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Seahorse Xf^{e}24 Extracellular Flux Analyzer-based analysis of cellular respiration in *Caenorhabditis elegans*

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Abstract

Mitochondria are critical for their role in ATP production as well as multiple nonenergetic functions, and mitochondrial dysfunction is causal in myriad human diseases. Less well appreciated is the fact that mitochondria integrate environmental and inter- as well as intracellular signals to modulate function. Because mitochondria function in an organismal milieu, there is need for assays capable of rapidly assessing mitochondrial health *in vivo*. Here, using the Seahorse XF^{e}24 Extracellular Flux Analyzer and the pharmacological inhibitors dicyclohexylcarbodiimide (DCCD, ATP synthase inhibitor), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, mitochondrial uncoupler) and sodium azide (cytochrome c oxidase inhibitor), we describe how to obtain *in vivo* measurements of the fundamental parameters (basal oxygen consumption rate (OCR), ATP-linked respiration, maximal OCR, spare respiratory capacity and proton leak) of the mitochondrial respiratory chain in the model organism *Caenorhabditis elegans*.

Keywords

Mitochondrial toxicity; Mitochondrial respiration; Seahorse XF^{e}24; *Caenorhabditis elegans*

INTRODUCTION

Mitochondria, best known for producing ATP via oxidative phosphorylation (OXPHOS), also house all or part of many other important metabolic pathways such as the Krebs cycle (Fernie et al., 2004) and fatty acid β-oxidation (Houten and Wanders, 2010), making them the nexus of intermediary metabolism. Therefore it is not surprising that mitochondrial dysfunction is causal and/or implicated in myriad human diseases, including cancer (Frezza and Gottlieb, 2009; Gogvadze et al., 2008; Wallace, 2012), neurodegeneration (Beal, 2005; Lin and Beal, 2006), and metabolic disorders (Smeitink et al., 2006). In fact, as many as 1 in

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5,000 individuals are estimated to be afflicted by mitochondrial disease (Schaefer et al., 2004). Furthermore, mitochondria integrate environmental and intercellular signals to meet cellular energy demands (Chan, 2012). Many pharmacological (Neustadt and Pieczenik, 2008) and environmental toxicants (Meyer et al., 2013) are known mitochondrial poisons that can interfere with this signaling. Understanding how genetics, toxicant exposure and gene-environment interactions affect mitochondrial function *in vivo* will be crucial in preventing, diagnosing and treating mitochondrial disease in the future.

The model organism *Caenorhabditis elegans*, a free-living, multicellular nematode, offers many advantages over other model organisms for studying mitochondrial function *in vivo*. Ease and low cost of maintenance, a short life cycle (~2 weeks), and a high reproductive rate (~300 offspring per wild-type nematode) make *C. elegans* a powerful model for medium-throughput experiments (Boyd et al., 2010; Boyd et al., 2009). Furthermore, a fully sequenced and annotated genome (Consortium, 1998), conserved biochemical pathways (Kaletta and Hengartner, 2006) and mitochondrial biology (Tsang and Lemire, 2003a), and availability of genetic mutants (Thompson et al., 2013) and simple RNAi knockdown technology (Fraser et al., 2000; Kamath et al., 2003) make *C. elegans* a good model for studying mitochondrial function *in vivo.* Current tools for assessing mitochondrial health in nematodes include *in vivo* analysis of mitochondrial morphology (Addo et al., 2010) and ATP levels (Lagido et al., 2008) using transgenic reporter strains, oxygen consumption via low throughput Clarke-type electrode oxygen meters (Braeckman et al., 2002), and time consuming biochemical analysis of extracts (Krijgsveld et al., 2003). Here, using the Seahorse XF^{e}24 Extracellular Flux Analyzer (Seahorse Bioscience, Massachusetts, USA) we describe how to measure the fundamental parameters of the electron transport chain (ETC): basal oxygen consumption rate (OCR), ATP-linked respiration, maximal OCR, spare respiratory capacity, and proton leak.

BASIC PROTOCOL 1: in vivo quantification of the fundamental parameters of the mitochondrial electron transport chain in larval stage four nematodes

Using the pharmacological inhibitors dicyclohexylcarbodiimide (DCCD, ATP synthase inhibitor), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, mitochondrial uncoupler) and sodium azide (cytochrome c oxidase inhibitor), we describe how to measure the fundamental parameters of the mitochondrial ETC, including basal OCR, ATP-linked respiration (basal OCR minus DCCD inhibited OCR), maximal OCR (FCCP-induced OCR), spare respiratory capacity (FCCP-induced OCR minus basal OCR), and proton leak (DCCD inhibited OCR minus sodium azide inhibited OCR), *in vivo*, in larval stage four (L4) *C. elegans*. Although we outline this assay for use with L4 nematodes, as the L3/L4 transition is accompanied by a metabolic switch from glycolysis to oxidative phosphorylation (Bratic et al., 2009; Leung et al., 2013; Tsang and Lemire, 2002; Tsang and Lemire, 2003b), this protocol can easily be adapted for analysis of mitochondrial function in older and/or younger nematodes, mutant strains, or for mitochondrial toxicity testing, as outlined throughout the protocol.

Materials

- XF Flux pack. Contents include:
  1. 18 XF^{e}24 extracellular flux assay kits (each kit contains a sensor cartridge, lid, hydrobooster, and 24-well utility plate)
  2. 500 mL XF Calibrant
  3. 20 XF cell culture microplates
- OP50 K-agar plates with synchronous populations of L4 nematodes (see support protocol 1)
- 2.0mM DCCD (dissolved in DMSO, store at −20°C)
- 1.25mM FCCP (dissolved in DMSO, store at −20°C)
- 80mM sodium azide (dissolved in unbuffered EPA water (see recipe), store at 4°C)
- 0.1% Triton X-100 (diluted in ddH_2O, store at room temperature)
- 10% Sodium dodecyl sulfate (SDS) solution (dissolved in ddH_2O, store at room temperature for up to 6 months)
- K-medium (see recipe)
- Unbuffered EPA water
- Thermo Scientific^{TM} Pierce^{TM} BCA^{TM} Protein Assay BCA Assay
- 15mL Centrifuge tubes
- Microscope Slides

Equipment

- Seahorse XFe24 Extracellular Flux Analyzer & computer (provided with flux analyzer with XF^{e}24 Wave software installed)
- Orbital shaker
- 20°C incubator
- Dissecting light microscope
- Ultrasonicator (Ultrasonic homogenizer, Model 3000, Biologics, Inc., Virginia, USA)
- Microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany, with filters capable of measuring absorbance in the range of 540–590nm)

Hydrating the Seahorse XF^{e}24 sensor cartridge

1 Pipette 1.0mL of XF Calibrant solution into each well of a 24-well utility plate, then place the hydro-booster (pink spacer) onto the utility plate, followed by the sensor cartridge and finally the lid. Place the sensor cartridge out of direct sunlight to hydrate for a minimum of 4 hours.

IMPORTANT. The sensor cartridge must be left hydrating for a minimum of 4 hours prior to use. We typically allow the sensor cartridge to hydrate overnight (12–18 hours) in a 25°C incubator, but have successfully used cartridges that have been left to hydrate for up to 4 days at 25°C.

A diagram of how to hydrate a sensor cartridge is provided by the manufacturer with each XF Flux pack.

Nematode culturing

Note: Nematodes are cultured on k-agar plates seeded with *Escherichia coli* OP50 at 20°C as previously described (Stiernagle, 1999). Synchronous populations of L1 nematodes are obtained by dissolution of gravid nematodes using a hypochlorite bleach solution as described in Support Protocol 1 and (Lewis and Fleming, 1995).

2 Using a Pasteur pipette, transfer synchronized populations of L1 nematodes, obtained by hypochlorite bleaching (Supporting Protocol 1), onto OP50 seeded k-agar plates at 20°C. Incubate the nematodes at 20°C until a synchronous population of L4 nematodes is obtained.

In order to obtain a synchronous population of L4 nematodes, the OP50 food source should never be allowed to become depleted; developing nematode populations should be transferred to fresh OP50 k-agar plates as necessary. This is of particular importance in the context of measurement of metabolic function, because nematodes respond to food scarcity by altering their metabolic programming.

We find that synchronous L1 wild-type N2 nematodes reach L4 after being incubated with OP50 at 20°C for 48 hours. However, if you are working with growth delayed strains, a longer incubation period may be required to obtain a synchronous population of L4s. For example, complex III mutant isp-1 (qm150) requires ~96h to reach L4 and outer/inner mitochondrial fusion-deficient fzo-1 (tm1133) and eat-3(ad426) nematodes require incubation with OP50 at 20°C for 72h.

Seahorse XFe24 Analyzer WAVE software set-up

3 Turn on the Seahorse XF^{e}24 and start the Wave software.

4 In the WAVE software click on the “New” tab, then highlight the “Blank” option and click “Design” to start designing a new assay.

5 Click the “Assay Conditions” tab, followed by the “Cell Type” tab, and then click “Add Cell Type Condition”. Add the appropriate information to the Name (strain, larval stage, treatment etc.), Cell Type (*C. elegans and/or strain name*) and Seeding Density (number of nematodes added per well) fields.

If more than one strain or treatment is being tested in the assay you can add additional cell types by clicking on the “Cell Type” heading and

then clicking “Add”. This allows you to name each cell type as necessary.

We typically run each Seahorse plate with 4 strains or treatments, such that each strain or treatment is run in 5 to 6 wells of an islet plate. Also note that two wells must be designated as blanks (we typically use wells A1 and D6, although any wells can be used for this purpose), leaving 22 wells available for samples.

6 Click on the “Injections” tab, followed by “Add Injection Strategy”, then highlight port A and click “Add”. Add the appropriate information to the Compound (i.e. FCCP, DCCD, or sodium azide), Concentration (final drug concentrations are 25μM, 20μM, and 10mM for FCCP, DCCD, and sodium azide, respectively), Solvent (i.e. DMSO) and Percent Solvent (final percent DMSO is 2, 1, or 0% for FCCP, DCCD, and sodium azide, respectively) fields.

*Drugs cannot be injected in tandem from the sensor cartridge. We have found that the magnitude of sodium azide’s effect is significantly reduced if injected post-FCCP (*Luz et al., In Press*). It may be possible to inject another complete respiratory inhibitor (i.e. rotenone and antimycin A or cyanide) post-FCCP injection, however this has not been tested. Likewise, it may also be possible to inject sodium azide after DCCD, increasing sample throughput.*

We have also found that sensor cartridges cannot be reused after drug injection. For example, basal OCR is elevated in nematodes when a sensor cartridge previously used for FCCP injection is used. Thus we test each drug in its own new sensor cartridge.

7 Click “Generate Groups” and then click on the “Plate Map” tab. Assign the groups to the plate map by clicking on the first group (i.e. control or wild-type strain) and then click on the wells of the 24-well plate map where you will load the group into the 24-well islet plate. Assign all of your groups an equal number of wells on the plate map, leaving 2 wells as blanks (again, we typically use A1 and D6).

Groups should be assigned randomly to an equal number of wells across the plate to avoid potentially confounding plate effects.

8 Select the “Instrument Protocol” tab to set up the OCR measurements protocol. Click on the “Edit Measurement Details” dropdown tab under the “Basal Measurement Cycles” heading and change the number of cycles to 8, the mix cycle to 1 (to oxygenate the micro-chamber), the wait cycle to 3 (to allow nematodes to settle) and the measure cycle to 3 minutes.

The mix cycle is very important. Oxygen levels will fall during the 3 minute measurement cycle, but should not fall far below 100mmHg. If oxygen levels do fall below 100mmHg, then they should recover after the mix cycle (~160mmHg). If oxygen levels do not recover, it is a

good indication that too many nematodes have been loaded per well (Kevin Bittman, Ph.D., Field Applications Scientist, Seahorse Biosciences, Inc., personal communication).

9 Click the "Injection" button, select the port that you will be injecting your drug from (typically port A), and name your injection strategy (i.e. 25μM FCCP, 20μM DCCD or 10mM sodium azide). Click on the "Edit Measurement Details" dropdown tab under the injection strategy heading and change the number of cycles.

The number of cycles will vary depending on which inhibitor you are injecting. We use 8, 14, or 4 measurement cycles to measure nematode response to FCCP, DCCD, or sodium azide respectively. The Mix (1 minute), Wait (3 minutes), and Measure (3 minutes) cycle times mentioned in step 8 do not change.

10 Click on the "Review and Run" tab. Name and save the assay.

Preparation of ETC inhibitors for injection (Summarized in Table 1)

11 Prepare stocks of 80mM sodium azide in EPA water, 1.25mM FCCP in DMSO, and 2mM DCCD in DMSO.

Store single use aliquots of 350μl 1.25mM FCCP and 200μl 2mM DCCD at −20°C to limit freeze thawing your stocks of the inhibitors. 80mM sodium azide should be stored at 4°C.

12 Dilute the ETC inhibitor stocks to the appropriate port concentration as described in Table 1.

13 Pipette 75μl (8X concentrated) of the desired ETC inhibitor into the appropriate injection ports of the sensor cartridge.

75μl of the appropriate ETC inhibitor are loaded into the sensor cartridge port at an 8X concentration. Thus, for example, when 75μl of 200 μM FCCP in 16% DMSO are injected into a well containing nematodes in 525μl EPA water, it is diluted to a final working concentration of 25μM in 2% DMSO (i.e. 1:8 dilution).

14 Under the "Review and Run" tab click "Start Run" and the Seahorse Analyzer's tray will slide out. Remove the lid and hydro-booster and place the sensor cartridge and utility plate on the Seahorse Analyzer's tray. Click "ready" to start calibrating the sensor cartridge.

The calibration process takes approximately 20 minutes. During this time, you will add your synchronous L4 nematodes to a Seahorse Analyzer islet plate (step 15).

Preparation of nematodes for Seahorse XF^{e}24 Analyzer assay

15 Using sterile k-medium wash your synchronized population of L4 nematodes (from step 2) off of an OP50 seeded k-agar plate into a 15mL centrifuge tube.

Centrifuge 30 seconds at 2200 RCF or let nematodes settle by gravity (this is most effective with larger nematodes). Aspirate the supernatant. Resuspend the nematodes in 10mL k-medium, and repeat the pelleting and rinsing process.

This step removes live OP50 from the medium that could otherwise confound OCR measurements. This issue could also be overcome by feeding nematodes either heat- or UVC-inactivated bacteria.

16 After the second k-medium rinse, resuspend the nematodes in 10mL k-medium and place the centrifuge tube on an orbital shaker at 20°C for 20 minutes.

This provides the nematodes time to clear live OP50 from their guts, which could confound OCR measurements.

17 After the 20 minute incubation, allow the nematodes to settle by gravity or centrifuge for 30s at 2200 RCF. Aspirate the supernatant. Resuspend the nematodes in unbuffered EPA water to a concentration of 1 ± 0.2 nematodes per microliter (step 18).

18 To estimate the number of nematodes per microliter, invert the centrifuge tube 2–3 times to suspend the nematodes, then pipette 20µl of nematodes onto a glass slide and count the number of nematodes in 3–4 20µl drops using a dissecting light microscope. Calculate the average number of nematodes per microliter and the volume required to obtain 75 and 1000 nematodes (i.e. if you have on average 0.9 nematode/µl, then 83µl and 1111µl will contain ~75 and ~1000 nematodes, respectively).

*We trim the tip of each pipette tip with scissors and use a a new tip rinsed in 0.1% triton X-100 (i.e. pipette and exspell the triton prior to pipetting nematodes) for each 20µl drop. The triton X-100 helps prevent nematodes from sticking to the pipette tips, while trimming the tips increases the circumference, allowing large worms to be pipetted without injury.*This helps ensure an accurate nematode/µl estimation.

19 Following the plate map (step 7), pipette the volume required for 75 L4 nematodes into each well of a 24-well Seahorse XF^{e}24 islet plate and bring the volume of each well to 525µl with unbuffered EPA water.

When adding nematodes to the islet plate, be sure to use a new, trimmed pipette tip rinsed in 0.1% triton X-100 for each well of the 24-well islet plate. Cap and invert the 15mL centrifuge tube 2–3 times inbetween wells to ensure nematodes are fully resuspended.

20 By this time the sensor cartridge will be done calibrating (step 14). In the WAVE software click “Ready” and the Seahorse Analyzer’s tray will slide out with the utility plate. Replace the utility plate with the nematode-containing islet plate (without lid) and click “Ready” to start the assay.

In this step only the utility plate slides out with the tray, the sensor cartridge does not.

With current Seahorse Analyzer settings the runtime for the FCCP, sodium azide, and DCCD plates are roughly 150, 120, and 210 minutes, respectively.

21 Using trimmed pipette tips rinsed in 0.1% triton X-100, pipet the volume required for ~1000 L4 nematodes into a microcentrifuge tube and freeze at −80°C until total protein analysis in step 24.

22 Upon completion of the assay, click “Okay” and the analyzer’s tray will slide out. Remove the sensor cartridge and islet plate from the tray, click “Okay”, and the tray will slide closed. You will next be asked if you want to open the results from the current assay, click “Yes”.

As the islet plate and sensor cartridge came into contact with toxic substances they should be disposed of according to your institution’s toxic waste disposal guidelines. The utility plate can be thrown directly into the trash.

23 Click on the small box in the upper left hand side of the 24-well plate map to display all OCR data in the graphed results section. Make sure background correction is turned on and then click on the “Display” dropdown and select “well”. Right click on the graph and select “Export Graph Data” to save the excel file.

This will generate an excel file containing all of the OCR measurements taken for each well of the plate. Wells will be labeled A1-D6 in the file, so be sure to relabel the data with the appropriate strain/treatment conditions for each well, as assigned in the plate map (step 7).

Total Protein Extraction

24 Remove the aliquot of 1000 nematode from the −80°C freezer (step 21). Let the samples thaw at room temperature and then spin at 2200 RCF for 1 minute. Remove the supernatant, resuspend the nematodes in 250ul 10% SDS, and chill on ice for 2–3 minutes.

10% SDS freezes rapidly when on ice. However, sonication (step 25) heats the samples, so it is best to start the sonication process with pre-chilled samples. If samples do freeze let them sit at room temperature until they re-thaw.

25 Ultrasonicate each chilled sample (on the lowest possible setting) for 30 seconds. Chill on ice for 2–3 minutes and then ultrasonicate for an additional 30 seconds.

After the second 30s sonication period, pipette 10ul of the homogenate onto a slide under a microscope to ensure complete homogenization. No large debris should be present. If necessary sonicate for an additional 30s.

IMPORTANT! Wipe the ultrasonicator micro-tip down with 70% ethanol in between every samples to prevent protein carryover between samples.

26 When complete homogenization is achieved, measure protein content using the BCA assay (Thermo Fisher Scientific, Rockford, IL), following manufacturer's instructions.

Be sure to use 10% SDS when preparing your standard curve (see manufacturer's instructions). This assay gives protein values in units of μg/mL, convert to mg/mL for normalization purposes.

The BCA assay requires access to a microplate reader with filters capable of measuring absorbance in the range of 540–590nm. We use a FLUOstar OPTIMA (BMG Labtech, Ortenberg, Germany); however, any microplate model, with the proper filters, will work.

Data Analysis

27 Open the excel file from step 23 and average the readings from each well as follows:

1. Basal OCR: Readings 1–8 for each well. Values tend to be variable over the initial 4 measurements, so average OCR measurements 5–8 to get an average basal OCR per well (units of pmol O_2/minute).
2. DCCD response: OCR readings 9–22 for each well are for the 14 DCCD injection readings. Nematodes do not respond to DCCD instantly. It typically takes 8 measurements before OCR levels fall and plateau; thus, we average the final 6 OCR measurements (measurements 17–22) to get an average DCCD response per well.
3. FCCP response: OCR readings 9–16 for each well are for the 8 FCCP injection readings. Nematodes do not respond to FCCP instantly; however, OCR tends to increase and plateau by the fourth measurement. Thus we average the final 5 OCR measurements (measurements 12–16) post-FCCP injection to get an average FCCP-response per well.
4. Sodium azide response: OCR readings 9–12 for each well are for the 4 azide injection readings. Nematodes respond to sodium azide essentially instantaneously, so we average all 4 OCR readings post-azide injection to calculate the average azide response per well.

28 Divide the average basal and the average drug-response OCR measurements by the number of nematodes added per well (i.e. 75) to convert OCR measurements to units of pmol O_2/min/nematode. Next, divide your OCR measurements for each well by the corresponding total protein values calculated in step 26 to get units of pmol O_2/min/mg protein.

Using this method we count each well measured as an n of one. We recommend repeating each experiment 2–3 separate times (i.e., with entirely separate cultures of nematodes).

NOTE: Depending upon the researcher's interests, nematode OCR data can be normalized a variety of ways, other than to total protein, including: worm number, worm volume, or mitochondrial mass or volume.

29 Calculate spare capacity, ATP-linked respiration, and proton leak as follows:

1. Spare respiratory capacity (Maximal OCR - Basal OCR) is the ability of an organism to respond to increasing energy demands. To calculate, subtract each well's average basal OCR from its corresponding average FCCP response (maximal OCR). Values from across experiments can be averaged for statistical analysis (step 30).
2. ATP-linked respiration (Basal OCR - DCCD response) is oxygen consumption linked directly to ATP production and is estimated by inhibiting ATP synthase. To calculate, subtract each well's average DCCD response from its corresponding average basal OCR. Values from across experiments can be averaged for statistical analysis (step 30).
3. Proton leak (DCCD response - azide response) is the dissipation of the proton gradient across the inner mitochondrial membrane independent of ATP synthase activity. Calculating proton leak is not straight forward, as we do not have sample wells where both DCCD and azide were injected. To overcome this, we subtract each azide response within an experiment from the corresponding average DCCD response within the same experiment.

Figure 1 shows representative Seahorse data for L4 N2 nematodes.

Statistical Analysis

30 Each mitochondrial parameter should initially be assessed with a one- two- or three-way ANOVA, depending upon how many variables are being assessed (i.e. strain, time, dose etc.). We carry out post-hoc analysis of between-group differences using the Student's t-test when justified by significant effects observed in our global ANOVAs.

SUPPORT PROTOCOL 1: Age-synchronizing nematodes via sodium hypochlorite treatment

Synchronous populations of L1 nematodes can be generated by treating gravid adult nematodes with sodium hypochlorite bleach solution. Larval and adult nematodes are sensitive to hypochlorite treatment; however, eggs are resistant. Thus hypochlorite treatment allows for the isolation of nematode eggs. Isolated eggs are then left to hatch overnight in the absence of food, generating a synchronous population of L1 nematodes (Lewis and Fleming, 1995).

Materials

- OP50 seeded k-agar plates containing gravid adult nematodes
- K-medium (see recipe)
- 15mL centrifuge tubes
- Dissecting light microscope
- Bunsen burner
- 70% Ethanol
- Glass L-shaped rod
- Sodium hydroxide bleach solution (see recipe)
- 20°C incubator
- Orbital shaker
- 50mL cell culture flask
- Complete K-medium (see recipe)

1. Wash gravid adult nematodes from k-agar plate, using k-medium, into a 15mL centrifuge tube.
2. Under a dissecting light microscope, carefully loosen eggs from the surface of the k-agar plates using a sterile L-shaped glass rod. Wash the loosened eggs from the k-agar plate into the centrifuge tube (containing gravid adults) using k-medium.

   The L-shaped glass rod can be sterilized by dipping the rod in 70% ethanol and then passing the rod through the flame of a Bunsen burner, allowing the ethanol to burn off.
3. Centrifuge at 2200 RCF for 2 minutes to pellet the nematodes and eggs.
4. Aspirate the supernatant off the pellet. Add 10mL of sodium hydroxide bleach solution (see recipe) and place the tube on an orbital shaker at 20°C for 8 minutes.

   Some strains are slightly more sensitive to this solution and should be incubated for less time in order to recover a large number of viable eggs. The goal is to incubate for the minimal time required to kill all stages except eggs.
5. After 8 minutes of shaking check the tube under a microscope to ensure adult nematodes have been dissolved. Centrifuge for 2 minutes at 2200 RCF. Aspirate the supernatant taking care not to disrupt the pellet.

   If adult nematodes are not fully dissolved they can be placed back on the shaker for up to 2 additional minutes.
6. Resuspend the pellet in 15mL k-medium and centrifuge for 2 minutes at 2200 RCF. Aspirate the supernatant taking care not to disrupt the pellet.

7. Resuspend the pellet in 1.5 mL complete k-medium (see recipe) and transfer into a sterile 50mL cell culture flask containing 8mL complete k-medium. Incubate the flask on an orbital shaker at 20°C overnight (12–20 hours).
8. Check under a microscope to ensure eggs have hatched. Transfer hatched L1 larvae to a 15 mL sterile centrifuge tube and centrifuge at 2200 RCF for 2 minutes. Aspirate the supernatant and transfer your age-synchronized population of L1 larvae onto an OP50 seeded k-agar plate.
9. Incubate your nematodes at 20°C. Transfer nematodes to fresh OP50 k-agar plates as necessary to avoid depletion of the bacterial food source. Culture nematodes until L4 stage is reached.

REAGENTS AND SOLUTIONS

K-medium

2.36g Potassium chloride (KCl)

3.0g Sodium chloride (NaCl)

1.0L ddH_2O

Autoclave to sterilize

Store at room temperature, indefinitely, if stored under sterile conditions

Complete k-medium

150µl 1M Calcium chloride ($CaCl_2$)

150µl 1M Magnesium sulfate ($MgSO_4$)

25µl 10mg/mL Cholesterol (dissolved in 100% ethanol and filter sterilized)

50mL sterile K-medium

Store at room temperature for up to 1 week

Unbuffered EPA water (Weber, 1991)

60mg Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)

60mg Calcium sulfate ($CaSO_4 \cdot 2H_2O$)

4mg Potassium chloride (KCl)

Store at room temperature, indefinitely, if stored under sterile conditions

Sodium hydroxide bleach solution

44mL ddH_2O

6mL Clorox Regular Bleach (non-germicidal*, 8.25% sodium hypochlorite)

5 Sodium hydroxide (NaOH) pellets (Avantor Performance Materials, Center Valley, PA)

Store at room temperature for up to 3 days

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

COMMENTARY

Background Information

Mitochondrial dysfunction has been implicated in myriad human diseases, including cancer (Frezza and Gottlieb, 2009; Gogvadze et al., 2008; Wallace, 2012), neurodegeneration (Beal, 2005; Lin and Beal, 2006), and metabolic disorders (Smeitink et al., 2006). Thus understanding how toxicant exposure or genetic mutations affect mitochondrial function will be critical in understanding mitochondrial disease. Historically, measurement of oxygen consumption has been a slow process, accomplished by using low-throughput Clarke-type electrode oxygen meters (Braeckman et al., 2002). The advent of the Seahorse XF24 and XF96 has not only allowed for rapid and high-throughput determination of basal OCR in *in vitro* models, but also allows for the determination of ATP-linked respiration, maximal OCR, spare respiratory capacity, and proton leak through injection of various inhibitors of the mitochondrial ETC. Due to the dual probe capacity of the XF^e24 and XF^e96, it is not only possible to measure OCR, but also extracellular acidification rates (ECAR) thus allowing researchers to identify metabolic shifts from OXPHOS to aerobic glycolysis.

Although the Seahorse XF^e24 Analyzer offers nematode researchers the ability to measure the fundamental parameters of the mitochondrial ETC *in vivo*, it does have limitations. For example, ETC inhibitors cannot be injected in tandem, as done in *in vitro* assays, limiting its throughput. We have previously demonstrated that the magnitude of response to sodium azide is reduced if injected post-FCCP (Luz et al., In Press); however, it is possible that injecting a different complete respiratory inhibitor (such as cyanide, or rotenone and antimycin A) post-FCCP would prove more effective. This limitation could be partially overcome by adapting the assay to the XF96 (or XF^e96), which has previously been used to measure basal respiration in nematodes (Andreux et al., 2014). Another issue with the XF^e24 Analyzer is that is lacks a cooling function; thus, the instrument tends to heat (up to ~25–26°C) as it operates. This issue could be overcome by housing the Seahorse Analyzer in a temperature and humidity controlled apparatus or be limited by maintaining the ambient lab temperature at 20°C. Simultaneous OCR and ECAR measurements have successfully demonstrated metabolic shifts from OXPHOS to aerobic glycolysis, otherwise known as the Warburg effect (Warburg, 1956), in the context of toxicant exposure in *in vitro* models (Zhao et al., 2014; Zhao et al., 2013). However, ECAR measurements appear to have little value in *C. elegans*, as mitofusin-deficient *fzo-1* (*tm1133*) nematodes, which exhibit elevated intracellular acidification rates due to increased glycolysis (Johnson and Nehrke, 2010), have similar ECAR rates as wild-type nematodes (Luz et al., In Press). Thus we hypothesize that nematodes do not extrude glycolytic by-products (such as lactate) in the same manner as cells in culture. One final issue with this assay is lack of a highly specific ATP synthase inhibitor. Oligomycin A, a highly specific inhibitor of the F_O subunit (Hong and Pedersen, 2008) fails to penetrate the nematode cuticle in the timeframe of the assay. Thus we optimized this assay for use with the less specific F_OF_1 inhibitor DCCD (Hong and

Pedersen, 2008), which can inhibit additional cellular ATPases that may contribute to changes in oxygen consumption. Interestingly, even in a cuticle deficient background (*bus-8* (*e2885*)), oligomycin fails to inhibit OCR to the same extent as DCCD (Luz et al., In Press). Thus we recommend researchers carefully consider the use of DCCD and oligomycin A prior to starting experiments, and confirm results via alternative methods, such as ATP assays.

In addition to these logistical limitations, it is important to keep in mind that a whole-organism approach such as this, while permitting analysis of mitochondrial function in the context of normal intercellular signaling, also results in analysis of respiration in all cells at once. Thus, cell-specific differences may be obscured if they are quantitatively minor, occur in a small number of cells, or are offset but directionally opposite changes in other cells.

Critical Parameters

Perhaps the most critical parameter in using the Seahorse XF^{e}24 Analyzer to measure mitochondrial respiration in *C. elegans* is choosing the optimal number of nematodes to add to each well of the islet plate. If too few nematodes are added basal OCR may fall below the instrument's limit of detection (40 pmol O_2/min). Likewise, even if basal OCR is above 40 pmol O_2/min, the addition of DCCD or sodium azide may cause OCR to fall below detection limits. Thus we recommend basal OCR rates be in the range of 200–400 pmol O_2/min. Conversely, if too many nematodes are added per well, OCR may rise above the analyzer's upper limit of detection (1400 pmol O_2/min) when FCCP is injected. However, even if FCCP-induced OCR does not rise above 1400 pmol O_2/min, OCR measurements may still be confounded if oxygen levels fall below 100mmHg or if the mix cycle fails to completely re-oxygenate the well (160–170 mmHg) before the next measurement is taken (see Table 2). This protocol has been optimized to work with 75 L4 nematodes per well; however, if mitochondrial mutant strains are being tested, more L4s may be required (we successfully used 150 L4 complex III mutant nematodes (*isp-1*(*qm150*)) per well (Luz et al., In Press)). Thus, measuring mitochondrial respiration in younger (L1–L3) or older nematodes will require more or less nematodes, respectively, to be loaded per well. As a general guide, we have successfully measured basal and maximal OCR using 35 eight day old wild-type (N2) nematodes and all mitochondrial parameters using 50 four day old germline-deficient (*glp-1* (*jk1107*)) worms; however, we recommend optimizing the number of nematodes added per well, such that basal OCR is in the range of 200–400 pmol O_2/min, prior to injection of ETC inhibitors.

Troubleshooting

Table 2 highlights some of the common problems encountered in Seahorse XF^{e}24 analysis of mitochondrial function in *C. elegans* and provides potential causes and solutions to these problems.

Anticipated Results

Basal OCR—Basal OCR is measured in every well over the first 8 measurements of the analyzer's run cycle. Typically, the initial 4 OCR measurements are highly variable, but

become less variable for the final 4 measurements. This however is not always the case, which is why we recommend running each experiment at least 3 times, separate in time.

DCCD-response—Nematodes do not respond instantly to DCCD. Typically OCR falls and plateaus by the ninth OCR measurement post-DCCD injection. Thus, the final 6 OCR measurements are averaged for each wells DCCD-response. See trouble shooting section (Table 2) if OCR fails to decrease and plateau in response to DCCD.

FCCP-response—Nematodes do not respond instantly to FCCP. OCR typically begins to rise by the second OCR measurement post-FCCP injection and should plateau by the fourth measurement. Thus, the final 5 OCR measurements are averaged for a wells FCCP-response. See trouble shooting section (Table 2) if OCR fails to increase and plateau in response to FCCP.

Sodium azide-response—Nematodes respond essentially instantly to sodium azide. OCR will fall and plateau by the time the first OCR is measured post-azide injection, we have never observed differently and recommend making a fresh 80mM sodium azide stock if OCR does not fall and plateau instantly after azide injection.

Time Considerations

It will take 3–4 days from hypochlorite treatment of gravid adult nematodes to measuring mitochondrial function in the Seahorse Analyzer. This, however, will depend upon the life stage, strain and/or exposure length of the nematodes being used in the study. For the purposes of this protocol, once a synchronous population of L4 nematodes is generated, it will take approximately 10 hours to analyze the nematodes' response to FCCP, sodium azide and DCCD. On the day of Seahorse XF^{e}24 analysis it will take approximately 1 hour to prepare ETC inhibitors for injection, set up the experiment in the WAVE software and add nematodes to the islet plate. The length of Seahorse analysis varies depending upon which drug is being injected, as the number of measurements varies for each drug. For example, analysis of nematodes response to FCCP, sodium azide, or DCCD takes approximately 2.5, 2, or 3.5 hours, respectively. We typically run our FCCP experiments first, followed by sodium azide, and finally DCCD. As analysis of the DCCD response takes the longest, the assay can be left to completion overnight. Typically, when 1 hour remains in a run we begin loading the next ETC inhibitor into a new hydrated sensor cartridge and load nematodes into a new islet plate. This way, when one Seahorse analysis finishes then next can be started immediately.

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LITERATURE CITED

Addo MG, Cossard R, Pichard D, Obiri-Danso K, Rötig A, Delahodde A. Caenorhabditis elegans, a pluricellular model organism to screen new genes involved in mitochondrial genome maintenance. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2010; 1802:765–773. [PubMed: 20580819]

Andreux PA, Mouchiroud L, Wang X, Jovaisaite V, Mottis A, Bichet S, Moullan N, Houtkooper RH, Auwerx J. A method to identify and validate mitochondrial modulators using mammalian cells and the worm C. elegans. Scientific reports. 2014:4.

Beal MF. Mitochondria take center stage in aging and neurodegeneration. Annals of neurology. 2005; 58:495–505. [PubMed: 16178023]

Boyd WA, McBride SJ, Rice JR, Snyder DW, Freedman JH. A high-throughput method for assessing chemical toxicity using a Caenorhabditis elegans reproduction assay. Toxicology and applied pharmacology. 2010; 245:153–159. [PubMed: 20206647]

Boyd WA, Smith MV, Kissling GE, Rice JR, Snyder DW, Portier CJ, Freedman JH. Application of a mathematical model to describe the effects of chlorpyrifos on Caenorhabditis elegans development. PLoS One. 2009; 4:e7024. [PubMed: 19753116]

Braeckman BP, Houthoofd K, De Vreese A, Vanfleteren JR. Assaying metabolic activity in ageing Caenorhabditis elegans. Mechanisms of ageing and development. 2002; 123:105–119. [PubMed: 11718805]

Bratic I, Hench J, Henriksson J, Antebi A, Bürglin TR, Trifunovic A. Mitochondrial DNA level, but not active replicase, is essential for Caenorhabditis elegans development. Nucleic acids research. 2009:gkp018.

Chan DC. Fusion and fission: interlinked processes critical for mitochondrial health. Annual review of genetics. 2012; 46:265–287.

Consortium S. Genome sequence of the nematode $ C. elegans $: A platform for investigating biology. Science. 1998; 282:2012–2018. [PubMed: 9851916]

Fernie AR, Carrari F, Sweetlove LJ. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Current opinion in plant biology. 2004; 7:254–261. [PubMed: 15134745]

Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature. 2000; 408:325–330. [PubMed: 11099033]

Frezza C, Gottlieb E. Mitochondria in cancer: not just innocent bystanders. Seminars in cancer biology. 2009

Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria in cancer cells: what is so special about them? Trends in cell biology. 2008; 18:165–173. [PubMed: 18296052]

Hong S, Pedersen PL. ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas. Microbiology and Molecular Biology Reviews. 2008; 72:590–641. [PubMed: 19052322]

Houten SM, Wanders RJ. A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation. Journal of inherited metabolic disease. 2010; 33:469–477. [PubMed: 20195903]

Johnson D, Nehrke K. Mitochondrial fragmentation leads to intracellular acidification in Caenorhabditis elegans and mammalian cells. Molecular biology of the cell. 2010; 21:2191–2201. [PubMed: 20444981]

Kaletta T, Hengartner MO. Finding function in novel targets: C. elegans as a model organism. Nature Reviews Drug Discovery. 2006; 5:387–399. [PubMed: 16672925]

Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, Le Bot N, Moreno S, Sohrmann M. Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature. 2003; 421:231–237. [PubMed: 12529635]

Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RH, Heck AJ. Metabolic labeling of C. elegans and D. melanogaster for quantitative proteomics. Nature biotechnology. 2003; 21:927–931.

Lagido C, Pettitt J, Flett A, Glover LA. Bridging the phenotypic gap: real-time assessment of mitochondrial function and metabolism of the nematode Caenorhabditis elegans. BMC physiology. 2008; 8:7. [PubMed: 18384668]

Leung MC, Rooney JP, Ryde IT, Bernal AJ, Bess AS, Crocker TL, Ji AQ, Meyer JN. Effects of early life exposure to ultraviolet C radiation on mitochondrial DNA content, transcription, ATP production, and oxygen consumption in developing Caenorhabditis elegans. BMC Pharmacology and Toxicology. 2013; 14:9. [PubMed: 23374645]

Lewis JA, Fleming JT. Basic culture methods. Methods Cell Biol. 1995; 48:3–29. [PubMed: 8531730]

Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature. 2006; 443:787–795. [PubMed: 17051205]

Luz AL, Rooney JP, Kubik LL, González-Hunt CP, Song DH, Meyer JN. Mitochondrial morphology and fundamental parameters of the mitochondrial respiratory chain are altered in Caenorhabditis elegans strains deficient in mitochondrial dynamics and homeostasis processes. PloS one. In Press.

Meyer JN, Leung MC, Rooney JP, Sendoel A, Hengartner MO, Kisby GE, Bess AS. Mitochondria as a target of environmental toxicants. toxicological sciences. 2013; 134:1–17. [PubMed: 23629515]

Neustadt J, Pieczenik SR. Medication-induced mitochondrial damage and disease. Molecular nutrition & food research. 2008; 52:780–788. [PubMed: 18626887]

Schaefer AM, Taylor RW, Turnbull DM, Chinnery PF. The epidemiology of mitochondrial disorders—past, present and future. Biochimica et Biophysica Acta (BBA)-Bioenergetics. 2004; 1659:115–120. [PubMed: 15576042]

Smeitink JA, Zeviani M, Turnbull DM, Jacobs HT. Mitochondrial medicine: a metabolic perspective on the pathology of oxidative phosphorylation disorders. Cell metabolism. 2006; 3:9–13. [PubMed: 16399500]

Stiernagle T. Maintenance of C. elegans. C. elegans. 1999; 2:51–67.

Thompson O, Edgley M, Strasbourger P, Flibotte S, Ewing B, Adair R, Au V, Chaudhry I, Fernando L, Hutter H. The million mutation project: a new approach to genetics in Caenorhabditis elegans. Genome research. 2013; 23:1749–1762. [PubMed: 23800452]

Tsang WY, Lemire BD. Mitochondrial genome content is regulated during nematode development. Biochemical and biophysical research communications. 2002; 291:8–16. [PubMed: 11829454]

Tsang WY, Lemire BD. The role of mitochondria in the life of the nematode, Caenorhabditis elegans. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2003a; 1638:91–105. [PubMed: 12853115]

Tsang WY, Lemire BD. The role of mitochondria in the life of the nematode,*Caenorhabditis elegans*. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2003b; 1638:91–105. [PubMed: 12853115]

Wallace DC. Mitochondria and cancer. Nature Reviews Cancer. 2012; 12:685–698. [PubMed: 23001348]

Warburg O. On the origin of cancer cells. Science. 1956; 123:309–314. [PubMed: 13298683]

Weber, CI. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency; 1991.

Zhao F, Malm SW, Hinchman AN, Li H, Beeks CG, Klimecki WT. Arsenite-induced pseudo-hypoxia results in loss of anchorage-dependent growth in BEAS-2B pulmonary epithelial cells. PloS one. 2014; 9:e114549. [PubMed: 25513814]

Zhao F, Severson P, Pacheco S, Futscher BW, Klimecki WT. Arsenic exposure induces the Warburg effect in cultured human cells. Toxicology and applied pharmacology. 2013; 271:72–77. [PubMed: 23648393]

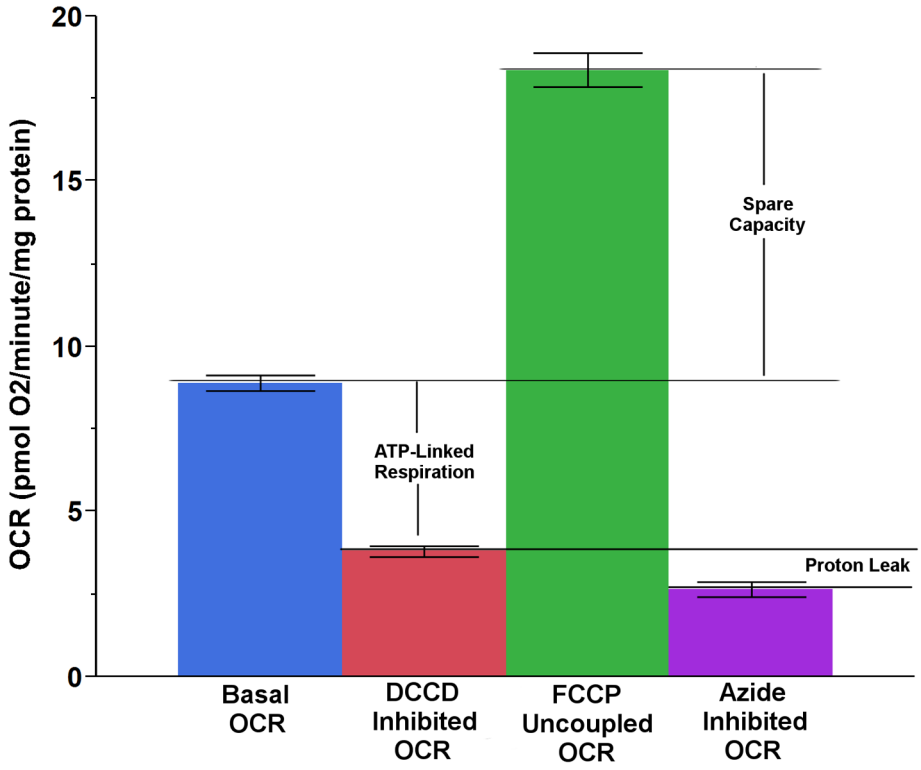

Figure 1.

Seahorse XF^{e}24 Analyzer output data for L4 N2 *C. elegans*. Fundamental parameters (basal OCR, ATP-linked respiration, maximal OCR, spare respiratory capacity, and proton leak) of the mitochondrial ETC, shown for L4 wild-type nematodes. Data taken from (Luz et al., In Press).

Table 1

Preparation of Electron Transport Chain Inhibitors

Drug (ETC target)	Stock Concentration	Sensor Cartridge Port Concentration (8x)	Final Well Concentration (1x)	OCR Measurements Post Injection
FCCP (mitochondrial uncoupler)	1.25mM dissolved in 100% DMSO (store at −20°C in 350µl aliquots)	200uM dissolved in 16% DMSO **To make**: Add 320ul 1.25mM FCCP (100% DMSO) to 1680ul unbuffered EPA water	25uM in 2% DMSO	8
DCCD (ATP synthase inhibitor)	2mM dissolved in 100% DMSO (store at −20°C in 200µl aliquots)	160uM Dissolved in 8% DMSO **To make**: Add 160ul 2mM DCCD to 1840ul unbuffered EPA water	20uM in 1% DMSO	14
Sodium Azide (cytochrome c oxidase inhibitor)	80mM dissolved in 100% unbuffered EPA water (store at 4°C)	80mM Dissolved in 100% unbuffered EPA water	10mM in 100% unbuffered EPA water	4

Table 2

Troubleshooting guide for Seahorse XFe24 analysis in *C. elegans*

Problem	Possible Cause	Solution
Worms do not respond to FCCP and/or FCCP response fails to plateau	FCCP precipitated out of solution	Be sure frozen 1.25mM FCCP stocks are warmed completely to room temperature prior to diluting in EPA water
	25uM FCCP is not efficiently uncoupling respiration	Test a higher concentration of FCCP
	Drug was loaded into wrong injection port	Check the injection ports to make sure the drug was injected and/or was not added to the wrong injection port
Worms do not respond to DCCD and/or DCCD response fails to plateau	DCCD precipitated out of solution	Be sure frozen 2.0mM DCCD stocks are warmed completely to room temperature prior to diluting in EPA water
	Drug was loaded into wrong injection port	Check the injection ports to make sure the drug was injected and/or was not added to the wrong injection port
Drug response is outside of the analyzer's range of detection (i.e. 40–1400 pmol/min)	Too few or too many nematodes per well	Adjust the number of nematodes added per well
Oxygen levels fall below 100mm Hg during OCR measurements	Too many nematodes loaded per well	Reduce the number of nematodes per well
	Mix cycle is not long enough	Increase the length of the mix cycle (or decrease the length of the measure cycle) to avoid subjecting the worms to anoxic conditions
Seahorse XFe24 Analyzer is overheating	Ambient lab temperature is not cool enough	Lower the laboratory thermostat (20°C is usually sufficient)
		Open the Seahorse tray, turn off the analyzer, and let the instrument sit idle for 30–60 minutes in between assays, giving it time to cool
		House the Seahorse analyzer in a temperature and humidity controlled apparatus