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Measurement of Oxygen Consumption Rates in Intact Caenorhabditis Elegans

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Cover Letter

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JOVE

Dear JOVE editorial staff and handling editor,

I am submitting a revised manuscript JoVE59277 entitled "Measurement of oxygen consumption rates in intact *Caenorhabditis elegans*" for evaluation. The submission has been modified to address the editorial concerns we received on our manuscript. In a separate rebuttal letter, we have addressed each concern individually. I greatly appreciate your consideration of our manuscript. Please do not hesitate to contact me if you require further information.

All the best
Kenneth Norman

TITLE:

Measurement of Oxygen Consumption Rates in Intact *Caenorhabditis Elegans*

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KEYWORDS:

C. elegans, respiration, oxygen consumption rate (OCR), respirometer, mitochondria, metabolism

SUMMARY:

Mitochondrial respiration is critical for organismal survival; therefore, oxygen consumption rate is an excellent indicator of mitochondrial health. In this protocol, we describe the use of a commercially available respirometer to measure basal and maximal oxygen consumption rates in live, intact, and freely-motile *Caenorhabditis elegans*.

ABSTRACT:

Optimal mitochondrial function is critical for healthy cellular activity, particularly in cells that have high energy demands like those in the nervous system and muscle. Consistent with this, mitochondrial dysfunction has been associated with a myriad of neurodegenerative diseases and aging in general. *Caenorhabditis elegans* have been a powerful model system for elucidating the many intricacies of mitochondrial function. Mitochondrial respiration is a strong indicator of mitochondrial function and recently developed respirometers offer a state-of-the-art platform to measure respiration in cells. In this protocol, we provide a technique to analyze live, intact *C. elegans*. This protocol spans a period of ~7 days and includes steps for (1) growing and synchronization of *C. elegans,* (2) preparation and loading of compounds to be injected and hydration of probes, (3) drug loading and cartridge equilibration, (4) preparation of worm assay plate and assay run, and (5) post-experiment data analysis.

INTRODUCTION:

Adenosine triphosphate (ATP), the main source of cellular energy, is produced in the mitochondria by enzymes in the electron transport chain (ETC) located in the inner mitochondrial membrane. Pyruvate, a key metabolite utilized for mitochondrial ATP production, is imported into the mitochondrial matrix where it is decarboxylated to produce acetyl coenzyme A (CoA). Subsequently, acetyl CoA enters the citric acid cycle resulting in the generation of nicotinamide adenine dinucleotide (NADH), a key electron carrier molecule. As electrons from NADH are passed to oxygen via the ETC, protons build up in the mitochondrial intermembrane space, which results in the generation of an electrochemical gradient across the membrane. These protons will

then flow from the intermembrane space across this electrochemical gradient back into the mitochondrial matrix through the proton pore of the ATP synthase, driving its rotation and the synthesis of ATP[1] (**Figure 1**).

Mitochondrial function is not limited to energy production but is also crucial for calcium homeostasis, reactive oxygen species (ROS) scavenging, and apoptosis, critically positioning their function in organismal health[2]. Mitochondrial function can be assessed using a variety of assays, including but not limited to analyses that measure mitochondrial membrane potential, ATP and ROS levels, and mitochondrial calcium concentrations. However, these assays provide a single snapshot of mitochondrial function and therefore might not provide a comprehensive view of mitochondrial health. Since oxygen consumption during ATP generation is reliant on a myriad of sequential reactions, it serves as a superior indicator of mitochondrial function. Interestingly, variations in oxygen consumption rates have been observed as a result of mitochondrial dysfunction[3-5].

Oxygen consumption rates (OCR) of living samples can be measured using techniques that can be broadly divided into two groups: amperometric oxygen sensors and porphyrin-based phosphors that can be quenched by oxygen[6]. Amperometric oxygen sensors have been used extensively to measure OCR in cultured cells, tissues, and in model systems, such as *C. elegans*. However, porphyrin-based phosphors containing respirometers possess the following advantages: (1) they allow for a side by side comparison of two samples in triplicate, (2) they require smaller sample size (e.g., 20 worms per well versus ~2,000–5,000 worms in the chamber)[7], and (3) the respirometer can be programmed to do four different compound injections at desired times throughout the experimental run, eliminating the need for manual application.

In this protocol, steps involved in using a porphyrin-based oxygen-sensing respirometer to measure OCR in live, intact *C. elegans* are described. While there is a written protocol for the use of the large format, high throughput respirometer[8], this protocol has been adapted for use with a more budget friendly, accessible, and smaller scale instrument. This protocol is particularly useful for assessing the difference in OCR between two strains, where high-throughput screening is not required and its use would be excessive.

PROTOCOL:

NOTE: **Figure 2** provides a schematic overview of the full protocol.

1. Growth and synchronization of nematode population[9,10]

1.1. Transfer L4 larvae of desired genetic backgrounds (e.g., N2 [wild type] and *sel-12* animals) onto nematode growth media (NGM) plates (see **Table 1** for recipe) freshly seeded lawn of *Escherichia coli* (OP50)[11]. Use at least two 100 mm or three 60 mm plates for each strain. Incubate the worms at the appropriate growth temperature (between 15–25 °C) for 3–4 days or until plates are concentrated with large number of eggs and gravid worms.

1.2. Wash the eggs and worms off the plates using approximately 6 mL of M9 buffer (see **Table 1** for recipe) per 100 mm plate of nematodes and transfer them with glass Pasteur pipettes into individual 15 mL centrifuge tubes for each strain. Spin these tubes down for 3 min at 6,180 x *g* and aspirate out the M9 buffer, retaining just the animals and eggs pellet.

1.3. Add 3–4 mL of bleach solution (see **Table 1** for recipe) to each tube and intermittently vortex for 6 min. Add M9 buffer to fill each tube and spin at 6,180 x *g* for 1 min and aspirate supernatant. Repeat this wash with M9 three times and move the egg pellet to a fresh 15 mL tube containing approximately 9 mL of M9 buffer.

1.4. Synchronize the freshly hatched worms by nutating at 20 °C for 16–48 h. Spin these tubes down at 6,180 x *g* for 1.5 min and put the animals down on individual NGM plates freshly seeded lawn of OP50 (approximately 6,000–10,000 animals per 100 mm plate) and keep at 20 °C.

1.5. These animals will reach the L4 larval stage after ~42 h. At this time, move the L4 larvae using a platinum pick to OP50 seeded NGM plates containing 0.5 mg/mL 5-fluoro-2'-deoxyuridine (FUdR) to prevent them from producing progeny.

NOTE: Make sure that within an experiment all strains are synchronized for a similar amount of time. FUdR treatment sterilizes the animals and prevents egg laying and progeny production, which could influence OCR. These sterilized animals will be analyzed the next day (day 1 adult animals at ~66 h) for the assay. FUdR has been reported to impact physiology and lifespan of certain mutant worms. Therefore, this should be taken into consideration when the drug is used for sterilization[12-14]. Worms can also be sterilized by the means of feminizing mutations such as *fem-1(hc17)* or *fem-3(e2006)*. However, these mutations might impact mitochondrial function.

2. Preparation and loading of compounds to be injected and hydration of probes

NOTE: During the assay run, both basal and maximal respiration rates of the nematodes are measured. Maximal respiration is triggered in the animals upon the addition of carbonyl cyanide-4 (trifluormethoxy)phenylhydrazone (FCCP), an uncoupling ionophore that disturbs the mitochondrial membrane potential and thus ATP synthesis by transporting protons through the mitochondrial membrane, while allowing proton pumping, electron transport, and oxygen consumption to proceed[4,15] uncoupled from ATP synthesis (**Figure 1**). The final step in the assay involves the addition of sodium azide (NaN_3), a drug that inhibits complexes IV and V in the ETC, allowing one to determine non-mitochondrial respiration[16] (**Figure 1**). The following steps can be performed the day before the actual assay run.

2.1. Prepare 1 mL stock solutions of the FCCP (10 mM in dimethyl sulfoxide [DMSO]: 1000x the final assay concentration) and NaN_3 (400 mM in dH_2O: 10x final assay concentration) and store at -20 °C.

NOTE: Run a concentration curve to optimize the concentration of FCCP required to elicit maximal OCR response for each instrument and laboratory setting.

2.2. Hydrate the sensor cartridge by adding 200 µL of dH_2O to each well (and the surrounding reservoir) in the plate, ensuring that the sensor probes are submerged in the dH_2O and store overnight at room temperature.

NOTE: The cartridge probes can be left submerged for up to 72 h but in the case of a prolonged hydration, the plates should be wrapped in paraffin film and stored at 4 °C. If overnight hydration is not possible, the sensor cartridge should be hydrated for at least 4 h.

2.3. Turn off the heating element within the respirometer interface and store the machine inside a 15 °C incubator overnight to lower core temperature within the machine to prevent the animals from overheating during the assay run.

NOTE: The respirometer is equipped with a heating element but cannot be cooled. Within a 15 °C incubator, the respirometer will have a stable temperature between 18–22 °C, which is a healthy temperature for *C. elegans* maintenance.

3. Drug loading and cartridge equilibration

3.1. Pipette out and discard the dH_2O used to hydrate the sensor probes and replace it with 200 µL of the calibrant solution (pH 7.4) in each well. Dilute the FCCP stock solution to 100 µM FCCP in dH_2O and add 20 µL of the diluted solution to the injection port A in the sensor cartridge. Add 22 µL of 400 mM sodium azide to port B of the senor cartridge.

3.2. Turn the respirometer on and on the home screen select **Start**; the templates page will appear. On the templates page, select **Blank** or a previously designed template; the Groups page will appear. On the Groups page, select the wells A and H as the background wells and assign the remaining 6 wells into appropriate groups according to the experimental plan.

3.3. Push the arrow on the lower right corner to go to the Protocol page. On the Protocol page, ensure that the **Equilibrate**, **Basal** and **Injections 1 and 2** buttons are selected. On this page, adjust the number of readings of basal OCR, as well as maximal OCR (after injection 1 with FCCP) and non-mitochondrial OCR (after injection 2 with sodium azide).

NOTE: Each measurement is preceded by a mix and wait step and the time frames for these parameters are shown in **Table 2**.

3.4. Select the arrow on the lower right corner and a prompt to load the sensor cartridge plate will appear. Ensure that the sensor cartridge plate is loaded in the right orientation, following the instructions on the reminder prompt screen. The respirometer will now equilibrate the cartridge.

NOTE: This equilibration provides ample time to place the animals to be assayed into appropriate wells of the cell plate.

4. Preparation of worm plate and assay run

4.1. Add 200 µL of M9 buffer into each of the 8 wells of the cell plate and into the reservoirs surrounding the wells.

4.2. Pick ~100 worms from each strain onto unseeded NGM plates and allow to rest for 2-3 min. Wet the end of the platinum pick with M9 buffer and pick 20 age-synchronized animals into wells B-G, leaving the background wells A and H empty.

NOTE: After loading each well, wait approximately 2 min to allow the animals to settle into the bottom of the wells. It is also advisable that a worm number curve be run to optimize the worm number per well for each instrument and laboratory setting.

4.3. By now, the respirometer should be calibrated and by clicking **OK** on the screen, the plate containing calibration buffer is ejected and can be removed from the respirometer, while the sensor cartridge stays inside the instrument. Replace the calibrant plate with the plate containing animals. Load plate and close the door by hitting **Continue** and allow the assay to run.

5. Post-experiment data analysis

5.1. Once the assay run is complete, follow the prompts on the screen and remove the cell plate and sensor cartridge and insert a flash drive into the USB port to save the run data in the .war format. Remove the sensor cartridge and allow the animals to settle to the bottom of the wells for approximately 2 min. Place the cell plates under a stereo dissecting microscope and count the number of animals per well.

5.2. Open the analysis software on the computer and open the normalization tab to normalize OCR to animal number. Apply appropriate labels for the various groups under the modify tab and export the file as a Prism file for further analysis.

NOTE: The average of the first five measurements, before the addition of FCCP is the basal OCR, while the average of the five measurements after FCCP addition is the maximal OCR and the average of the last two measurements after sodium azide addition is the non-mitochondrial respiration rate. The assay should be repeated three times to ensure reproducibility.

REPRESENTATIVE RESULTS:

Using the protocol described herein, OCR of wild type animals and three different *sel-12* mutant strains were determined. *sel-12* encodes the *C. elegans* ortholog of presenilin[17]. Mutations in human presenilin are the most common genetic aberration associated with the development of familial Alzheimer's disease[18]. Our studies have shown elevated mitochondrial calcium levels in *sel-12* mutant animals compared to wild type animals[3]. Since calcium dysregulation can result in

altered mitochondrial function[3,19,20], OCR in *sel-12* mutant and wild type animals were measured to examine the effect of *sel-12* mutations on mitochondrial function and health. Wild type animals consistently showed basal OCR rates below 5 pmol/min/worm, while all three strains of *sel-12* mutants showed significantly elevated OCR of ~7 pmol/min/worm (**Figure 3** and **Figure 4**). Upon the addition of FCCP, as expected, there was an increase in the OCR of wild type as well as *sel-12* mutants (**Figure 3** and **Figure 5**). Wild type animals showed maximum OCR of ~7 pmol/min/worm, while *sel-12* mutants had OCR of ~10 pmol/min/worm (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the major players involved in cellular respiration and the effect of FCCP and sodium azide. Transfer of electrons from NADH to the ETC complex I results in the generation of an electrochemical gradient across the inner mitochondrial membrane as protons get pumped across it. Protons flowing back into the mitochondrial matrix from the intermembrane space via complex V results in ATP synthesis. Addition of FCCP results in the uncoupling of this process by disrupting the mitochondrial membrane potential and thereby ATP synthesis, while oxygen consumption continues, allowing for the measurement of maximal OCR. Sodium azide (NaN_3) is an inhibitor of complexes IV and V, thereby allowing for the measurement of non-mitochondrial respiration.

Figure 2: Schematic of steps involved in the measurement of OCR in *C. elegans*. The five steps involved in the assay setup and run (**left**) and a timeframe for each step (**right**).

Figure 3: Characteristic OCR profile in *C. elegans* respirometry. The five initial readings show the basal respiration, followed by five readings of maximal and five readings of non-mitochondrial respiration after FCCP and sodium azide injections, respectively. Error bars represent the standard error of measurement (SEM).

Figure 4: Basal respiration in wild type and various *sel-12* mutants. Average basal respiration in Day 1 adult age-matched wild type and *sel-12* mutant animals. Data compiled from three assay repeats. Error bars represent SEM and **** indicates $p < 0.0001$. *p* values were calculated using a two-tailed *t*-test.

Figure 5: Maximal respiration in wild type and various *sel-12* mutants. Average maximal respiration in Day 1 adult age-matched wild type and *sel-12* mutant animals after FCCP injection. Data compiled from three assay repeats. Error bars represent SEM and **** indicates $p < 0.0001$. *p* values were calculated using a two-tailed *t*-test.

Table 1: Recipes for NGM plates, M9 buffer, and bleach solution.

Table 2: Characteristic assay parameters in *C. elegans* respirometry.

DISCUSSION:

Mitochondrial respiration is an insightful indicator of mitochondrial function; therefore, being able to measure the oxygen consumption rates in a biological system, whether in vitro or in vivo

is highly valuable. Respirometers sense oxygen levels using porphyrin-based phosphors that get quenched by oxygen or via amperometric oxygen sensors that rely on the generation of an electric current proportional to oxygen pressure. Clark electrode falls into the latter category and has been used extensively in literature, especially while analyzing respiration in *C. elegans*. However, the need for a large sample size and the inability to assess more than one sample at a time makes amperometric oxygen sensors inefficient.

This protocol provides a simple guide to measure OCR in live, intact *C. elegans* without isolating mitochondria, a process that could potentially impact the mitochondrial membrane potential and, therefore, the OCR. Given that animals exhibit different OCR through various life stages, animals used in this protocol should be age synchronized. Younger animals have lower OCR compared to adult animals and the OCR levels can drop again as animals age further[3]. *C. elegans* are also sterilized by the use of FUdR to ensure that OCR are not confounded by the presence of progeny. Nevertheless, if animals of varying sizes are to be examined, strategies for normalization need to be addressed.

This protocol utilizes a platinum pick to transfer animals to the assay plate. In contrast to liquid transfer of animals, this manipulation enables the researcher to carefully examine and determine the health of the animals before and during the transfer. Also, it allows for better control of worm number and prevents the introduction of eggs and carcasses. Since the animals are alive and active during the assay run, variability is likely to arise from probe positioning. Assays should therefore be done in triplicate and should be repeated a minimum of three times. Also, double checking animal count post analysis is important for normalizing to animal number. A major drawback of this protocol is the inability to compare more than two samples at once, without compromising the number of replicates within a single assay. Despite this limitation, this protocol can be a very powerful research tool in analyzing OCR when comparing two genotypes or conditions. Moreover, this protocol could be easily adapted to examine the OCR of animals grown on different food sources, supplements, or drug treatments.

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DISCLOSURES:

The authors have nothing to disclose.

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Homeostasis Impacts Mitochondrial Function and Morphology in Caenorhabditis elegans. *Genetics.* **201**, 1453-1466, doi:10.1534/genetics.115.182808 (2015).

Figure 1

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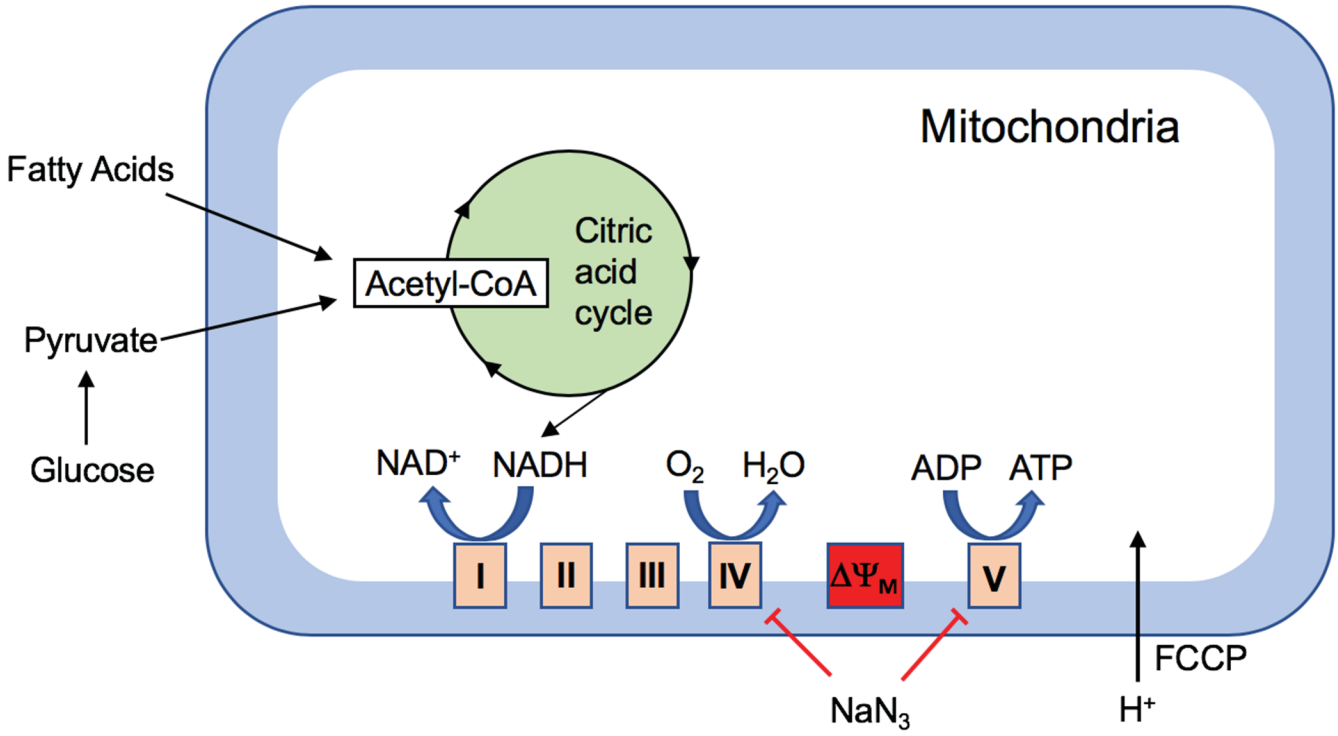

Figure 2

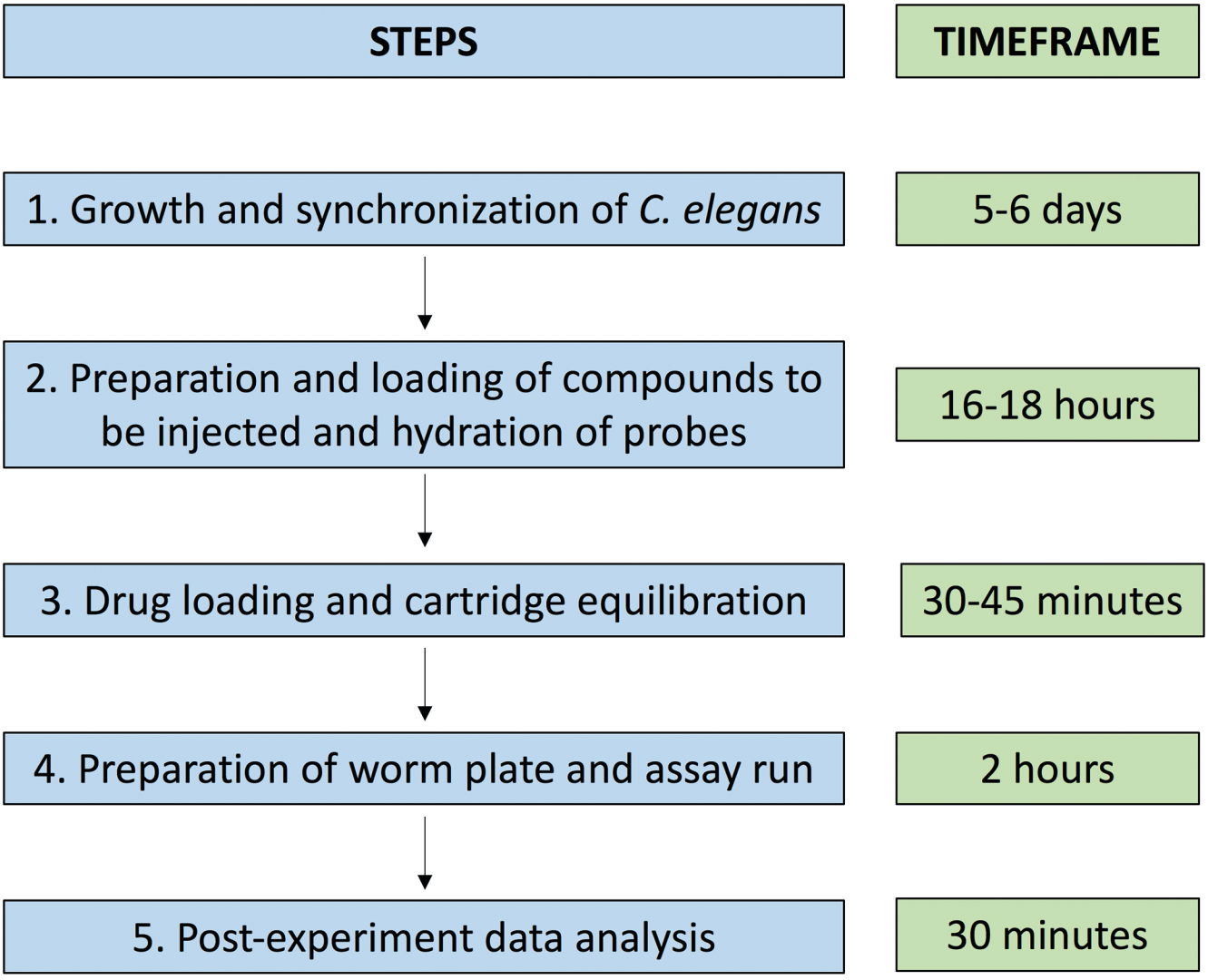

Figure 3

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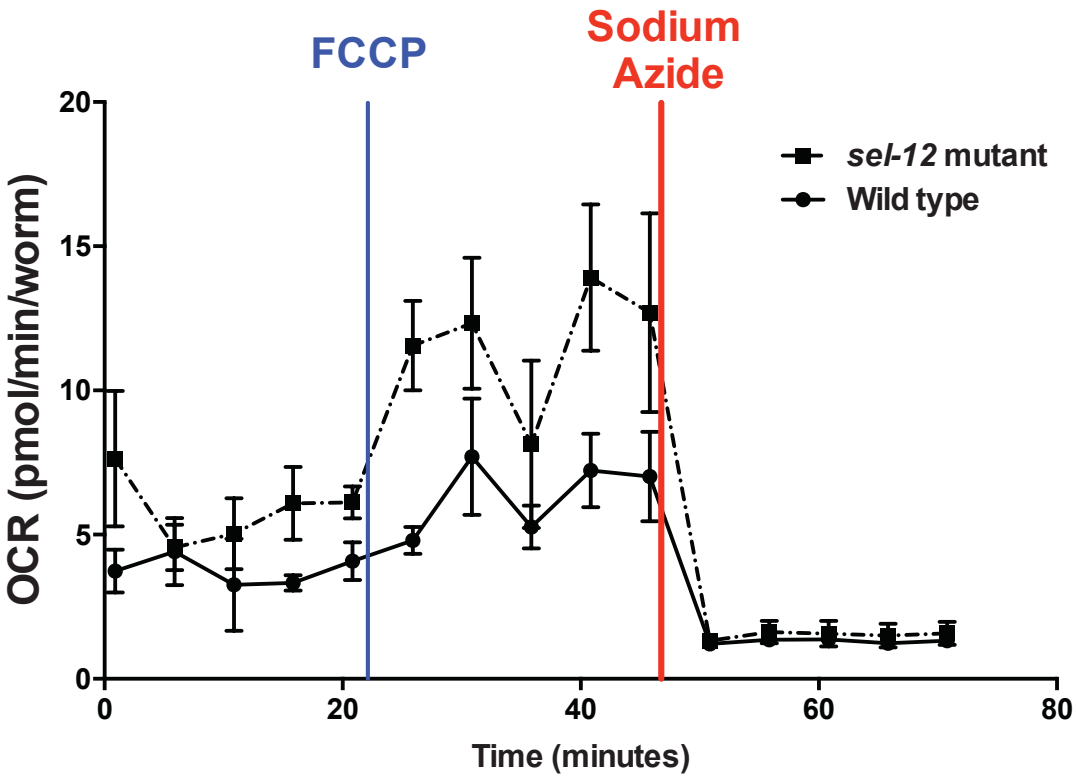

Figure 4

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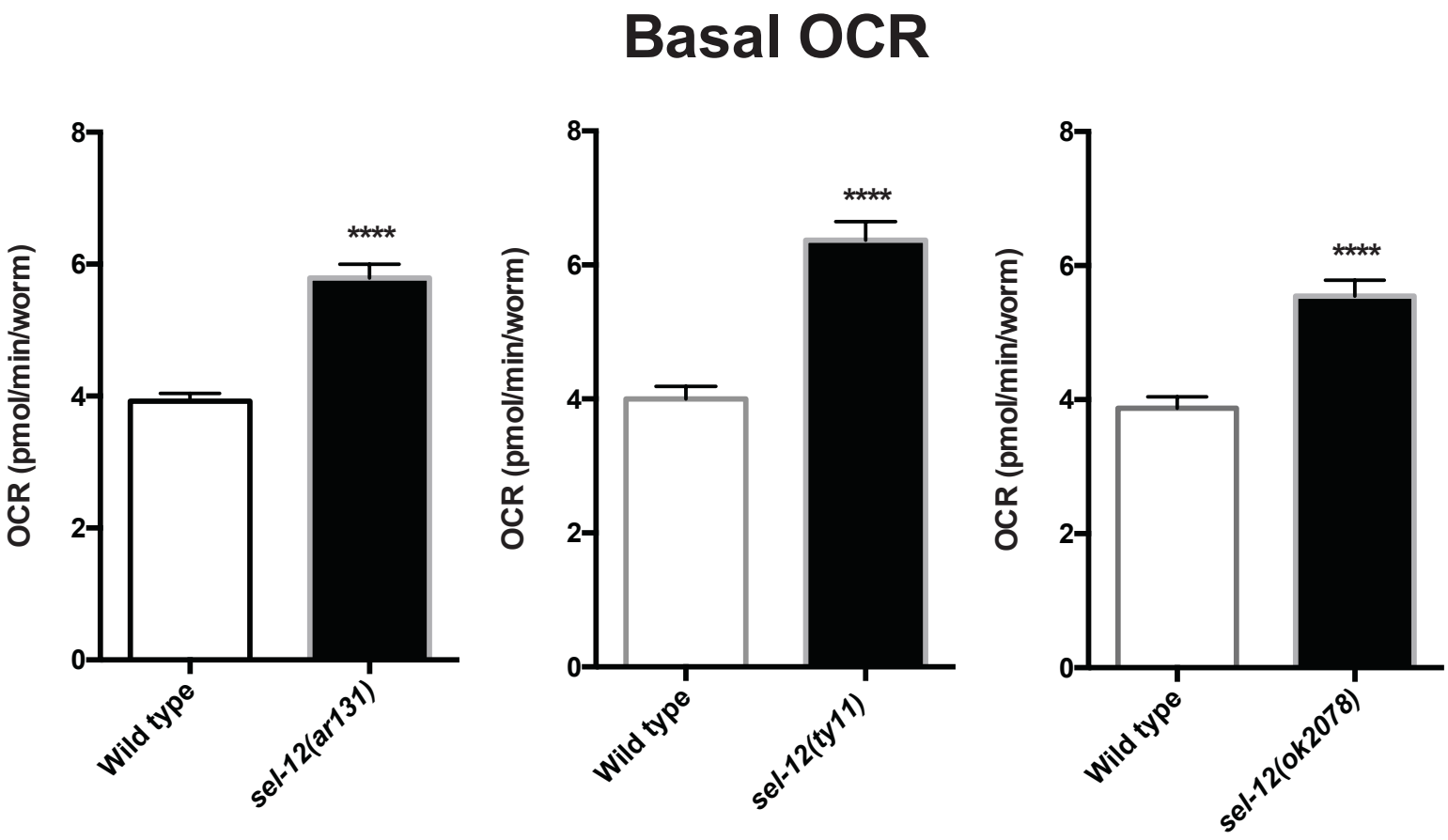

Figure 5

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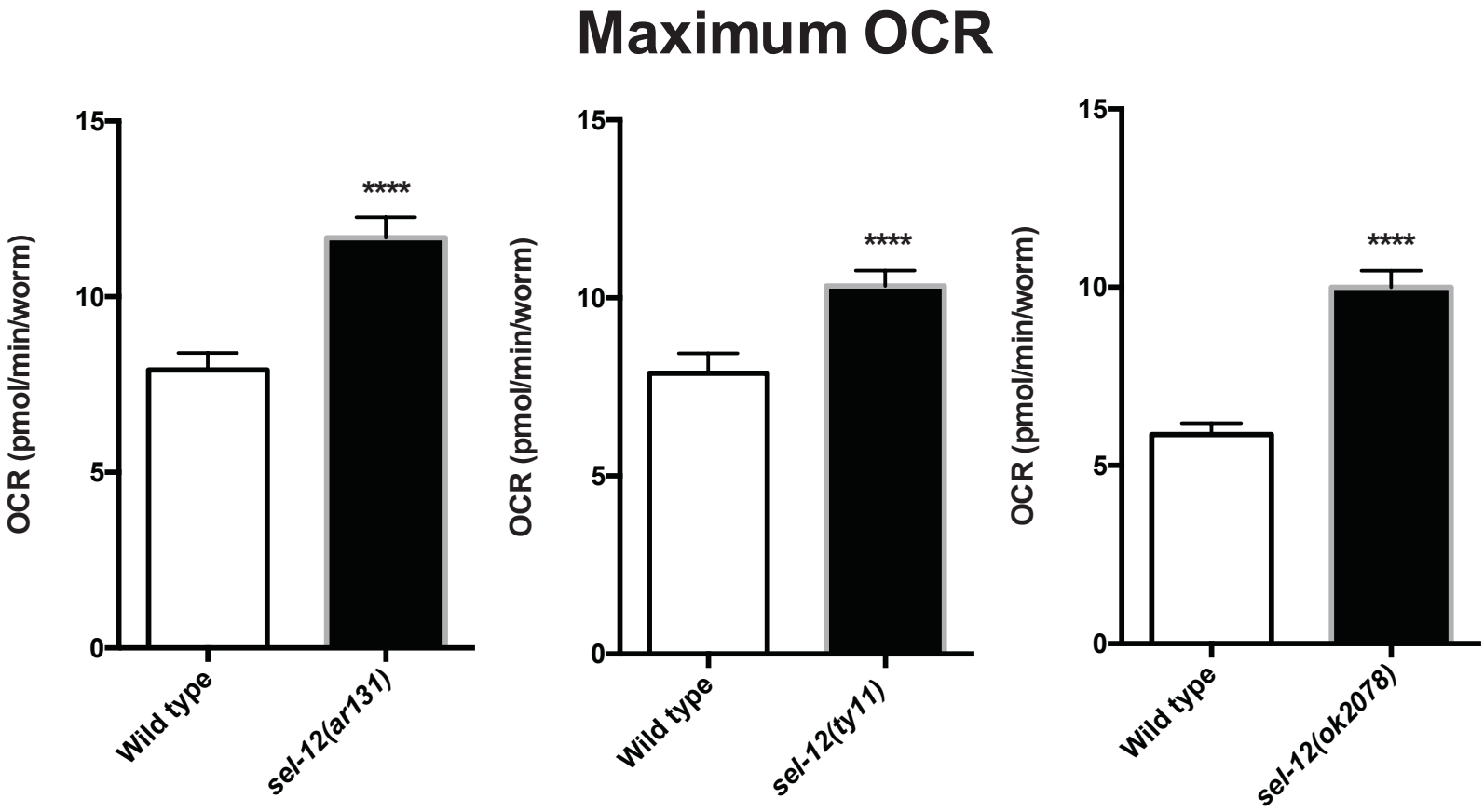

M9 buffer (1 L)

dH_2O	1,000 mL
NaCl	5 g
KH_2PO_4	3 g
Na_2HPO_4	6 g
1 M $MgSO_4$	1 mL *add after autoclaving

Split between 2 bottles: 500 mL each. Autoclave on liquid cycle (15 min exposure)

Bleach solution (50 mL)

dH_2O	36 mL
Bleach	14 mL
10 N NaOH	800 µL

Standard Nematode Growth Media (NGM) plates

	1 L	0.5 L	0.25 L
NaCl	3 g	1.5 g	0.75 g
Bacto-agar	17 g	8.5 g	4.25 g
Bacto-peptone	2.5 g	1.25 g	0.625 g

a. Autoclave on liquid cycle (45 min exposure), allow to cool to ~60 °C and then use sterile technique and add the following:

	1 L	0.5 L	0.25 L
1 M $CaCl_2$	1 mL	0.5 mL	0.25 mL
1 M $MgSO_4$	1 mL	0.5 mL	0.25 mL
1 M KPO_4	25 mL	12.5 mL	6.25 mL
5 mg/mL cholesterol	1 mL	0.5 mL	0.25 mL

b. Swirl to mix thoroughly after each addition. After all additions are made, pour plates

Table 2

Calibration	**Basal**	**FCCP**	**Sodium Azide**
		Port(s): A	Port(s): B
Equilibration: Yes	Mix: 00:02:00	Mix: 00:02:00	Mix: 00:02:00
	Wait: 00:00:30	Wait: 00:00:30	Wait: 00:00:30
	Measure: 00:02:00	Measure: 00:02:00	Measure: 00:02:00
	Cycles: 5	Cycles: at least 5	Cycles: 2–5
	Duration: 00:22:30	Duration: 00:22:30	Duration: 00:09:00

Name of Material/ Equipment	Company	Catalog Number
100 mm, 60 mm Petri dishes	Kord-Valmark Labware Products	2900, 2901
1.5 mL centrifuge tubes	Globe Scientific	6285
15 mL conical tubes	Corning	430791
22 × 22 mm coverslip	Globe Scientific	1404-10
50 mL conical tubes	Corning	430829
Agar	Fisher Scientific	BP1423-2
Bacto peptone	BD, Bacto	211677
Bacto tryptone	BD, Bacto	211705
Bacto yeast extract	BD, Bacto	212705
Bleach	Generic	
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Fisher Scientific	C79-500
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	Abcam	ab120081
Cholesterol	Fisher Scientific	C314-500
Deionized water (dH_2O)		
Dimethyl sulfoxide (DMSO)	Thomas Scientific	C987Y85
Glass Pasteur pipettes	Krackeler Scientific	6-72050-900
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Fisher Scientific	BP213-1
Potassium phosphate dibasic (K_2HPO_4)	Fisher Scientific	BP363-1
Potassium phosphate monobasic (KH_2PO_4)	Fisher Scientific	P285-500
Sodium chloride	Fisher Scientific	BP358-10
Sodium hydroxide (NaOH)	Fisher Scientific	BP359-500
Sodium phosphate dibasic anhydrous (Na_2HPO_4)	Fisher Scientific	BP332-1
Seahorse XFp Analyzer	Agilent	
Seahorse XFp FluxPak	Agilent	103022-100
Sodium Azide	Sigma-Aldrich	S2002

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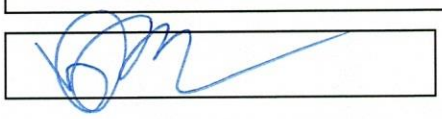
Signature:	
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