Click on the injection tab. Make sure all sample containing wells are set to have 50 μ l of luminescence buffer injected. Click Okay.

28. Click on Measure and select the appropriate protocol for measuring luminescence. Name the current run in the pop-up menu. Click start.

When the luminescence measurement finishes the results automatically save in the OPTMA software.

29. When finished with the instrument, wash the injector tubing. First, remove the injector needle from the plate reader and place it into the waste container. Place the injector tubing into ddH₂O, and rinse the tubing with 3ml ddH₂O, followed by 3ml 70% ethanol. Finally, dry the injector tubing by back flushing 3ml into the waste container.

Back flushing the injector pushes air through the line to dry it out; any remaining ethanol in the line should then evaporate.

30. Open results in the OPTIMA Software. Blank correct each well's GFP and luminescence values by subtracting the EPA H₂O blank GFP and luminescence values, respectively. An example of how to normalize data is provided in Supplemental File 1.

Each well's blank-corrected luminescence can be divided by the corresponding blank-corrected GFP value. Alternatively, we generate normalization factors for each well by dividing each wells blank-subtracted GFP value by the average GFP value for the entire plate. Blank-corrected luminescence values can then be divided by the corresponding normalization factor as detailed in Supplemental File 1.

Statistical Analysis

31. Assuming normally distributed data (which has been our experience), assess the effects of each drug initially with a one-, two-, or three-way ANOVA, depending upon how many factors you have (i.e. time, strain, dose, etc.). If different developmental stages are compared, ATP levels may vary enough that logarithmic transformation of the data is necessary to permit comparison of

exposure-or strain-related differences across ages. When warranted, post-hoc analysis can be performed.

SUPPORT PROTOCOL 1

Preparing OP50 seeded k-agar plates—Nematodes are cultured on *Escherichia coli* OP50 seeded k-agar plates. The preparation of OP50 seeded K-agar plates has previously been described (Lewis and Fleming, 1995; Stiernagle, 1999), and is briefly outlined below.

Materials: Potassium chloride (KCl),

Sodium chloride (NaCl)

Bacto-peptone

Bacto-agar

1M Calcium chloride (CaCl₂, dissolved in ddH₂O and autoclaved)

1M Magnesium sulfate (MgSO₄, dissolved in ddH₂O and autoclaved)

10 mg/ml Cholesterol (dissolved in ethanol and filter sterilized)

5mL 1.25 mg/ml Nystatin (dissolved in ethanol)

LB broth (see recipe)

E. coli OP50 (which can be purchased from the *Caenorhabditis* Genetics Center, University of Minnesota)

Erlenmeyer flask (2L or larger)

Magnetic spin bar

Magnetic hot plate

Autoclave tape

Autoclave

Serological pipette (25 or 50 mL)

Motorized pipette aid (e.g. Drummond)

Petri dishes (100 × 50mm)

Inoculating loop

37°C Shaking incubator

Repeating pipette (e.g. Eppendorf) & 10 mL displacement tips

Glass hockey stick spreaders

Rotating pedestal

Bunsen burner

Pouring k-agar plates

1. Weigh and add 2.36g potassium chloride, 3.0g sodium chloride, 2.5g bacto-peptone, and 20g bacto-agar to a 2L Erlenmeyer flask containing 1L ddH₂O and a magnetic spin bar. Cover the flask with foil and add autoclave tape.

Prior to autoclaving the agar can be mixed gently on a magnetic spin plate; however, this is not required, as autoclaving will dissolve all ingredients.

2. Autoclave to sterilize.

A 30 minute liquid cycle at 121°C and 17psi is sufficient for sterilization.

3. Place sterilized k-agar on a magnetic spin plate with the spin bar turned on and let the agar cool to ≈55°C.

4. Once cooled, add 1ml 1M CaCl₂, 1ml 1M MgSO₄, 1 ml 10 mg/ml cholesterol, and 5 ml 1.25 mg/ml nystatin (dissolve in 100% EtOH) to the k-agar.

The nystatin is an anti-fungal used to help prevent contamination.

5. Using a 50 mL serological pipet, carefully pipette 17mL k-agar into a 100×50mm sterile petri dish. Gently swirl the agar to ensure the entire surface of the plate is covered.

Caution! K-agar plates are easily contaminated. To minimize contamination risk use sterile technique. One liter of k-agar fills approximately 60 petri dishes.

6. Let the k-agar plates cool and solidify overnight (12-18 hours).

Growing *E. coli* OP50

7. Using sterile technique, inoculate 50mL sterile LB broth (see recipe) with *E. coli* OP50 using a sterile inoculating loop.
8. Incubate the LB broth at 37°C, while shaking at 250 rpm, overnight (16 hours).

Seeding K-agar plates with OP50

9. Using sterile technique pipette 300μl of OP50 culture onto the center of each k-agar plate.

We use a 10mL Eppendorf repeating pipettor, which minimizes the number of times that you will have to pipette directly from the OP50 culture, thus minimizing the risk of contamination.

10. Using a sterile glass hockey stick and a rotating pedestal spread the OP50 on the surface of the k-agar.

To sterilize the glass hockey stick dip it in 70% ethanol, and then pass it through the flame of a bunsen burner and allow the ethanol to burn off. Re-sterilize the hockey stick every five plates.

11. Let the OP50 seeded k-agar plates dry at room temperature (~48 hours). Dry OP50 plates can then be stored at 4°C for up to 3 months.

SUPPORT PROTOCOL 2

Age-synchronizing nematodes via sodium hypochlorite treatment—

Synchronous populations of L1 nematodes can be generated by treating gravid adult nematodes with sodium hypochlorite solution. Nematode eggs are resistant to sodium hypochlorite, while gravid adults are not, which allows for the isolation of eggs. Eggs are then allowed to hatch overnight (12-18 hours) in the absence of food, resulting in synchronous populations of L1 nematodes (Lewis and Fleming, 1995). Note that the timing may need to be reduced if using RNAi or crossing luciferase reporter strains with strains carrying mutations, because some genetic manipulations sensitize eggs to this treatment.

Materials: OP50 seeded k-agar plates (see Support Protocol 1) containing gravid adult nematodes

K-medium (see recipe)

70% ethanol

15 mL Centrifuge tubes (e.g. Corning Falcon)

Centrifuge (e.g. Beckman Coulter equipped for 15mL tubes)

Glass hockey stick spreader

Bunsen burner

Orbital shaker

Pasteur pipette

50ml cell culture flask

Dissecting light microscope

1. Using sterile k-medium wash gravid adult nematodes from the k-agar plate into a new 15 mL centrifuge tube. Pellet nematodes by centrifuging at 2200 RCF for 2 minutes at room temperature. Discard the supernatant.

2. Pipette 2-3ml k-medium onto k-agar plate and gently loosen eggs from the surface of the agar using a sterile glass hockey stick spreader. Carefully pour the loosened eggs into the 15 ml centrifuge tube containing the gravid adult nematodes, and spin at 2200 RCF for 2 minutes. Discard the supernatant.

Glass hockey stick spreaders can be sterilized by dipping them in 70% ethanol, and then passing them through the flame of a Bunsen burner, allowing the ethanol to burn off.

3. Carefully add 10ml of sodium hydroxide bleach solution (see recipe) to the nematode pellet.
4. Place the centrifuge tube on an orbital shaker in a 20°C incubator for 8 minutes.

8 minutes is sufficient time for the sodium hydroxide bleach solution to disintegrate adult nematodes, but not eggs, allowing for the isolation of a large quantity of nematode eggs. After 8 minutes, if adult nematodes remain, place the tube back on the orbital shaker for up to 2 additional minutes.

5. After 8 minutes, spin the centrifuge tube at 2200 RCF for 2 minutes, discard the supernatant, and resuspend the pelleted eggs in 15 ml k-medium.

Take care not to disturb the egg pellet when removing the bleach solution; however, no more than 50-100µl of bleach solution should remain prior to resuspension in k-medium.

6. Spin the resuspended eggs for an additional 2 minutes at 2200 RCF. Discard all but 0.5-1.0ml of the supernatant.
7. Using a sterile glass Pasteur pipette, resuspend the pelleted eggs, and transfer them to a sterile 50ml cell culture flask containing 8ml complete k-medium (see recipe).
8. Incubate the flask overnight (12-18 hours) on an orbital shaker at 20°C to obtain a synchronous population of L1 nematodes.
9. Under a dissecting light microscope check to make sure the majority of the eggs have hatched. Pour the L1 nematodes into a sterile 15 mL centrifuge tube, and centrifuge at 2200 RCF for 2 minutes. Discard the supernatant.
10. Transfer the nematodes to an OP50 seeded k-agar plate (see support protocol 1) using a Pasteur pipette and incubate the nematodes at the appropriate temperature until the desired life stage is reached.

REAGENTS AND SOLUTIONS

Complete k-medium

150µl 1M calcium chloride (CaCl_2 ; sterilized via autoclave)

150µl 1M magnesium sulfate (MgSO_4 ; sterilized via autoclave)

25µl 10mg/ml cholesterol (dissolve in 100% ethanol and filter sterilize)

50mL sterile k-medium

Store at room temperature for up to one week

K-medium

2.36g Potassium chloride (KCl)

3g Sodium chloride (NaCl)

1L ddH₂O

Autoclave to sterilize

Store at room temperature, indefinitely, under sterile conditions

LB broth

0.5g Tryptone

0.25g Yeast extract

0.5g Sodium chloride (NaCl)

50mL ddH₂O

Autoclave to sterilize

Store at room temperature, indefinitely, under sterile conditions

Luminescence Buffer

6.925ml 0.2M Na₂PO₄ (store at room temperature)

3.075ml 0.1M Citric acid (store at room temperature)

100µl DMSO

100µl 5% Triton X-100 (diluted in ddH₂O)

100µl 10mM Luciferin salt (dissolved in ddH₂O; store in 100µl aliquots at -20°C ; protect from light)

Make fresh prior to ATP measurements

Sodium hydroxide bleach solution

6mL Clorox bleach (non-germicidal, regular bleach*, 8.25% sodium hypochlorite)

5 Sodium hydroxide pellets (NaOH; Avantor Performance Materials)

44mL ddH₂O

Shake until NaOH pellets are completely dissolved

Each pellet weighs ~89mg.

Store at room temperature for up to 3 days.

*It is important to use non-germicidal bleach, as some bleaches contain germicides that are toxic to nematodes.

Unbuffered EPA water (Weber, 1991)

60mg Magnesium sulfate ($\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$)

60mg Calcium sulfate ($\text{CaSO}_4 \cdot 2 \text{H}_2\text{O}$)

4mg Potassium chloride (KCl)

1L ddH₂O

Store at room temperature, indefinitely, under sterile conditions

COMMENTARY

Background Information

The protocol described in this unit details how to rapidly assess the contribution of different pathways to steady-state ATP levels following drug or toxicant exposure in the model organism *C. elegans*. As mitochondrial function is dependent upon cellular context (Chan, 2012; McBride et al., 2006), this approach offers the advantage of an *in vivo* model, as well as all of the other benefits associated with working with nematodes. For example, simple RNAi gene knockdown technology (Kamath et al., 2003), and/or outbreeding the PE255 transgene into any of thousands of genetically deficient strains (Thompson et al., 2013) could extend this protocol's utility to include genetic and gene-environment interaction studies.

Although this protocol offers many advantages over other assays capable of assessing mitochondrial respiratory chain health, it also has limitations. This protocol cannot distinguish between direct enzyme inhibition, substrate limitation, or changes in overall enzyme content as causative of observed changes in inhibitor based ATP-depletion. Instead, this protocol offers an economical way to thoroughly assess mitochondrial health that can then be followed up with more targeted assays, such as metabolomics, Seahorse Analysis, and gene or protein expression studies. Another drawback of this protocol is that it does not directly measure changes in steady-state ATP levels, but instead measures changes

bioluminescence generated by the ATP-powered firefly luciferase enzyme. However, targeting of mitochondrial respiratory chain genes by RNAi gave a bioluminescence response that correlated with steady-state ATP levels in PE255 nematodes (Lagido et al., 2015; Lagido et al., 2008). Finally, differences in ATP depletion between control and exposed nematodes may be due to compensatory increases in ATP production via other routes, such as glycolysis. However, if this is the case then toxicant-exposed nematodes would be expected to be less sensitive to ATP depletion induced by all ETC inhibitors; thus, toxicant-induced changes at only one or two of the ETC complexes is highly suggestive of altered function at the site of inhibition rather than a compensatory increase in ATP production via an alternative route.

In addition to the inhibitors optimized for this protocol, inhibitors of other metabolic pathways could also be used to further assess toxicant-induced mitochondrial dysfunction. For example, dichloroacetate (DCA), a pyruvate dehydrogenase kinase (negative regulator of the Krebs cycle) inhibitor could be used to assess changes in Krebs cycle activity. We have previously tested this inhibitor in the context of arsenite exposure, but observed no significant changes in bioluminescence under any conditions tested (1-6 hour exposure to either 0.5 or 1.0mM DCA) (Luz et al., Submitted). Likewise, Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), a glutaminase (GLS1) inhibitor, could be used to test for toxicant-induced changes in glutamine metabolism (Shukla et al., 2012). However, not all metabolic inhibitors will inhibit their intended targets in nematodes. For example, the mitochondrial uncoupler 2,4-dinitrophenol and the ATP synthase inhibitor oligomycin A do not inhibit mitochondrial respiration in nematodes (Luz et al., 2015a). It is probable that the nematode cuticle, a thick collagenous barrier, limits the uptake of these inhibitors, as oligomycin can inhibit mitochondrial respiration in cuticle-deficient (*bus-8*) nematodes (Luz et al., 2015a); thus, we use the less-specific ATP synthase inhibitor, DCCD, in the current protocol.

Finally, under certain conditions the ETC complex II inhibitor TTFA can also function as a mitochondrial uncoupler. However, 1mM TTFA did not increase mitochondrial respiration (Supplemental Figure 13), suggesting that TTFA-induced depletion of ATP (Supplemental Figure 3) is due to complex II inhibition. Nonetheless, we have also optimized malonate, a competitive inhibitor of complex II for the current protocol (Thorn, 1953). Exposure to 15mM malonate for one hour reduces ATP by approximately 50% (Supplemental Figure 14A), and reduces mitochondrial respiration (Supplemental Figure 15); however, higher concentrations of malonate were inexplicably found to reduce GFP fluorescence (Supplemental Figure 14B), which under certain conditions may confound results. Thus, we recommend researchers consider the caveats to working with TTFA and malonate prior to performing experiments.

Critical Parameters

Number of nematode per well—This protocol has been optimized to work with 50 nematodes per well of a 96-well plate, and has successfully been used with both L4 and 8 day old adult nematodes. Fewer nematodes (25) have been tested, but tend to give variable luminescence values (data not shown). Alternatively, if this assay is to be used with L2 or L3

nematodes, the experimenter will have to load >50 nematodes per well, but must be careful not to overload the wells as this could result in anoxic conditions.

Age and genetic background of nematodes—Early and later life stages are widely considered more sensitive to certain exposures. Thus, concentrations of inhibitors may need to be adjusted depending upon the life stage being investigated. In agreement with this, we observed increased sensitivity to ATP depletion with rotenone, antimycin A, and FCCP in 8 day old PE255 N2 nematodes (Figures 3-5). In contrast, reduced sensitivity to TTFA was observed in 8 day old PE255 N2 nematodes (Figure 6), while no age related sensitivities were observed for azide, DCCD, or 2-DG (Supplemental Figures 10-12).

Genetic deficiencies can also sensitize (or protect) an organism from toxicity following toxicant exposure. Therefore, if this protocol is adapted to RNAi studies or if the PE255 transgene is crossbred into other genetically-deficient strains, inhibitor concentrations may need to be adjusted. However, we have crossbred the PE255 transgene into mitochondrial fission-deficient nematodes (*drp-1*), and observed similar responses to inhibitors as with PE255 N2 nematodes (data not shown). Interestingly, young adult PE255 *glp-4* nematodes appear less sensitive to TTFA than either L4 or 8 day old PE255 N2 nematodes, as 1mM TTFA reduced luminescence ~50% in *glp-4* (Supplemental Figure 3) and 80-99% in N2 nematodes (Figure 6). On the other hand, young adult PE255 *glp-4* nematodes appear more sensitive to sodium azide than either L4 or young adult PE255 N2, as 0.25mM azide reduced luminescence ~50% in *glp-4*-deficient nematodes (Supplemental Figure 5), but did not have a statistically significant effect on luminescence in PE255 N2 (Supplemental Figure 10). Thus, concentrations of TTFA (500µM is effective for N2 versus 1000µM for *glp-4*) and sodium azide (500µM is effective for N2 versus 250µM for *glp-4*) will need to be adjusted depending upon the genetic background.

Troubleshooting

Table 2 highlights some of the common problems encountered with this protocol, indicates potential causes, and outlines potential solutions to these problems.

Anticipated Results

ETC Inhibitors—Incubation with inhibitors of the mitochondrial ETC (i.e. rotenone, TTFA, antimycin A, sodium azide, DCCD, FCCP) should dramatically (40-60%) reduce nematode luminescence, as these inhibitors directly interfere with ATP production via oxidative phosphorylation. Therefore, if prior toxicant exposure alters the activity of one or more ETC complexes the magnitude of ATP depletion will be significantly altered compared to unexposed nematodes. For example, if toxicant exposure reduces complex I activity the magnitude of ATP depletion following a one-hour incubation with rotenone will be reduced compared to unexposed nematodes.

2-Deoxy-D-Glucose—If drug or toxicant exposure increases glycolysis, then incubation with the glycolysis inhibitor 2-DG should reduce nematode luminescence, whereas 2-DG should have little to no effect on untreated nematode bioluminescence because of the relatively small baseline contribution of glycolysis to ATP.

Perhexiline—Perhexiline, a prophylactic anti-anginal medication, prevents mitochondrial fatty acid oxidation (FAO) by inhibiting mitochondrial carnitine palmitoyltransferase-1 (CPT-1), thus preventing the transport of long chain fatty acids into mitochondria (Kennedy et al., 1996). Inhibition of FAO with perhexiline results in a shift in cardiac metabolism from the utilization of fatty acids to glucose, which is beneficial because glucose oxidation requires less oxygen per unit of ATP generated. Thus, perhexiline increases cardiac efficiency (Kjekshus and Mjøs, 1972; Mjos and Kjekshus, 1971). Like cardiac myocytes (Stanley et al., 1997), germline-deficient nematodes elevate fatty acid oxidation (Ratnapan et al., 2014). Thus, we hypothesized that treatment of PE255 *glp-4*-deficient nematodes with perhexiline would increase nematode luminescence by increasing the efficiency of ATP production, which our findings support (Supplemental Figure 8). In the context of toxicant exposure the effect of perhexiline on luminescence may prove more difficult to interpret. We postulate that toxicant exposures that disrupt glucose catabolism may result in increased FAO, in which case perhexiline would be expected to decrease nematode luminescence by inhibiting FAO. This would provide initial evidence for toxicant-induced changes in FAO that can be confirmed with gene expression or metabolomics studies.

Time Considerations

It will take approximately 48 hours to culture a synchronous population of L4 PE255 nematodes; however, the overall duration of nematode culturing will depend upon the desired larval stage when toxicant exposure is to be initiated. Likewise, the length of toxicant exposure will vary from experiment to experiment. However, following toxicant exposure the entire assay can be performed in approximately six hours (1.5 hours to prepare inhibitors, load nematodes into 96-well plates and start inhibitor exposures, and 1 - 4.5 hours of inhibitor exposure).

When performing assays inhibitors must be pipetted into the appropriate wells of the 96-well plate in a timely manner. This is especially important for the 60 minute inhibitor incubation, as delays will introduce variability into the experiment. The addition of all inhibitors should take no more than 3-4 minutes when using a single channel pipette; however, this can be further reduced by using a multi-channel pipette or by splitting samples onto multiple 96-well plates. However, the addition of inhibitors should be staggered by 15-20 minutes if multiple 96-well plates are being run to ensure no overlap on the microplate reader.

Approximately 10-15 minutes prior to the end of the inhibitor exposure the plate reader can be prepped for the pending GFP and luminescence measurements. Depending upon the plate reader, it will take 5-10 minutes to gain adjust and measure GFP per 96-well plate. Thus to avoid extended incubation periods we typically begin GFP measurements 5-10 minutes before the end of the incubation period. This allows luminescence to be measured immediately following the end of the 1 or 4.5 hour incubation period, which will help limit variability between experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Workflow for assessing toxicant-induced mitochondrial dysfunction in luciferase expressing PE255 *C. elegans*.

Figure 2.
Example of how toxicant exposed nematodes can be loaded into a 96-well plate for inhibitor exposure.

Figure 3.

Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 20 μ M rotenone than 2 day old nematodes (2 way ANOVA, main effects of time ($p=0.006$), treatment ($p<0.0001$), and their interaction ($p=0.008$)). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey's HSD). $N=3-4$. Bars $\pm$ SEM.

Figure 4.

Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 150 μ M antimycin A than 2 day old nematodes (2 way ANOVA, main effect of treatment ($p < 0.0001$), and time*treatment interaction ($p = 0.002$), but not time ($p = 0.19$)). Asterisk denotes statistical significance ($p < 0.05$) for post-hoc comparison (Tukey's HSD). $N = 3-4$. Bars $\pm$ SEM.

Figure 5.

Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 25 μ M FCCP than 2 day old nematodes (2 way ANOVA, main effects of time ($p=0.02$), treatment ($p<0.0001$), and their interaction ($p=0.02$)). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey's HSD). $N=3-4$. Bars $\pm$ SEM.

Figure 6.

Eight day old PE255 N2 nematodes are less sensitive to ATP depletion following a one hour exposure to 1mM TTFA than 2 day old nematodes (2 way ANOVA, main effects of time ($p=0.01$), treatment ($p<0.0001$), and their interaction ($p=0.008$)). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey's HSD). $N=3$. Bars $\pm$ SEM.

Table 1**Preparation of Inhibitors**

Inhibitor (Target)	Stock Concentration	Working Concentration (8x)	Final Concentration (1x)	Incubation period (Hours)
Rotenone (ETC Complex I)	2mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)	160µM Dissolved in 8% DMSO To make: Add 24µl 2mM Rotenone (100% DMSO) to 276µl unbuffered EPA H ₂ O	20µM in 1% DMSO	1
* TTFA (ETC Complex II)	100mM dissolved in 100% DMSO (store at 4°C in 30µl aliquots)	8mM Dissolved in 8% DMSO To make: Add 24µl 100mM TTFA to 276µl unbuffered EPA H ₂ O	1mM in 1% DMSO	1
‡ Malonate (ETC Complex II)	120mM dissolved in 100% unbuffered EPA H ₂ O (store at 4°C in 1mL aliquots)	120mM dissolved in 100% unbuffered EPA H ₂ O	15mM in 100% unbuffered EPA H ₂ O	1
Antimycin A (ETC Complex III)	15mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)	1.2mM Dissolved in 8% DMSO To make: Add 24µl 15mM Antimycin A (100% DMSO) to 276µl unbuffered EPA H ₂ O	150µM in 1% DMSO	1
** Sodium Azide (ETC Complex IV)	2mM dissolved in 100% unbuffered EPA H ₂ O (store at 4°C in 1mL aliquots)	2mM dissolved in 100% unbuffered EPA H ₂ O	250µM in 100% unbuffered EPA H ₂ O	1
DCCD (ATP synthase)	2mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)	160µM Dissolved in 8% DMSO To make: Add 24µl 2mM DCCD (100% DMSO) to 276µl unbuffered EPA H ₂ O	20µM in 1% DMSO	1
FCCP (Mitochondrial uncoupler)	2.5mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)	200µM Dissolved in 8% DMSO To make: Add 24µl 2.5mM FCCP (100% DMSO) to 276µl unbuffered EPA H ₂ O	25µM in 1% DMSO	1
Perhexiline (Fatty acid oxidation)	10mM dissolved in 100% DMSO (store at 4°C in 30µl aliquots)	800µM Dissolved in 8% DMSO To make: Add 24µl 10mM Perhexiline (100% DMSO) to 276µl unbuffered EPA H ₂ O	100µM in 1% DMSO	1
2-DG (Glycolysis)	400mM dissolved in unbuffered EPA H ₂ O (store at 4°C in 30 µl aliquots)	400mM dissolved in unbuffered EPA H ₂ O	50mM in 100% unbuffered EPA H ₂ O	4.5

Concentrations of ETC inhibitors listed in Table 1 caused roughly a 40-60% reduction in bioluminescence in young adult PE255 *glp-4* deficient nematodes (Supplemental Figures 2-7), and with the exceptions of TTFA and sodium azide, cause similar reductions in both L4 and 8 day old PE255 N2 nematodes.

* 500µM TTFA reduces luminescence approximately 50% in PE255 N2 nematodes (data not shown), while 1000µM causes an 80-99% reduction in PE255 N2 bioluminescence (Figure 6).

** 250µM Sodium azide has no significant effect on PE255 N2 bioluminescence, while 500µM azide reduces bioluminescence ~50%.

‡ Malonate, a competitive inhibitors of ETC complex II, can be used in place of TTFA at the discretion of the experimenter. Pros and cons of this are discussed in the Background Information section.

TABLE 2

Trouble Shooting

Problem	Possible cause	Solution
Inhibitor caused no, or only a minor decrease in luminescence	Concentration of inhibitor is too low.	Increase inhibitor concentration.
	Inhibitor precipitated out of solution	It is imperative that inhibitor stocks equilibrate to room temperature prior to their addition to the white 96-well plates, as the rapid temperature change may cause the inhibitors to precipitate out of solution.
	Luminescence optic not installed properly	Check that the luminescence optic has been installed properly.
	Luciferin was not added to the luminescence buffer.	Re-prepare the luminescence buffer taking care to add luciferin to the buffer.
	Luminescence buffer failed to inject	Ensure that your plate reader's injector needle has been properly installed and/or that luminescence buffer was properly added prior to measuring luminescence.
Inhibitor resulted in greater than a 90% loss of luminescence.	Inhibitor concentration is too high.	Decrease the concentration of inhibitor used.