A screening-based platform for the assessment of cellular respiration in *Caenorhabditis elegans*

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Mitochondrial dysfunction is at the core of many diseases ranging from inherited metabolic diseases to common conditions that are associated with aging. Although associations between aging and mitochondrial function have been identified using mammalian models, much of the mechanistic insight has emerged from *Caenorhabditis elegans*. Mitochondrial respiration is recognized as an indicator of mitochondrial health. The Seahorse XF96 respirometer represents the state-of-the-art platform for assessing respiration in cells, and we adapted the technique for applications involving *C. elegans*. Here we provide a detailed protocol to optimize and measure respiration in *C. elegans* with the XF96 respirometer, including the interpretation of parameters and results. The protocol takes ~2 d to complete, excluding the time spent culturing *C. elegans*, and it includes (i) the preparation of *C. elegans* samples, (ii) selection and loading of compounds to be injected, (iii) preparation and execution of a run with the XF96 respirometer and (iv) postexperimental data analysis, including normalization. In addition, we compare our XF96 application with other existing techniques, including the eight-well Seahorse XFp. The main benefits of the XF96 include the limited number of worms required and the high throughput capacity due to the 96-well format.

INTRODUCTION

Mitochondria have long been considered the crucial organelles responsible for the production of ATP[1]. To perform their key roles in energy production, mitochondria use an intricate system that encompasses the breakdown of substrates—such as glucose, fatty acids and amino acids—coupled to the generation of ATP via oxidative phosphorylation[2,3] (**Fig. 1a**,**b**). In the past few years, it has become increasingly apparent that mitochondria, the 'powerhouses' of the cell, are not only involved in the production of cellular energy, but are also intimately involved in a variety of processes and signaling pathways including, but not limited to, apoptosis and calcium homeostasis (as reviewed in refs. 4–6). As a consequence, it does not come as a surprise that mitochondrial dysfunction is associated with a broad spectrum of diseases, including inherited metabolic diseases, as well as common conditions such as aging, neurodegenerative diseases, cancer and diabetes[7–14].

Numerous *in vitro*, *in situ* and *in vivo* methodologies have been developed to assess various aspects of mitochondrial function[1,15,16]. Assays include the estimation of mitochondrial membrane potential[17], the determination of the production and/or concentration of ATP with bioluminescence[18] and the determination of calcium retention[19]. Although these assays measure one specific aspect of mitochondrial function, they do not cover the full extent of mitochondrial health. The oxygen consumption rate (OCR) of mitochondria provides an additional layer of complexity that is dependent on many sequential reactions in mitochondria. As such, the measurement of mitochondrial respiration at the level of the electron transport chain (**Fig. 1b**) has emerged as a proxy for mitochondrial health[20–22]. Indeed, alterations in the rate of oxygen consumption are described as an informative indicator of mitochondrial dysfunction[23]. It was the introduction of the Seahorse extracellular flux analyzers (hereafter called XF respirometers) that allowed measurement of O_2 consumption in multiple parallel wells without the need to lyse cells or isolate mitochondria[1,24].

Although initially developed for cultured or isolated cells, XF respirometers can also be used for whole organisms such as *C. elegans*, when taking some important adjustments into account[25–28]. *C. elegans* is often used as a model organism to study mitochondria in the context of longevity[29]. It is the ease of genetic modifications (e.g., RNAi, CRISPR) that renders the exploration of mitochondria-related pathways in worms readily accessible[30–32], especially considering that many of the genes involved in human metabolism are conserved in *C. elegans*[33–36]. Consequently, we can use *C. elegans* as a simple model to study the complex interplay between genes and mitochondrial function, which is usually measured at the level of gene expression (e.g., heat-shock protein induction) or phenotypic characterization of live animals (e.g., locomotion or pharyngeal pumping rates). Nevertheless, the progress of the mitochondrial field in *C. elegans* was long delayed by the limited options for the dedicated measure of metabolism, in particular mitochondrial activity. Hence, we set out to develop a platform using Seahorse respirometry, which was successfully applied in several projects[15,26,27]. Here we describe in detail how Seahorse XF96 respirometers can be used to study mitochondrial biology in *C. elegans*. Moreover, we also provide detailed information about how parameters can by optimized for individual worm strains.

Theoretical background of XF96 respirometers

The XF96 respirometers provide researchers with the possibility of performing real-time measurements of the OCR and extracellular acidification rate (ECAR). The OCR is predominantly the result of mitochondrial respiration through oxidative phosphorylation (**Fig. 1b**), whereas ECAR is predominantly the result of glycolysis (**Fig. 1a**). The system uses a 96-well plate combined with a sensor cartridge containing an equal number of individual sensor probes.

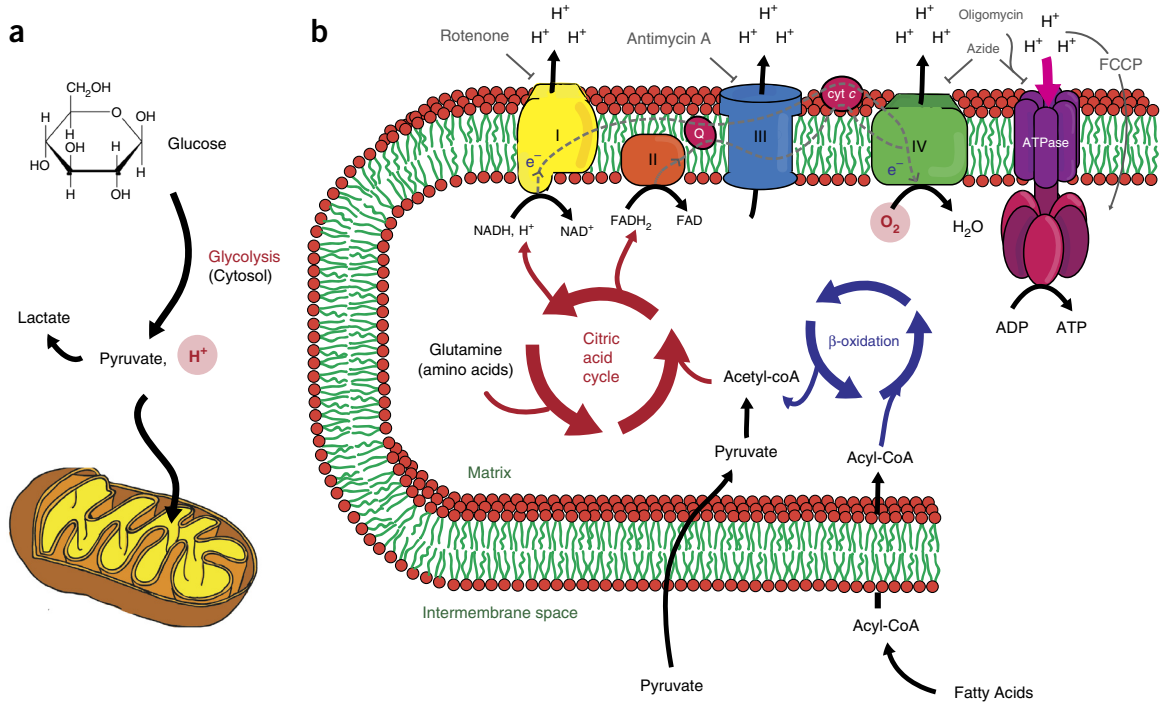

Figure 1 | A schematic overview of glycolysis and oxidative phosphorylation. (**a**) The mitochondrion with its characteristic structure and the translocation of pyruvate, which is the result of glycolysis. During glycolysis, H^+ is generated, which causes extracellular acidification. (**b**) Energy-related metabolic pathways that take place in the mitochondrial matrix. Oxidative phosphorylation requires the consumption of O_2. Compounds known to inhibit the different complexes (as shown with the roman numerals) are listed in gray (rotenone, antimycin A, oligomycin and azide). FCCP is an uncoupler reagent and transfers H^+ back to the mitochondrial matrix without generating ATP. Acyl-CoA, Acyl-Coenzyme A.

At the tip of each of the sensor probes, there are two separate polymer-embedded fluorophores that are sensitive to either O_2 or H^+. The sensitivity of theses probes is based on the quenching chemistry of the fluorophores in response to O_2 and H^+ in the assay medium. The concentrations of O_2 and H^+ are therefore directly linked to the intensity of the fluorescence signal relative to a standard solution. Moreover, any changes in the signal can be inferred to be proportional to changes in O_2 or H^+ concentrations, and therefore rates of change in O_2 or H^+ concentrations can be obtained by following the fluorescent signal over time.

During a measurement cycle, the sensor cartridge is lowered into the wells, creating a transient microchamber of a defined volume (**Fig. 2a**). Fiber-optic bundles are inserted into the probes, and light is pumped into the sensor to excite the embedded fluorophores. The resulting emitted light is transmitted back through the fiber-optic bundle and measured by the detector. The fluorescent emission is measured for a specified period (measuring time; **Fig. 2b**). The slope of the linear decline (for O_2 concentration over time) or incline (for H^+ concentration over time) provides the basis for calculating the OCR (in picomoles per minute) and ECAR (milli pH units per minute), respectively (**Fig. 2b**). The exact algorithm that is used to calculate the OCR in XF respirometers is described in Gerencser *et al.*[22].

After several minutes of continuous measurements, the sensor cartridge is raised and subsequently allowed to move up and down for a specified time interval, which causes the larger volume of medium above to mix with the medium in the transient microchamber, thereby restoring the O_2 and H^+ concentrations (mixing time; **Fig. 2b**). To get multiple, steady estimations of the OCR and ECAR, the measuring and mixing cycle can be repeated (looped).

In addition, the presence of an integrated drug delivery system in the sensor cartridge plate allows sequential addition of up to four compounds/solutions per well at user-defined time points. Selection of the compounds to be added, and the sequence in which they are added, makes it possible to differentiate between several aspects of cellular respiration; for example, non-mitochondrial respiration can be determined by blocking mitochondrial respiration with a compound (e.g., rotenone or antimycin A)[37–40] (**Fig. 1b**).

Comparison with other *in vivo* respirometry methods

Several methods are available that measure respiration of living samples; these can be divided into two general groups: methods that involve O_2-dependent quenching of porphyrin-based phosphors (using a Seahorse Bioscience XF respirometer and Luxcel MitoXpress) and those that involve amperometric O_2 sensors (using Clark electrodes, including the widely adopted Oroboros system)[1,41]. Historically, the amperometric approach has been the main method used to assess mitochondrial respiration in *C. elegans*. For the amperometric approach, nematodes are delivered into a single respiratory chamber, which is separated from two half-cells by O_2-permeable material. In this way, only O_2 can diffuse from the assay medium through the membrane. When a small voltage is applied to the half-cells, O_2 is reduced by electrons at the cathode, yielding hydrogen peroxide. Subsequently, H_2O_2 oxidizes the Ag (silver) of the Ag/AgCl anode, which results in an electrical current that is proportional to the O_2 pressure—and thus concentration—in the experimental respiratory chamber.

Apart from the detection modality, differences between XF respirometric methods appear in relation to the number of worms per assay, number of replicates, multiple or real-time measurements,

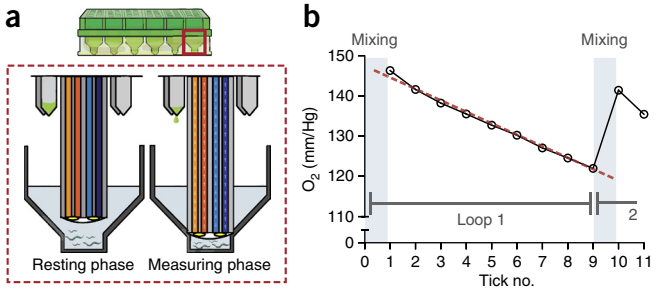

Figure 2 | The measurement units of XF respirometers. (**a**) Dashed red box shows a close-up of the probes of the sensor cartridge (solid red box). During the measuring phase, probes of the sensor cartridge are lowered, thereby creating a transient microchamber (from resting phase to measuring phase). Light is transmitted through the fiber-optic bundles to excite the embedded fluorophores, which are sensitive to O_2 and H^+, at the tip of the probe. Emitted light then passes back up the optic bundle to the detector. Compounds can be injected during the experimental assay via ports at the base of the probe. (**b**) During a measuring cycle, oxygen is consumed and the concentration of the analyte drops. A representative measurement is shown. The mixing phase represents the stage at which the probe is repeatedly moving between the resting position and the measuring position, allowing for mixing of the medium. During the measuring phase, the O_2 concentration is estimated nine times per loop, when measuring for 2 min. These nine estimations of the oxygen concentration (or ticks, as directly derived from the 'level data' in the Excel file) together are used to calculate one OCR per loop. Here, the linear decline of O_2 concentration (red dotted line) provides the basis for the OCR estimation.

and the ability to inject compounds during an experiment (**Table 1**). The Clark electrode approach requires thousands (~2,000–5,000) of worms in a single chamber to obtain an estimation of the OCR[42]. Performing multiple measurements and biological replicates, and comparing conditions provide the biggest challenges within the Clark electrode method, as the traditional setup allows the measurements of only one sample at a time. By contrast, an XF96 respirometer requires ~10–20 worms per well to acquire a reproducible OCR; measurements can be easily and quickly (in the order of minutes) repeated in an automated way; and as XF respirometers can analyze whole plates at the same time, ~96 conditions/replicates can be tested at once. An additional difference is the presence of drug-injection ports that can be programmed to inject compounds into all 96 wells at time points that are specified *a priori* during an XF respirometer experiment. Clark electrode systems also allow injection of compounds, and even offer flexibility with respect to the timing, dosing and number of additions, as compounds are injected manually during the course of the assay. However, precise timing of manual additions between replicate experiments may be challenging.

More similar to the Seahorse XF respirometer method is the Luxcel MitoXpress O_2 consumption assay, which relies on O_2-dependent quenching of porphyrin-based phosphor. The MitoXpress kits provide a way of performing real-time analysis of cellular respiration via an oxygen-quenching fluorophore system. Worms are placed into the wells of a 96- or 384-well plate, the kit reagents are added and measurements are made in a fluorometric plate reader. Multiple conditions and replicates can be tested side by side in the wells of a single plate, but repeated measurements over time are more challenging, as there is typically no automatized mixing system integrated into the plates or plate readers to restore basal O_2 levels. In addition, single estimation of the OCR takes >90 min, whereas careful estimations of the OCR in the XF respirometer approach take only 2–5 min of measuring time. Finally, the use of compounds to assess multiple aspects of mitochondrial function related to oxygen consumption is limited, as the compounds need to be injected manually immediately before the start of the experiment.

Advantages and limitations of XF respirometry

On the basis of the comparisons with other respirometric methods, the XF respirometer approach offers important benefits for the measurement of mitochondrial respiration in *C. elegans*, especially when considering the ease of performing repeated measurements, comparing conditions and replicates, and investigating several aspects of mitochondrial function. This allows a higher throughput for screening-based applications—e.g., genome-wide screens to find genetic interactors affecting mitochondrial bioenergetics and compound screens for mitochondrial toxicity. Indeed, XF respirometry has already been successfully applied to study the effects of compounds or gene knockdowns on mitochondrial function in *C. elegans*[15,26,27].

In vivo assessment of mitochondrial function in *C. elegans* also has, however, some clear limitations. First of all, oxygen consumption of an intact organism (e.g., basal respiration) does not strictly reflect mitochondrial respiration. For instance, cells possess a variety of oxygenases that also consume oxygen and thereby contribute to the OCR[43]. Hence, residual respiration should be estimated in the presence of effective electron transport inhibitors in order to distinguish non-mitochondrial respiration from mitochondrial respiration (**Fig. 3a**). In cells, the use of four compounds (making use of the drug injection ports; **Fig. 3b**) to

TABLE 1 | Differences between *in vivo* respirometric methods.

	Clark electrode	Seahorse XF96 Flux Analyzer	Luxcel MitoXpress
Type of approach	Amperometric	Porphyrin-based phosphors	Porphyrin-based phosphors
Required no. of worms	~2,000–5,000	~2–25	~2–25[a]
Time required for measurement of OCR	2–15 min	2–3 min	>90 min
Multiple measurements/loops	No	Yes, automated	No
Maximal conditions per run	1	96 wells per plate (92 without background-correction wells)	96 or 384 (92 or 380 without background-correction wells)
Injection of compounds during run	Yes, manual[b]	Yes, automated	No[c]

[a]Provides an estimation of the number of worms per well, as we did not test this method ourselves and specific numbers are not mentioned in the literature yet. Numbers are derived from those used with the XF96 respirometer. [b]Compounds can be injected manually, not in an automated manner. [c]The influence of compounds can be assessed in this method, but they should be injected before the start of the assay.

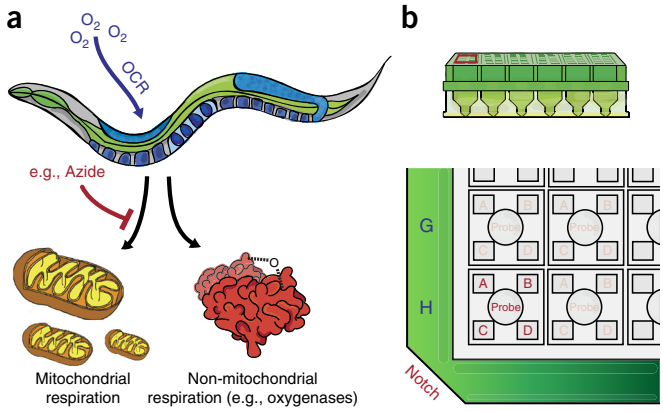

Figure 3 | Distinguishing non-mitochondrial respiration from mitochondrial respiration. (**a**) The oxygen consumption in cells and organisms is not due to mitochondrial respiration only. Therefore, one should add a compound (e.g., sodium azide) to block mitochondrial respiration, in order to distinguish mitochondrial oxygen consumption from other oxygen-consuming processes. (**b**) Layout of the drug injection ports per well; indexed as A–D when considering the notch at the bottom left.

distinguish between and to quantify several aspects of cellular respiration is well established. These aspects include, but are not limited to, the following: (i) respiration linked to ATP production (using oligomycin); (ii) maximal respiration (using carbonyl cyanide-4(trifluormethoxy)phenylhydrazone: FCCP); and (iii) mitochondrial and non-mitochondrial respiration (using antimycin A and rotenone; **Table 2**; **Fig. 1b**; refs. 37–40). Nevertheless, *C. elegans* has traditionally been considered a poor candidate for drug-related assays because of the relatively inefficient uptake of compounds caused by impermeability of the cuticle to non–water soluble compounds[44–46]. Consequently, it does not come as a surprise that, except for FCCP, the compounds typically used in cultured cells are inefficient in acutely inhibiting specific aspects of mitochondrial function in *C. elegans* (**Supplementary Fig. 1**)[28]. However, there are alternative compounds, such as the complex IV/V inhibitor sodium azide, that can be used to help decipher aspects of mitochondrial respiration in *C. elegans*[28,47] (**Fig. 1b**).

One can argue that isolating mitochondria of *C. elegans* would represent a more sophisticated way to assess mitochondrial function. Isolated mitochondria make exploration of the molecular (super)complexes involved in oxidative phosphorylation possible[48]. Changes in respiratory chain complexes and anaplerotic enzymes can be identified using combinations of substrates and inhibitors specific to the isolated mitochondria[49], which allows careful dissection of the respiratory chain. Nevertheless, the clear advantages of isolated mitochondria are also accompanied by several disadvantages. First of all, existing methods often require large amounts of sample because of a poor yield of mitochondrial content, and they do not prevent biased selection toward specific mitochondrial populations[50–53]. Second, the isolation process may decrease mitochondrial membrane integrity and disrupts both the mitochondrial environment and the cellular/organismal context, which affects mitochondrial morphology, respiration and reactive oxygen species (ROS) production[52,53]. In addition, mitochondrial isolation procedures are technically challenging in *C. elegans* compared with tissue samples or even cells, precluding the use of such a strategy for routine biochemical analysis[54]. As such, it is clear that the XF respirometers provide the user with a platform for performing relatively quick high-throughput screens in an organismal context. In this way, interactions of the mitochondria with the rest of the cell are maintained, and so is the physiological relevance.

Finally, in addition to the aforementioned considerations for using XF respirometers for assessing mitochondrial function in *C. elegans*, some critical technical 'limitations' should be mentioned. As XF respirometers were originally developed for cells, the temperature control/heater of the machine is designed to work at 37 °C. Considering that nematodes are typically cultured at 20–25 °C, and their physiology is dependent on the environmental temperature, the lack of tight temperature control could affect the outcome of the experiments. Depending on the environmental setting of the XF respirometer, turning the heater off will result in temperatures of ~20–25 °C, which will increase during the run by ~2–4 °C. We have found that temperatures in the range of 20–25 °C will not cause any significant changes in the respiration rate of wild-type *C. elegans* (**Fig. 4**). Nevertheless, it is strongly advised to compare strains within only one plate to account for masked effects of temperature on respiration. Another aspect to consider is that ECAR cannot be assessed in worms when using the protocol that we describe here, as worms are assayed in a buffered medium (M9, see below) that precludes changes in H^+ concentration. A method for measuring ECAR in *C. elegans* with an XFe24 respirometer has been described recently that uses unbuffered water as the assay medium[28]. Nonetheless, even when it is possible to estimate the ECAR, it is unclear whether this represents a valid

TABLE 2 | Compounds used to measure key parameters of mitochondrial function.

Compound	Function	Description
Oligomycin[a]	ATP synthase inhibitor	Inhibits ATP synthesis by blocking the proton channel of the F0 portion of ATP synthase (complex V)
FCCP[a]	ETC accelerator	An ionophore that disrupts the mitochondrial membrane potential and thus ATP synthesis while still allowing proton pumping, electron transport and oxygen consumption
Antimycin A and rotenone[a]	Mitochondrial inhibitors	Rotenone is a complex I inhibitor and antimycin A is a complex III inhibitor. Together they inhibit total mitochondrial respiration
Sodium azide[b]	Complex IV and V inhibitor	Inhibits complex IV (cytochrome c oxidase) and ATP synthase (complex V)

[a]They are commonly used in cells (Mito Stress Test Kit, Bioscience). [b]It is not used in cell assays, but it is used to inhibit mitochondrial respiration in *C. elegans* and is a good alternative to antimycin A and rotenone (which are inefficient in *C. elegans*).

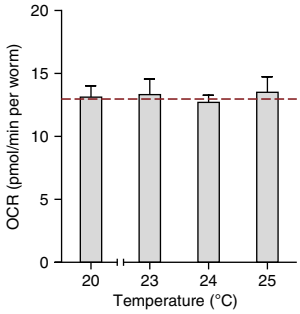

Figure 4 | Temperature does not influence the OCR of *C. elegans*. Up to 25 °C, the basal respiration of nematodes (N2) is not influenced by the temperature. Bars represent mean ± s.e.m. One-way ANOVA (not significant), $n = 7–9$ wells. The dashed red line represents the OCR at 20 °C.

marker for glycolytic rate. Further validation of these parameters will hopefully shed light on this option and expand the toolkit for measuring metabolism in *C. elegans*.

Alternative applications

The XF96 respirometer method provides a flexible platform for performing genome-wide screens or compound screens in relation to mitochondrial respiration. As the experimental setup requires only that organisms be viable in liquid culture and have a size that fits the transient microchamber (XF96: 3 µl and XF24: 7 µl), mitochondrial respiration can also be estimated in species other than *C. elegans*. Therefore, Seahorse XF respirometers might open new avenues for other well-established or upcoming species that may be used in metabolic or aging studies, such as the flatworm *Macrostomum lignano*[55], *Danio rerio* embryos[56,57] and even *Nothobranchius furzeri* embryos[58]. Although XF respirometers are, to our knowledge, not used in *M. lignano* or *N. furzeri* research yet, it has been shown that respiratory kinetics in embryos from *D. rerio* can be investigated with XF respirometers[57].

Overview of the procedure

Performing an XF respirometer experiment with *C. elegans* is technically a simple procedure that consists of six main steps: (i) preexperimental procedures; (ii) preparation of the XF respirometer; (iii) loading of compounds; (iv) collection and loading of worm samples; (v) the actual XF experiment; and (vi) postexperimental data analysis. An overview of the experimental procedure is given in **Figure 5**. Optimization of steps i–iv is required in order to obtain reliable estimations of the OCR and to reduce variation within runs. Some of the preparatory steps are very similar to those used in cells[49]. However, many of the exact parameters and steps cannot be translated one-on-one to *C. elegans*, and it is therefore highly recommended to take note of our proposed optimization steps before continuing to the PROCEDURE section.

(i) Pre-experimental procedures. The pre-experimental procedures should be performed 1 d before the actual XF experiment, but it will in general take more than 1 d of preparation. Comparing OCR of different worm strains requires strict age synchrony, as respiration changes significantly during development and aging (**Fig. 6**). Hence, it is important that careful phenotyping be performed before the respiration experiments in order to anticipate potential differences in the rate of development and worm size. When assessed, worm strains can be age-synchronized (hypochlorite treatment, or egg laying) several days before the experiment[59]. To avoid offspring or eggs from hatching, one can use plates containing 5-fluoro-2′-deoxyuridine (FUdR; starting from young adults)[60]. Here, it is important to realize that FUdR can alter the biology of the worms, as evidenced by reported changes to life span, worm size and metabolite levels[61,62]. Other techniques for maintaining synchronized worm cultures are described in Gruber *et al.*[63]. In addition to preparing the worm samples, the cartridge needs to be prepared in advance in order to hydrate the sensor probes. The sensor cartridges should be incubated with Seahorse

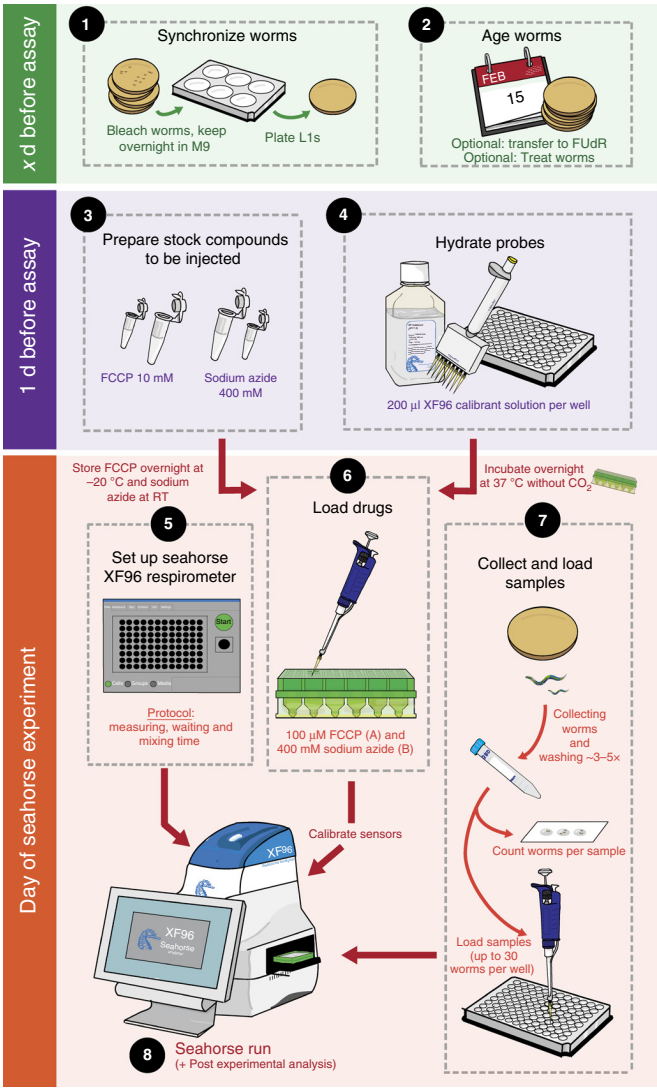

Figure 5 | A schematic overview of an XF respirometer experiment. Overview of experimental procedures involved in preparing and performing an XF assay with *C. elegans*. The procedure consists of (1) synchronizing worms; (2) aging worms; (3) preparing compounds to be injected; (4) hydrating the probes of the cartridge (Steps 1–4 in the PROCEDURE); (5) preparing the XF respirometer (Steps 5–14 in the PROCEDURE); (6) loading the compounds in the injection ports (Steps 15–20 in the PROCEDURE); (7) collecting, washing and loading worm samples (Steps 21–35 in the PROCEDURE); and (8) executing the actual respirometer run and performing data analysis (Steps 36–46 in the PROCEDURE). See the main text for a more detailed explanation of all steps involved.

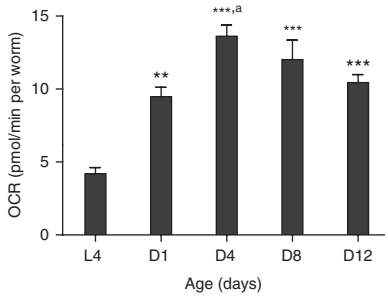

Figure 6 | Respiration rates change during development and with age in *C. elegans*. Basal respiration changes during development (from L4 to adult stage) and aging in N2 worms. All adult stages show significantly higher respiration than the larval stage L4. In addition, respiration is significantly higher at day 4 of adulthood (D4) as compared with day 1 (D1; $P < 0.05$), as indicated by 'a'. Bars represent mean ± s.e.m. One-way ANOVA ($P < 0.001$) with *post hoc* Tukey test, $n = 6–8$ wells. $^{**}P < 0.01$, $^{***}P < 0.001$.

Bioscience XF96 calibrant solution, pH 7.4, at 37 °C without CO_2 (to keep the calibration temperature constant and optimal for XF respirometers). It is recommended that the probes be hydrated overnight, but a minimum hydration time of 4 h is required, and, if necessary, the hydration can be prolonged to 72 h, in which case we advise sealing the plate with Parafilm and storing it at 4 °C. 1 d before the assay, one can also prepare stock solutions of the compounds to be injected during the Seahorse experiment, including FCCP (10 mM in DMSO: 1,000× the intended working concentration) and sodium azide (400 mM in dH_2O: 10× working concentration). It is important to realize that compounds are ten times diluted when they are injected in the wells (because of the volume already present). Therefore, stock solutions need to have a concentration that is ten-fold higher than the intended working concentration. Moreover, for compounds that are dissolved in DMSO, it is strongly suggested that one make a stock that is 1,000× concentrated, to ensure that the DMSO concentration is ≤1% (dilute 100× to get the stock required for injection)[64].

(ii) Preparation of the XF respirometer. In anticipation of the respiration measurements, the XF96 software should be prepared and programmed. Although the exact steps are described in the PROCEDURE, there are some important aspects that require clarification. As XF respirometers were initially developed for cell-related research, the machine has an automatic heater and temperature control system to maintain a temperature of 37 °C. For Seahorse experiments with *C. elegans*, it is essential that the temperature control system and heater be turned off in advance, thus allowing the machine to adapt to room temperature (~20–25 °C). Be aware that every time you restart the respirator, the temperature control system and the heater have to be turned off.

The 'assay wizard' embedded in the Seahorse software provides a user-friendly interface for generating an experimental template with all the commands to control the XF96 respirometer during the experiment. Many of the tabs and fields are filled at the user's discretion, including, for instance, user info, medium info and plate layout. Although these items can be helpful in tracing the experimental conditions, they do not affect the XF run. However, it is important to adjust three tabs: 'General', where you fill in the date and name of your experiment; 'Background correction', where you select the wells that function as correction wells (these contain buffer only); and 'Protocol', where you specify the order and duration of mixing, measuring and waiting time, as well as the injection of compounds. The 'Protocol' tab provides you with several commands that can be executed by the machine. Although most of them are self-explanatory, we provide here a brief overview:

- *Calibrate.* This is always the first command of each XF assay; it calibrates the sensors for the planned experiment. This takes ~15–20 min.
- *Equilibrate.* This is always the second step after calibration. It consists of three loops of 2 min each of mixing and waiting. Equilibration is performed to stabilize the temperature of the plate.
- *Mix.* This command sets the mixing of the contents of the wells by gentle up/down motion of the sensor sleeves. Mixing is necessary to restore analyte (O_2 and H^+) distribution and concentration in the wells and to mix injected compounds.
- *Wait.* This command instructs the machine to wait for *x* number of minutes.
- *Measure.* This command instructs the machine to do the actual measurements to determine the OCR and ECAR.
- *Inject.* This command instructs the machine to inject compounds that are loaded in the ports into the wells. Here you can also choose which port should be injected (A, B, C or D).
- *Loop.* This command is used to repeat a subset of commands *x* number of times.

The duration of the commands 'Mix', 'Wait' and 'Measure' should be carefully optimized to ensure a stable OCR over time; see Optimization of parameters. In addition, multiple loops should be performed to obtain not one but multiple estimations of the OCR. When the experimental template is ready, the actual run can be directly started; however, to avoid unnecessary heating, it is important to start the run only when the samples are almost ready.

(iii) Loading compounds. The benefit of working with XF96 respirometers is the possibility of using the integrated drug delivery system that allows sequential injection of up to four compounds. With the use of specific mitochondrial toxins, a more complete profile of mitochondrial function can be generated. When an XF experimental template is generated, it is possible to include the command 'Injection of port *x*', where *x* refers to a port indexed with a specific letter: A–D. The drug ports are organized in the following way around each probe: A = top left, B = top right, C = lower left and D = lower right (**Fig. 3b**). To facilitate the loading procedure, the sensor cartridge is accompanied by two loading guides—i.e., lids with holes that allow easy access to pipette only in the port of choice. When the sensor cartridge is loaded with the compound(s), the plate can be placed back in an incubator at 37 °C without CO_2. It is important to understand that the injection is initiated through a pneumatic mechanism. As a consequence, when using a specific injection port, this port should be filled for all 96 wells, even if those wells are not used for the oxygen consumption measurement.

There are several compounds that can be injected to assess different aspects of respiration. A drug that we have extensively tested and that is often used in combination with XF96 respirometers is FCCP. FCCP is a proton ionophore, which means that it is able to transport hydrogen ions across membranes. FCCP is therefore an uncoupling agent, as it disrupts ATP synthesis

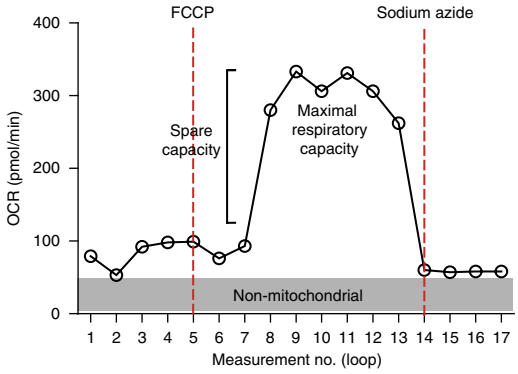

Figure 7 | Typical example of OCR profiles in *C. elegans* respirometry. Starting with the basal respiration, the spare capacity and maximal (mitochondrial) respiratory capacity (plateau phase) can be estimated by injecting FCCP. Non-mitochondrial respiration can be distinguished from mitochondrial respiration by injecting sodium azide. Dashed red lines indicate injections of compounds.

by transporting hydrogen ions across the inner mitochondrial membrane instead of the proton channel of the ATP synthase (complex V)[65]. Consequently, administration of FCCP results in the collapse of the mitochondrial membrane potential, thereby inducing a rapid increase in the consumption of energy and oxygen without the generation of ATP. FCCP injection can be used to calculate the maximal and spare respiratory capacity of worms. The latter is defined as the quantitative difference between the initial basal OCR and the maximal uncontrolled OCR (induced by FCCP; **Fig. 7**). Another drug that we have tested is sodium azide. Sodium azide blocks both cytochrome c oxidase (complex IV) and the ATP synthase (complex V), thereby shutting down the whole electron transport chain[47]. Therefore, we use sodium azide to estimate non-mitochondrial respiration—i.e., respiration caused by other cellular processes than oxidative phosphorylation (**Figs. 3a**, **7**).

(iv) Collecting and loading worm samples. When collecting the nematodes for the XF respirometric assay, several aspects should be taken into account. First, before removing worms from their plates, it is important to make note of any abnormalities—e.g., infections by fungi and the amount of food present (starvation). These observations may be critical in the interpretation of the data. Furthermore, collected worms should be washed multiple times to get rid of *Escherichia coli*. However, we have shown that the contribution of the *E. coli* to respiration is marginal and therefore will not affect the respiration data extensively (**Supplementary Fig. 2**). By estimating the number of worms per volume unit, one can estimate the volume required for a specific number of worms per well. This number needs to be optimized per strain and age; see Optimization of parameters.

Worms are loaded on a freshly opened Seahorse Bioscience cell culture microplate (the utility plate), keeping the total volume per well at 200 µl (= microliters of worms + microliters of M9). Use an XF96 plate layout (**Supplementary Fig. 3**) to keep track of which samples were placed in which wells. Visual inspection of the wells should show approximately the same number of worms in each experimental well and no worms in the background correction wells (typically the four corner wells: A1, A12, H1 and H12). The time between collecting the worms from their plate and loading them into a XF96 utility plate should be as short as possible, but not shorter than 30 min, to ensure that the worms have cleared their gut and the marginal oxygen consumption due to *E. coli* is further reduced. The OCR in the worms has a slight tendency to drop over time; therefore, a much longer waiting time, e.g., 2 h, should be avoided as much as possible.

(v) Starting the XF experiment. When the XF respirometer is prepared in advance, one can start the assay directly. We suggest starting the XF experimental assay when worms are collected, but not yet loaded (PROCEDURE), to avoid unnecessary heating of the machine before running the actual experiment. Calibration, the first command of an experimental template, takes ~20 min, during which the worms can be transferred to the utility plate. Just before starting calibration, we advise that one briefly place the sensor cartridge with calibrant solution at 37 °C, so that the calibration will take place at ~37 °C, which is the optimal temperature for the instrument's determination of actual oxygen concentrations. However, as the protocol is set to room temperature (i.e., the heater is turned off), the interior of the wells and machine will actually be ~20–25 °C during the measurements. Once the worms are loaded into the wells and the calibration has completed, the experimental part of the XF assay can start. The results of the XF assay can be followed in real time, but it should be mentioned that the OCR is represented per well and not per worm or µg protein. In addition to that, the XF software can be unstable during the assay, causing it to crash, which is why we discourage using the software during the run.

(vi) Postexperimental data analysis. In order to interpret the data and compare strains or treatments, the OCR per well should be converted to OCR per worm (or OCR per microgram of protein). Therefore, the number of worms per well needs to be counted. This can be done directly, or one can capture images of each well using a microscope and count the number of worms per well at a later date. Use the XF96 plate layout sheet (**Supplementary Fig. 3**) to fill in the number of worms per well. Some mutant or RNAi-treated worms are smaller than the age-matched controls. In this case, correcting for protein levels can provide additional information, although mitochondrial deficiency through RNAi leads to a reduced OCR with both normalization protocols[66]. In addition, differences in mitochondrial activity can be picked up by normalization to mtDNA content[67].

While inspecting the wells and/or images, also make note of any anomalies observed in the wells, such as dust or dirt, bacteria, embryos or larvae, as these may be important when interpreting the respiration data in the next step. An Excel spreadsheet is generated by the program after the XF assay is done. There will be different tabs in this spreadsheet, starting with 'Data viewer'. Although most of the information within the tabs is self-explanatory, a quick overview is given:

- *Data viewer.* When the Seahorse XF96 software is installed on the same computer that you use for analysis, there will be a Seahorse plugin in the 'Data viewer' tab (Windows only). Here, the data representation is identical to that on the XF respirometer computer. Although it gives a quick summary of the run, it is not corrected for worm number or protein concentration.

TABLE 3 | Optimized parameters for N2 worms.

Parameter/protocol	Optimized values
XF template	(1) Calibrate, (2) equilibrate, (3) loop five times[a], (3a) mix for 2 min, (3b) wait for 0.5 min, (3c) measure for 2 min and (4) end loop. (Optional) (5) inject port A with FCCP, (6) loop nine times, (6a) mix for 2 min, (6b) wait for 0.5 min, (6c) measure for 2 min, (7) end loop, (8) inject port B with sodium azide, (9) loop four times, (9a) mix for 2 min, (9b) wait for 0.5 min, (9c) measure for 2 min, (10) end loop, and (11) end program
Maximal loops	12–20[b]
Number of worms/well	Minimum of 2 worms. Maximum for L4: up to 50; D1: up to 25; D3: up to 30; D5: up to 30; D8: up to 30
Drug concentrations	FCCP: final concentration, 10 µM (injection concentration: 100 µM); sodium azide: final concentration 40 mM (injection concentration 400 mM)

[a]When interested only in basal respiration, one can increase five loops to, e.g., ten. However, this is not necessary; five loops are sufficient to get a reliable estimation of the OCR. [b]When a loop consisting of 2 min of measuring, 2 min of mixing and 0.5 min of waiting is used, the OCR remains stable up to 20 loops, although there might be some fluctuation. The OCR is consistently stable up until 12 loops.

- *Assay configuration.* The XF assay template is summarized here. The protocol, groups, background wells and all other parameters that were filled in are collected in this overview tab.
- *Levels.* This is one of the most important tabs, as it provides an overview of the oxygen concentration, pH and temperature per well and per time point.
- *Rate data.* This is the tab with the actual measurements of OCR and ECAR per well and time point. For *C. elegans*, only the OCR column is of importance. Wells 1–12 are at the first row of the plate (A1–A12), wells 13–24 are on the second row (B1–B12) and so on. Please note that as wells 1, 12, 85 and 96 are empty, these values should all be at zero.
- *Rate data* (*Plate view*). This tab also shows the actual measurements of OCR and ECAR, but this time in a plate view, in which respiration rates match the appropriate wells.
- *Time events.* This gives a written overview of all the steps in the Seahorse protocol.
- *Calibration.* This gives an overview of the calibration parameters.

The 'Rate data' tab is needed to do the actual calculations. The OCR values per well and time point can be divided by the number of worms in a specific well. Please note that the OCR and ECAR values are typically not stable until the second or third measurement cycle (or loop); thus, it is strongly recommended that the first basal measurement be excluded from analysis in order to establish a consistent baseline. Take the average of all the basal measurements per well for a good estimation of the OCR.

Experimental design

Overview of parameter optimization. There might be differences between *C. elegans* strains with respect to size and respiration rates. Therefore, it is essential to first optimize some important parameters for the strains of interest. These parameters include, but are not limited to, the timing for commands in the XF respirometer experimental template (i.e., "Measuring', 'Mixing' and 'Waiting time'), the number of worms per well, the stability of the OCR over time and the concentrations of compounds to be injected. By taking into account the parameters and optimization steps, one can adjust the protocol to each individual worm strain and also adapt the protocol to other organisms. The step-by-step PROCEDURE can easily be used to do optimization runs when parameters in specific steps are adjusted, as described in the following sections.

Table 3 shows the suggested start conditions, including optimal drug concentrations and timing, the XF template and the number of worms, when working with the N2 strain. This set of parameters also works for worms treated with RNAi, the *mev-1*(*kn1*) mutant and several other mutants.

Optimization of assay timing. Optimizing the timing of the XF assay commands 'Mixing', 'Measuring' and 'Waiting' requires interpretation of two aspects of the OCR: the course of the changing oxygen concentration and the stability of the OCR over time. On the basis of our experience, we advise one to start with the following protocol: (1) calibrate, (2) equilibrate, (3) loop *x* times, (3a) mix for 2 min, (3b) measure for 3 min, (4) loop end and (5) program end. Set *x* in loops to 20, pipette 10–25 worms per well (Optimization number of worms), start the run (PROCEDURE) and look for the factors mentioned above (the course of the changing oxygen concentrations and the stability of the OCR over time).

When investigating the course of changing oxygen concentrations, it is essential to determine whether the parameters within a loop (measuring, mixing and, when applicable, waiting time) are optimal. During the measurement step, oxygen is consumed in the transient microchamber, and the oxygen concentration drops. During mixing, the oxygen concentration should go back to baseline level (**Fig. 8a**). When the O_2 concentration fails to reach the baseline after each mixing cycle, this often indicates that the mixing time is too short. Alternatively, if the oxygen concentration drops to zero during the measurement phase (**Fig. 8b**), this may cause an underestimation of the OCR, and the measuring time or the number of worms per well should be decreased. The raw data concerning oxygen concentration per time unit can be found in the 'Levels' tab of the generated Excel result sheet. Add a filter to the column 'Well' in order to follow the changes in oxygen concentration over time per specific well.

The stability of the OCR over time can be studied by looking at the OCR estimations over the duration of the experiment. As no compounds are injected during the optimization runs, basal respiration should remain stable over time (at least up to 60 min, **Fig. 8c**, red dotted line). Small fluctuations in OCR typically occur across the different time points, usually without any overall upward or downward trend, but more drastic variation indicates that something is not optimal (**Fig. 8c**, black line). Outlier time points may be attributed to worms that occasionally stick to the probe

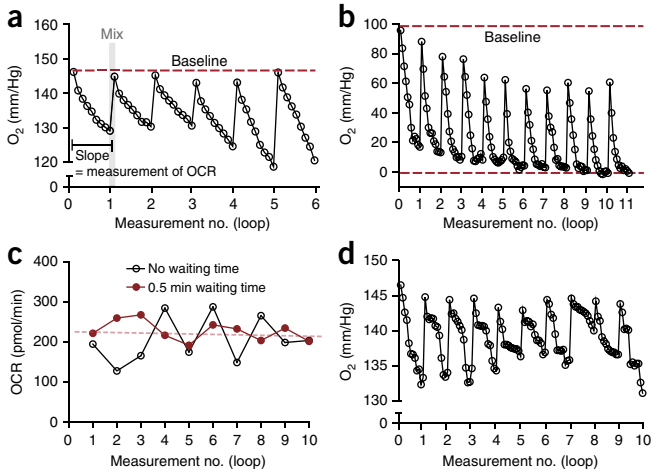

Figure 8 | Detailed tracing of oxygen levels is required to identify experimental issues. (**a**) The oxygen concentration at the start of each loop (measurement cycle) should be the same—i.e., it should touch the imaginary baseline (red dotted line). The O_2 concentration is estimated nine times per loop, when measuring for 2 min. These nine measurements (ticks) together are used to calculate the OCR per loop. After each measurement cycle, mixing takes place (as indicated by the gray area) to restore the initial O_2 concentration. (**b**) The graph shows data from a single well, in which the oxygen concentration does not return back to baseline after a loop because of an excess of worms. Moreover, in the last three loops, the oxygen concentration drops below 0, which causes an underestimation of the OCR. Loop: 2 min of mixing; 3 min of measuring. (**c**) The OCR over time when a protocol with or without waiting time is used (both with 2 min of mixing and 2 min of measuring). Clearly, introducing a waiting step decreases the fluctuation over time. Moreover, when applying linear regression to the OCR data with a waiting step (red dotted line), the slope does not significantly differ from 0, indicating that the OCR is stable over time (single-well data). (**d**) Data from a single well that shows clear fluctuation in the steepness of the slopes per loop. Loop: 2 min of mixing; 3 min of measuring. The variation in these slopes is probably caused by worms not always being in the transient microchamber.

during mixing, or remain outside the transient microchamber during measuring (**Fig. 8d**). To reduce this possibility, one can introduce the command 'Waiting time' between the mixing and measuring time, allowing the worms to sediment after mixing and ensuring that all worms are in the transient microchamber during the measurements. In our experience, a 'Waiting time' of 30 s is optimal for N2 worms.

Optimization of worm number. When the values for the commands 'Measuring', 'Mixing' and 'Waiting' are optimized, the influence of the number of worms per well can be studied. Using too many worms will exhaust the oxygen pool, thereby contributing to an underestimation of the OCR. To establish the appropriate number of worms per well, load a 96-well plate with different numbers of worms—e.g. ~5, 10, 15, 20, 25 and 30 worms per well. Start an XF assay with the optimized XF template (measuring, mixing and waiting time) and loop approximately ten times. Calculate the OCR per worm for each well (average over the last nine loops), and plot this against the number of worms. For N2 worms, adult day 1 (D1), a clear regression with a good fit can be generated (**Fig. 9a**), although two nonstochastic subpopulations can be distinguished (**Fig. 9b**). With segmented regression, one can distinguish a clear cutoff ~23 worms per well, implying that the OCR is reliable up to 23 worms, at least for N2 worms at D1 (**Fig. 9c**,**d**). The optimal number does change with age—e.g., for L4s, up to 50 worms can be used (**Supplementary Fig. 4**).

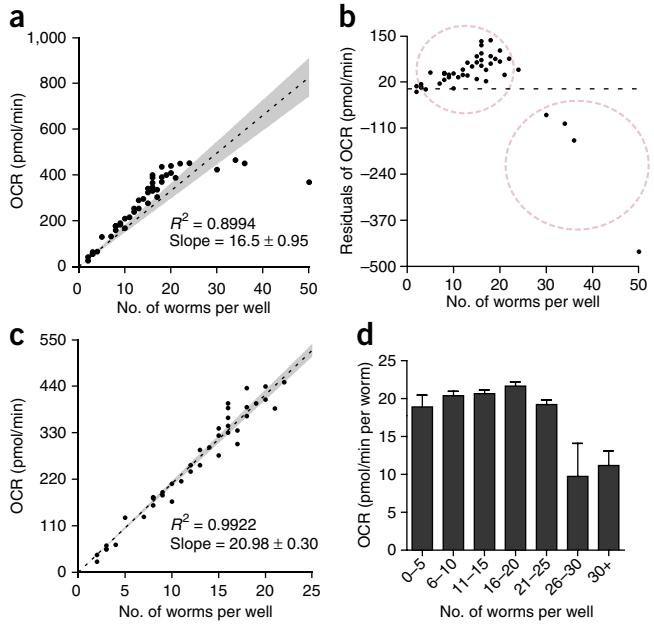

Figure 9 | The OCR correlates with the number of worms. (**a**) Linear regression of the OCR as a dependent variable of the number of worms per well, $n = 48$ wells. Although the R^2 value is relatively high, the fit is not optimal, as shown in **b**. (**b**) The residual plot. There is no stochastic distribution, but a clear biased distribution toward two populations (red dotted circles). (**c**) When excluding the negative residual population, the fit of the regression line is much better. (**d**) More than 25 worms per well (D1) renders OCR measurement inaccurate. In **a** and **c**, the gray area shows the confidence interval (95%) and the dotted line shows the linear regression. Bars represent mean ± s.e.m.

Optimization of compound concentrations. Titration assays for compounds of interest should be performed before XF assays, to ensure the optimal working concentration. When using new compounds, one can often find commonly used concentrations of the drug of interest in cells, worms or other organisms. Use this concentration as a starting point, and generate a dose–response curve (half-logarithmic scale concentrations) around this value, keeping in mind that worms often require higher doses. Program the machine to loop five times (with the just-optimized numbers for mixing, waiting and measuring and worm number), inject the compound and loop another 15 times to see its effect on respiration. It is strongly suggested that the specific drug concentration that maximizes the effect at the lowest concentration possible be chosen. By looping 15 times, the kinetics of the compound effect can be determined. As previously mentioned, *C. elegans* has a cuticle that shows clear impermeability toward non–water soluble compounds. Consequently, if a compound is injected and no clear respiratory response is observed, it does not exclude involvement of the targeted cellular process in *C. elegans* respiration; it may just be a matter of uptake efficiency. Here, the use of *bus* mutants, which have increased cuticle permeability[68], may help distinguish

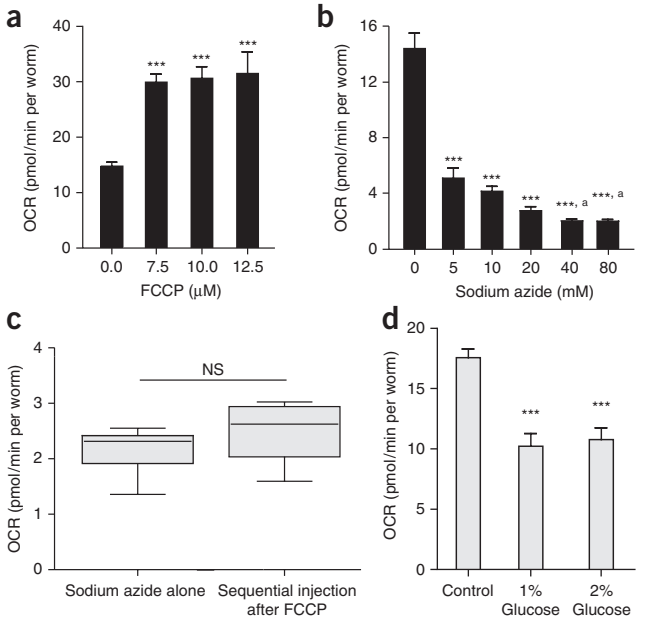

Figure 10 | The effects of specific compounds on the OCR in *C. elegans*. (**a**) Titration of FCCP and its effect on the OCR. 7.5–12.5 μM FCCP causes a significant increase in the OCR. One-way ANOVA ($P < 0.001$) with *post hoc* Tukey test, $n = 8$–16 wells. (**b**) Titration of sodium azide and its effect on the OCR. 5–80 mM sodium azide causes a significant decrease in the OCR. One-way ANOVA ($P < 0.001$) with *post hoc* Tukey test. [a] indicates that 40–80 mM causes a significant decrease in OCR compared with 5 mM sodium azide treatment, $n = 14$–16 wells. (**c**) There is no significant difference in the effect of sodium azide between when the compound was injected alone and when it was injected in sequence with FCCP; Student's *t*-test, $n = 15$–16 wells. (**d**) Feeding worms from L1 on with different concentrations of glucose (in the agar medium) causes a significant decrease in basal respiration at D4. Bars are mean ± s.e.m. One-way ANOVA ($P < 0.001$) with *post hoc* Tukey test, $n = 8$ wells. ***$P < 0.001$. NS, nonsignificant.

between these two possibilities. In addition, actual uptake efficiency can be determined with HPLC or liquid chromatography–tandem mass spectrometry.

We have already shown that the optimal concentrations of FCCP and sodium azide in N2 worms are 10 μM and 40 mM, respectively (**Fig. 10a**,**b**). Other compounds, such as rotenone and antimycin A, that are typically used for XF experiments with cultured cells appear to be highly inefficient in our Experimental Setup (**Supplementary Fig. 1**). Possible interference of sequential treatments should be carefully assessed when new compound combinations are used. For instance, it was reported that sodium azide is less effective when injected in sequence with FCCP[28], although we were unable to reproduce this effect (**Fig. 10c**). Finally, we have tested the effect of glucose pretreatments on the OCR of *C. elegans*. In multiple model organisms, increasing the glucose content causes a shift from oxidative phosphorylation toward glycolysis, resulting in a decreased OCR[42,69]. With the XF96 respirometer, we could also detect a decreased OCR when worms were fed with 1 or 2% (wt/vol) glucose starting from the L1 stage until D4 (**Fig. 10d**). Clearly, prefeeding with compounds can affect cellular respiration and therefore provide researchers with a long-term intervention method whose effects can be analyzed with XF respirometers.

Controls and replicates. Experimental control groups are helpful for determining whether the experiment worked and to assess variability from assay to assay. Typically, including an established mutant/RNAi condition with decreased respiration helps to determine whether the experimental setup is sensitive enough. Moreover, such a control may facilitate the relative comparison between different experiments, although we discourage directly comparing conditions between different plates or experiments. Established controls are *gas-1*(*fc21*)/*CW152, mev-1*(*kn1*)/*TK22* and *mrps-5* RNAi. ~6–8 replicates per group (per individual experiment) are sufficient to estimate the OCR with relatively small well-to-well intra-assay variability. It is strongly recommended that experimental setups be repeated at least three times, so one does not have to rely on one experiment with 6–8 technical replicates when drawing conclusions.

MATERIALS

REAGENTS

- Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; e.g., Abcam, cat. no. ab120081) **! CAUTION** FCCP can be acutely toxic even at low doses. Personal protective equipment should be worn at all times while handling this reagent; wear gloves and protective clothing.
- Calibration solvent, pH 7.4 (Seahorse Bioscience, cat. no. 100840-000)
- Sodium azide (e.g., Sigma-Aldrich, cat. no. S2002) **! CAUTION** The electron transport chain (ETC) inhibitor sodium azide can be acutely toxic even at low doses. Personal protective equipment should be worn at all times while handling this reagent; wear gloves and protective clothing. Sodium azide requires extra caution, as it changes rapidly into a toxic gas when mixed with water or acids.
- DMSO (e.g., Thomas Scientific, cat. no. C987Y85)
- KH_2PO_4 (e.g., Merck Millipore, cat. no. 1048731000)
- Na_2HPO_4 (e.g., Acros Organics, cat. no. 424380010)
- NaCl (e.g., Merck Millipore, cat. no. 1064041000)
- KCl (e.g., Sigma-Aldrich, cat. no. P9333-500)
- $MgSO_4$ (e.g., Fisher Chemicals, cat. no. M1000/60)
- dH_2O
- Triton X-100 (e.g., Thermo Fisher Scientific, cat. no. 215680010)
- *C. elegans* N2 worms (Caenorhabditis Genetics Center: *C. elegans* wild isolate) **▲ CRITICAL** N2 worms are used in the described protocol, but the protocol can be used for any other strain or for N2 worms treated with RNAi from the Ahringer library[70,71] (Source BioScience)
- *C. elegans mev-1*(*kn1*) (Caenorhabditis Genetics Center: cat. no. TK22)

EQUIPMENT

- Bioscience Seahorse XF96 Flux Analyzer (or XFe96)
- Heratherm incubator (Thermo Fisher Scientific, model no. 50125882 or similar)
- XFe96 extracellular flux assay kits (Seahorse Bioscience, cat. no. 102416-100)
- XF96 Cell Culture Microplates (Seahorse Bioscience, cat. no. 101085-004)
- Centrifuge (e.g., Thermo Fisher Scientific, model no. SL40R)
- Tubes, 15 ml (e.g., Cellstar, cat. no. 188271)
- Tubes, 1.5 ml (e.g., Greiner Bio-One, cat. no. 616201)
- Serological pipettes, 10–25 ml (e.g., Sarstedt, cat. nos. 86.1254.001 and 86.1685.001)
- Multichannel pipette, 20–200 μl (e.g., Eppendorf, cat. no. N21475D)
- Pipettes, 20, 200 μl
- Freezer (−20 °C)
- Vortex
- Dissection microscope
- (Optional) Parafilm **▲ CRITICAL** For equipment and protocols related to *C. elegans* maintenance, kindly refer to Stiernagle *et al.*[72].

REAGENT SETUP

M9 buffer (1×) Add 3 g of KH_2PO_4, 6 g of Na_2HPO_4 and 5 g of NaCl to 1 L of H_2O. Sterilize the buffer by autoclaving, and add 1 ml of 1 M $MgSO_4$ after cooling. The buffer can be stored at room temperature for several months.

FCCP stock (10 mM and 100 µM) Mix 2.54 mg of FCCP in 1 ml of DMSO and make 25-µl aliquots; these can be stored at −20 °C for up to 2–4 months. Dilute 25 µl of the FCCP stock (10 mM) in 2,475 µl of M9 buffer (= 100 µM) up to 24 h before use; ~2.5 ml of 100 µM FCCP solution is required per assay (25 µl of the stock in DMSO).

Sodium azide stock (400 mM) Add 26 mg of sodium azide per 1 ml of dH_2O. The solution can be stored at room temperature for up to 24 h before use. 2.5 ml of 400 mM of sodium azide solution is required per assay.

1× PBS + 0.05% (vol/vol) Triton X-100 To make PBS, add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 (pH 7.4) to 1 l dH_2O. Store PBS at room temperature or at 4 °C for up to 6 months. Add 0.5 ml of Triton X-100 to 1 l of PBS (0.05%); this solution can be stored at 4 °C for up to 1 month.

PROCEDURE

▲ **CRITICAL** *C. elegans* age-synchronization should take place in advance depending on the age of interest. Before starting the experimental procedures on day 2 of the protocol, make sure that the worms are at the appropriate age.

Hydration of probes ● TIMING 12–24 h; day 1

1| Open an unused flux package (green lid; XFe96 extracellular flux assay kit). In the package, you will find a utility plate with a green sensor cartridge on top, a lid and two drug-loading guides.

2| Remove the green sensor cartridge, but place it upside down so that the probe surface will not be scratched.

3| Use a multichannel pipette to add 200 µl of the Seahorse Bioscience XF96 calibrant (pH 7.4) solution to each well of the utility plate.

4| Place the sensor cartridge back on the utility plate so that the probes dip into the wells. Place the lid on top and incubate the cartridge overnight at 37 °C without CO_2.

▲ **CRITICAL STEP** One should be careful not to disturb the sensor probes' surface.

▲ **CRITICAL STEP** Cartridges cannot be reused. When performing multiple runs, make sure to hydrate multiple cartridges.

■ **PAUSE POINT** Hydration can take as little as 4 h and as long as 72 h (when hydrating for >24 h, seal the plate with Parafilm and store it at 4 °C. Rewarm to 37 °C before use).

Preparation of the XF96 respirometer: temperature control and background correction ● TIMING 5 min; day 2

5| Launch the XF software, and when prompted, select the 'Standard' software settings. Either create a new account or proceed by clicking on 'Seahorse guest'.

▲ **CRITICAL STEP** Always use the standard software package and not the software associated with one of the so-called 'stress kits'.

6| Turn the heater and temperature control off. Click on the instrument icon in the lower-right corner and subsequently select 'Administration' > 'Temperature' > 'Heater and temperature control'. The buttons should be gray when they are turned off.

▲ **CRITICAL STEP** This is a critical step for working with *C. elegans*, as use of the heater is designed for cell culture at 37 °C. The temperature of the machine can be directly followed on its display.

? TROUBLESHOOTING

7| In the same screen (administration), click on 'Background correction'. Make sure that background correction is on and that the correction wells are set to A1, A12, H1 and H12.

8| Leave the instrumental setup mode by clicking on 'End instrument set-up mode'.

Preparation of the XF96 respirometer: creating an XF assay ● TIMING 10–20 min; day 2

9| Click on the 'Assay wizard' icon (next to the instrument icon). In the 'General' tab, fill in at least the assay name and results file name, for instance, using the following format: 'YearMonthDate_Strains/conditions'—e.g., '20160515_N2_L4'.

10| The tabs 'Cells', 'Media' and 'Compounds' can be ignored. However, make sure to record the compounds to be injected, their concentrations and the port in which they will be loaded.

11| Proceed to the 'Background correction' tab and double-check that the correction is 'on' and the corner wells are selected (A1, A12, H1 and H12).

TABLE 4 | Stock, working and final concentrations of FCCP and sodium azide.

Compound	Final well concentration	Concentration at drug injection port	Stock concentration	Stock volume required per plate (96 wells)[a]
FCCP	10 µM	100 µM (dilute stock 1:100 in M9)[a]	10 mM in DMSO[b]	25 µl
Sodium azide	40 mM	400 mM[a]	400 mM in dH_2O	2.5 ml

[a]The volume required per assay depends on the volume that is pipetted per drug injection port. We normally use 22 µl per port for FCCP and 24 µl per port for sodium azide when injected sequentially. As there are 96 wells/ports, ~2.5 ml of the drug (with a concentration equal to the concentration at the drug injection port is required per full plate. [b]The stock concentration of compounds in DMSO should be higher to ensure that the final DMSO concentration is <1% DMSO.

12| (Optional) Wells can be labeled in the 'Groups and labels' tab. Select the wells with technical replicates and give them a name and color. During the run, you can see the average OCR of the groups involved. When labeling is not done before the assay, it can still be done afterward.
▲ **CRITICAL STEP** The OCR values that can be followed in real time are not corrected for the number of worms and are therefore not directly comparable.

13| Go to the 'Protocol' tab. By clicking on the commands, you can add instructions to the current protocol. For the commands 'Measure', 'Mix' and 'Wait', one must also fill in the duration. Always start with (1) 'Calibrate' and (2) 'Equilibrate'. For N2 worms, use the parameters shown in **Table 3**. For advice on designing assay protocols for different strains, see Experimental design.
▲ **CRITICAL STEP** When not injecting, or when injecting multiple compounds, make sure to exclude or include these commands in your protocol, respectively.

14| When the appropriate protocol is generated, go to the last tab, 'End', and click the button 'End Assay Wizard'. A screen with a green start button will appear under the 'Run' tab.
▲ **CRITICAL STEP** Do not click the 'Start' button yet. To avoid unnecessary heating of the machine, you should postpone starting the machine until <20 min before the worm assay plate will be ready.

Loading of compounds to be injected (optional) ● **TIMING 30 min; day 2**
15| Take the hydrated utility plate from Step 4 with the sensor cartridge from the incubator.

16| If desired, thaw one 25-µl aliquot of FCCP (10 mM) and dilute it 100-fold in M9 buffer (2,475 µl), as described in the Reagent Setup (**Table 4**).

17| Slowly pipette 22 µl of diluted FCCP (100 µM) into each 'port A' on the cartridge (**Table 4**), including those belonging to the background correction wells. During pipetting, insert the pipette tips into the holes and dispense the content. (Optional) The loading guide can be used to ensure pipetting into the correct port.
▲ **CRITICAL STEP** A multichannel pipette can be used for this purpose. Make sure to avoid allowing the liquid to adhere to the sides of the port and avoid creating bubbles, as compounds are injected into the XF96 in a pneumatic manner. In addition, be aware that the ports have a hole at the bottom for injection.
▲ **CRITICAL STEP** As the compounds are injected using a pneumatic mechanism, it is essential to add the same volume of liquid to all injection ports—i.e., when injecting FCCP into port A, all of the other ports should contain the same amount of a compound or a vehicle.
▲ **CRITICAL STEP** Do not use automated pipettes to load the compounds, as this will increase leakage through the bottom of the ports.

18| Load 24 µl of sodium azide (400 mM) in the same manner as described for FCCP into each 'port B' of the green sensor cartridge.
▲ **CRITICAL STEP** As FCCP is injected first, the volume in the well is not 200 µl anymore, but it is 220 µl. Therefore, the injection volume of sodium azide is higher to ensure a correct final concentration.

19| Visually inspect the injection ports A and B for even loading; the liquid should be down at the bottom of the port.

20| Place the lid on top of the cartridge. Carefully place the sensor cartridge (still on top of the utility plate) back into the non-CO_2 incubator at 37 °C, and keep it there until calibration is started.
▲ **CRITICAL STEP** Keep the plate for at least 10 min at 37 °C to ensure that the compounds are warmed up. In this way, calibration will take place at ~37 °C, which is the optimal calibration temperature for XF respirometers (the heater is still off).

Preparation/loading of samples and starting of the XF assay ● TIMING 2–3 h; day 2

21| Make note of the developmental stages seen on the worm agar plates.

▲ **CRITICAL STEP** Strict age synchrony is required for reliable estimations of the OCR. Make sure that worms are synchronized (by hypochlorite treatment, or egg laying and/or FUdR) and that they are of the age of interest on the day of the XF assay.

22| Use sufficient (2–4 ml per 9-cm plate) M9 buffer to rinse the worms off the plate with gentle agitation, and collect the worms for each sample in a 15-ml tube.

23| Spin the worms down in a centrifuge (20 °C, ~500*g* for 2 min).

24| Remove the supernatant and add fresh M9 buffer. Repeat Steps 23 and 24 two more times.

25| Because there may be eggs or even small larvae present in your samples when you are testing aged worms (even when using FUdR), add M9 to the tube, mix gently and allow the worms to settle for a few minutes by gravity. When the worms have sunken into a loose pellet, aspirate the supernatant, which usually contains smaller animals and eggs.

26| Repeat Step 25.

27| Resuspend the worms in 1.5–3 ml of M9, depending on the density and the size of the worm pellet.

28| Mix the worm suspension carefully and pipette two 3-µl drops onto an empty Petri dish, then count the number of worms per drop using a microscope.

▲ **CRITICAL STEP** Wash the pipette tip with PBS + 0.05% (vol/vol) Triton X-100 before you pipette the worms, or use low-retention tips to avoid worms sticking to the tip. Otherwise, you may use wide-bore tips or tips with the ends cut off using a razor blade.

29| Calculate the volume of sample that should be loaded to acquire 10–20 worms per well. Subtract this volume from 200 µl (the maximal volume per well); this is the volume of M9 that will be added per well.

▲ **CRITICAL STEP** The volume of M9 that should be loaded into the wells needs to be calculated for each sample separately. Make a clear pipetting scheme and fill in the scheme shown in **Supplementary Figure 3**. In addition, include control strains (e.g., *mev-1* mutant, *gas-1* mutant and *mrps-5* RNAi) to ensure correct interpretation of the results later on.

30| Before loading the worms, click 'Start' to start the actual XF assay. Be sure to make the appropriate entries in the 'Save directory' and 'Save name' commands before you click 'Start' again.

31| Follow the prompts on the screen for sensor cartridge calibration. When the loading door opens, take the sensor cartridge from the non-CO_2 incubator (Step 20) and place it on the tray.

▲ **CRITICAL STEP** The notch of the plate should point toward the front of the machine.

▲ **CRITICAL STEP** Make sure to remove the lid before inserting the plate into the machine.

? TROUBLESHOOTING

32| Open a fresh cell culture microplate (utility plate; blue lid) and load the appropriate volume of M9 per well, followed by the calculated volume of the samples (Step 29).

▲ **CRITICAL STEP** Wash the pipette tip with PBS + 0.05% (vol/vol) Triton X-100 before pipetting the worms, or use low-retention tips to avoid worms sticking to the tip. Otherwise, you may use wide-bore tips or tips with the ends cut off using a razor blade.

33| Pipette 200 µl of M9 into the background correction wells: A1, A12, H1 and H12.

34| By visual inspection with a dissection microscope, inspect all wells and make sure that the wells A1, A12, H1 and H12 are empty and that the experimental wells contain ~10–25 worms (see **Table 3** for appropriate worm numbers per well).

35| Wait at least 2 min from when the worms are pipetted until the plate is loaded, to allow the worms to settle by gravity to the bottom of the wells.

36| By this point, the calibration should be completed. *Note*: when calibration is completed before the samples are ready, the instrument stays closed and waits until samples are ready to load. Open the loading door by clicking 'OK' on the screen; this will release the utility plate containing calibration buffer from the XF respirometer, while the cartridge stays inside the machine. Replace the calibrant plate with the worm plate.
▲ **CRITICAL STEP** The green sensor cartridge is not ejected; it stays in the machine. Only the utility plates are swopped. Do not forget to remove the lid of the worm plate.

37| Close the door by clicking on 'Continue'; the equilibrate step of the protocol will now be executed.

38| Once the run is completed, follow the prompts to remove the cell plate and the sensor cartridge from the XF96 respirometer.

39| Keep the worm plate and use a dissection microscope to count the number of worms per well. Make note of the numbers on the scheme shown in **Supplementary Figure 3**. While inspecting the number of worms, also make note of any anomalies observed in the wells (e.g., larvae, embryos and bacteria), as this probably influenced the OCR measurements. (Optional) Capture images of each well using a microscope, and count the number of worms per well at a later date.
▲ **CRITICAL STEP** When sodium azide is injected during the XF assay, worms are paralyzed, which makes counting easier. Without injection of sodium azide, it is suggested that the plate be cooled down before counting the worms—as this also immobilizes the worms—or, alternatively, you can add sodium azide to the wells manually.
? TROUBLESHOOTING
■ **PAUSE POINT** At this point the experimental part of the PROCEDURE is completed; the postexperimental analysis can be performed at any moment from here on.

Postexperimental analysis ● TIMING 1–2 h
40| Open the Excel spreadsheet that is automatically exported to the directory selected by the user.
▲ **CRITICAL STEP** When you are performing the analysis on a computer without XF software, copy the important values in the 'Rate data' tab to another Excel file to avoid error messages.
? TROUBLESHOOTING

41| Copy the OCR values per well (with all separate measurement points) to a new Excel tab in such a way that the OCR values of a single well are collected below each other (in a column).

42| Divide all OCR values for each well at each time point by the number of worms in that well. (Optional) Determine the protein content with a BCA assay in exactly 1,000 worms to estimate the protein concentration per nematode. Subsequently divide the OCR per worm by this number.
▲ **CRITICAL STEP** When nematode strains differ in size, the optional protein determination step is recommended, although we suggest reporting both OCR per worm and OCR per milligram of protein.

43| Estimate the basal OCR for each well by averaging the OCR per worm of measurement points (loops) 2–5.
▲ **CRITICAL STEP** The basal OCR values are typically not stable until the second measurement loop. In addition, the first measurement after injection is typically not stable. Exclude these measurement points when calculating average OCRs.
▲ **CRITICAL STEP** The measurement points that should be used for calculations of the OCR are dependent on the selected protocol. Our calculations are based on our proposed protocol, as shown in **Table 3**.

44| To estimate the FCCP-induced OCR per worm, find a stable plateau phase of three points within measurements 7–14, and average these numbers.
? TROUBLESHOOTING

45| To calculate non-mitochondrial respiration per worm (sodium azide–induced), take the average of measurements 17 and 18.
? TROUBLESHOOTING

46| Use the values obtained in Steps 43–45 to calculate mitochondrial respiration for each well by subtracting the average sodium azide–induced OCR from the average basal OCR. To calculate the maximal respiratory capacity, subtract the sodium azide–induced OCR from the FCCP-induced average OCR. To calculate spare respiratory capacity, subtract the average of the basal OCR from the FCCP-induced average OCR. Non-mitochondrial respiration is defined as the sodium azide–induced OCR.
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

TABLE 5 | Troubleshooting table.

Step	Problem	Possible reason	Solution
6	The temperature is too high	The machine's heater is still on	Make sure to turn both the heater and the temperature control off before starting the experiment
31	The cartridge is not recognized/ barcode cannot be scanned	Condensation within the machine sometimes interferes with reading the barcode	Restart the device; make sure to turn off the heater/temperature control again
39, 45	Worms still move after injection of sodium azide	The concentration of the compound is too low	Increase the sodium azide concentration. Make a dose–response curve for sodium azide in the strain of interest
		The drug was loaded in the wrong port	Check whether the drug is still in one of the injection ports. Make sure to load the compound in the right port next time
		The drug was not properly loaded into the injection ports	Visual inspection can show whether the compounds are still in the injection ports. Make sure to pipette carefully, in one stream, and avoid inducing air bubbles
		The drug has gone bad	Make a fresh stock from recently purchased reagent in sterile deionized H_2O
40	The temperature markedly increases during the experimental assay	The environmental temperature is too high	Place the XF respirometer in a temperature-controlled room or the change the lab temperature
	The basal OCR heavily fluctuates over time/decreases over time	Nematodes are outside of the transient microchamber during the measuring time	Introduce a waiting step, or increase the duration of the waiting step
		Mixing time is insufficient	Increase the mixing time or decrease the measuring time
		The time between collecting the worms and the end of the assay is too long	Increase the working speed or ask someone to help you prepare the samples. Alternatively, reduce the length of the experimental protocol (number of loops)
	Oxygen levels drop to 0 mm/Hg during a measure cycle	Mixing time is insufficient	Increase the mixing time
		Too many worms in the well	Decrease the number of nematodes per well
		Measuring time is too long	Decrease the measuring time
	The OCR is much lower in the second measuring cycle	The first estimation of the OCR is in general not reliable	Exclude the first measuring cycle from analysis
44	Worms do not respond to FCCP injection	The FCCP concentration used is too low	Increase the concentration of FCCP. Make a dose–response curve for FCCP in the strain of interest
		The starting oxygen concentration—before the start of the measuring cycle—is too low. During the cycle, the oxygen concentration falls to zero and the OCR is underestimated	Decrease the measuring time or decrease the number of worms per well

(continued)

TABLE 5 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
44		The drug is loaded in the wrong injection port	Check whether the drug is still in one of the injection ports. Make sure to load the compound in the right port next time
		The drug is not properly loaded into the injection ports	Visual inspection can show whether the compounds are still in the injection ports. Make sure to pipette carefully, in one stream, and avoid inducing air bubbles
		The drug has gone bad	Make a fresh stock from recently purchased reagent in a freshly opened anhydrous vial of DMSO
46	The variation between technical replicates (within one run) is large	There are too many or too few worms in some of the wells	Plot the total OCR versus the number of worms and add a linear trend line. Create a residual plot and look for abnormalities. Exclude outliers caused by a high or low number of worms

● TIMING

Age-synchronization and aging of worms: ~2–20 d.
Steps 1–4, hydration of probes: 12–24 h (day 1)
Steps 5–8, preparation of the XF96 respirometer: temperature control and background correction: 5 min (day 2)
Steps 9–14, preparation of the XF96 respirometer: creating a XF assay: 10–20 min (day 2)
Steps 15–20, loading of the compounds to be injected (optional): 30 min (day 2)
Steps 21–39, preparation/loading of samples and starting of the XF assay: 2–3 h (day 2)
Steps 40–46, postexperimental analysis: 1–2 h

ANTICIPATED RESULTS

Interpretation and considerations

The goal of using the XF96 respirometers for *C. elegans* is to estimate the OCR, which mirrors cellular respiration of the worm. Clearly, including established mitochondrial mutants such as *gas-1* or *mev-1* or *mrps-5* RNAi in the experimental setup allows the user to interpret whether the assay is sensitive enough to detect differences in respiration. Basal OCR should therefore differ between N2 and mitochondrial mutant worms (**Fig. 11a**,**b**). Note that absolute OCR values can differ between assays because of changes in temperature and the use of different sensor cartridges. Therefore, mutants are ideally analyzed side by side on the same plate and on the same day. The injection of FCCP typically increases the OCR two- to three-fold, but the effect is somewhat delayed when compared with experiments performed with cells, which is likely due to a difference in uptake efficiency. The OCR in N2 worms drops ~75–90% when sodium azide is added, which highlights that most of the respiration is caused by

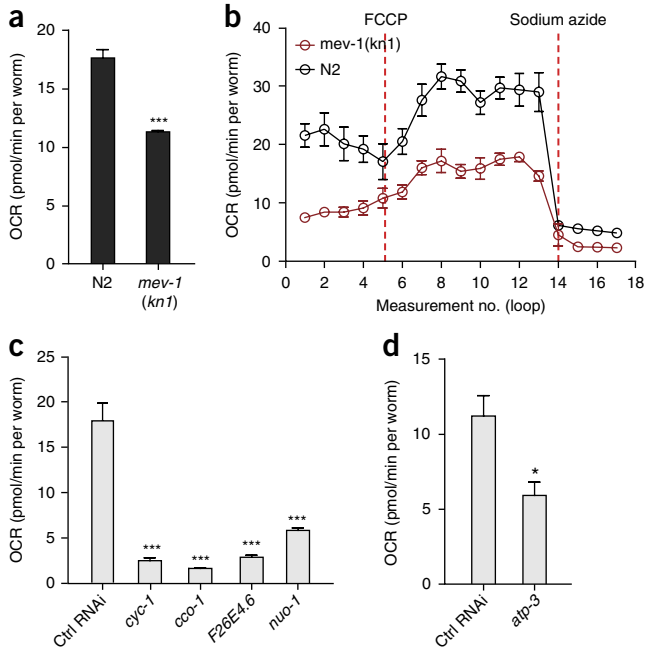

Figure 11 | Genetic manipulation of the electron transport chain lowers the OCR in *C. elegans*. (**a**) Basal respiration is significantly lower in the *mev-1* strain compared with N2 worms. Student's *t*-test ($P < 0.001$), $n = 8$ wells. (**b**) *mev-1* mutants have lower basal respiration, lower maximal respiration, lower spare capacity, but comparable non-mitochondrial respiration ($n = 8$ wells). Dashed red lines indicate injections of compounds. (**c**) Basal respiration is significantly lower when proteins in the electron transport chain are knocked down. One-way ANOVA ($P < 0.001$) with *post hoc* Tukey test, $n = 6–8$ wells. (**d**) A knockdown of *atp-3* in complex V causes a significant decrease in basal respiration. Bars are mean ± s.e.m. Student's *t*-test ($P = 0.032$), $n = 8$ wells. Ctrl, control. $^*P < 0.05$; $^{***}P < 0.001$.

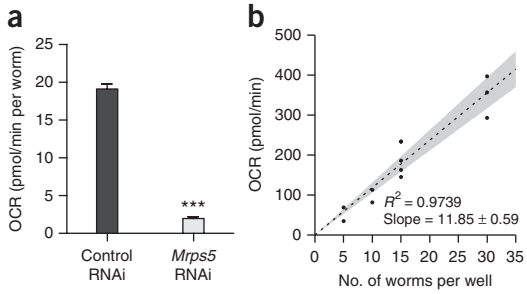

Figure 12 | XFp respirometers provide an alternative to XF96 respirometers. Data acquired in XFp respirometers. (**a**) Nematodes treated with *mrps5* RNAi show significant lower respiration than worms treated with control RNAi. Student's *t*-test ($P < 0.0001$). This is comparable to what was previously described in XF96 respirometers[26]. Bars are mean ± s.e.m. (**b**) Linear regression of the OCR as a dependent variable of the number of worms per well. Data from two separate runs are combined ($n = 10$ wells). Gray area shows the confidence interval (95%) and the dotted line shows the linear regression.***$P < 0.001$.

mitochondrial function (**Fig. 11b**). The quality of the different OCR readings can be verified by looking at the raw data—e.g., the variation in different time points and the course of the oxygen concentration, as described in detail in the Optimization section. The variation between different time points should be small (<15%), except for the initial time points after compound injection (particularly for FCCP), and the oxygen concentration should restore itself with each loop (**Fig. 8**).

To further verify and test the ability of XF96 respirometers to measure oxygen consumption and especially differences in the OCRs, we tested RNAi directed toward several proteins in the electron transport chain, including *cyc-1* (cytochrome c1, complex III), *cco-1* (cytochrome *c* oxidase, complex IV), *nuo-1* (NADH:ubiquinone oxidoreductase, complex I), *atp-3* (ATP synthase subunit δ, complex V) and *F26E4.6* (cytochrome *c* oxidase subunit VIIc, complex IV; **Figs. 1b**, **11c**,**d**). Clearly, similar to compounds that inhibit specific electron transport chain proteins (**Fig. 1b**; **Table 2**), knocking down proteins associated with oxidative phosphorylation causes a striking decrease in basal respiration. These results underline the strength of XF respirometers in combination with *C. elegans*, as the effects of genetic interventions on cellular respiration can be assessed side by side in a quick and straightforward manner when following our developed protocol.

One important consideration that should be taken into account is the interpretation of mitochondrial respiration, spare respiratory capacity, maximal respiratory capacity and non-mitochondrial respiration. These terms were once coined to describe respiration effects in isolated mitochondria with exogenously controlled energy sources. It is likely that results from using the more complex intact animal should be interpreted differently than those from isolated mitochondria in a defined medium. The question that directly arises is whether treatment with FCCP and sodium azide indeed accurately reflects these different aspects of cellular respiration. This argues caution against overinterpretation of the OCR values in terms of selective association with aspects such as maximal respiratory capacity and would argue for relying on basal respiration only. Clearly, basal respiration provides the user with the most readily interpretable number from a XF96 experiment.

Scaling down: from an XF96 to an XFp respirometer

The possibility of assessing respiration in 96 parallel wells makes the XF96 respirometer attractive for moderately large screens and the comparison of many conditions at the same time. However, when, for example, only two conditions need to be compared, 8–12 wells may be sufficient. In this specific case, it is not efficient to fill only a small fraction of the 96 wells, and it is not advisable to reuse plates. Here, the recently launched XFp respirometers provide an alternative. The XFp respirometer works with eight-well plates (including two wells for background correction) in which the separate wells are the same size as the wells of the XF96 plates. The user interface of the XFp respirometer is intuitive, the machine is easy to handle and, because of the limited number of wells, the time for manual transfer of worms to the eight-well plate and the eventual starting of the assay is relatively short. Using the XFp, which was operated in a cold room so that the operating temperature was stable at 20 °C, we have confirmed that knockdown of *mrps-5* reduces OCR in *C. elegans* (**Fig. 12a**), similar to the effects we had observed earlier using the XF96 respirometer[26]. Furthermore, the optimal FCCP concentrations are similar to those used in XF96 respirometers, and the number of worms correlates well with the OCR (**Fig. 12b**). The main limitation of the XFp respirometer is, however, the number of assay wells. Sample-to-sample variation in the XFp respirometer is similar to that observed in XF96 respirometers, but the limited number of wells (six experimental wells) can preclude sufficient technical replicates when analyzing mobile samples such as worms. As a consequence, although massive differences in respiration are easily picked up using the XFp respirometer (**Fig. 12a**), small changes in OCR might be missed. As such, the XF96 is more practical than the XFp respirometer for measuring worms, and these benefits outweigh the difference in investment that must be made when purchasing the XF96 respirometer and its consumables. As a final consideration, the same optimization steps can be used to measure respiration with the 24-well respirometer (XF24), which has larger wells that require more worms per well[73].

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS M.K. performed most of the experiments, optimized the final experimental pipeline and wrote the manuscript together with R.H.H., and with contributions from all authors. H.M. and R.K performed experiments using the XFp/XF96 respirometers. L.M., R.H.H., B.M.D. and J.A. pioneered the use of XF respirometers with *C. elegans* and developed the initial protocols. M.K., H.M., E.A.A.N., B.M.D. and R.H.H. have been extensively involved in discussions and interpretations of results.

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