

Analysis of mitochondrial function using phosphorescent oxygen-sensitive probes

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Mitochondrial dysfunction has been associated with a variety of currently marketed therapeutics and has also been implicated in many disease states. Alterations in the rate of oxygen consumption are an informative indicator of mitochondrial dysfunction, but the use of such assays has been limited by the constraints of traditional measurement approaches. Here, we present a high-throughput, fluorescence-based methodology for the analysis of mitochondrial oxygen consumption using a phosphorescent oxygen-sensitive probe, standard microtitre plates and plate reader detection. The protocol describes the isolation of mitochondria from animal tissue, initial establishment and optimization of the oxygen consumption assay, subsequent screening of compounds for mitochondrial toxicity (uncoupling and inhibition), data analysis and generation of dose-response curves. It allows dozens of compounds (or hundreds of assay points) to be analyzed in a single day, and can be further up-scaled, automated and adapted for other enzyme- and cell-based screening applications.

INTRODUCTION

Recent years have seen a resurgence in interest in mitochondrial biology and in the links between mitochondrial dysfunction and disease^{1–6}. It has also been recognized that many adverse drug reactions can be attributed to mitochondrial dysfunction. Mitochondrial toxicity has been reported for antivirals, statins, anti-lipidemic and anti-diabetic drugs, as well as for some chemotherapeutic agents and antibiotics^{7–10}. These observations strongly suggest that the analysis of mitochondrial function and mitochondrial toxicity testing should be more widely deployed and positioned at earlier stages of the drug discovery process.

A variety of cellular parameters have been used as indicators of mitochondrial function, including: the redox state of mitochondrial cytochromes; cellular ATP levels; reactive oxygen species (ROS) production; mitochondrial membrane potential ($\Delta\Psi_m$); and oxygen consumption^{11–16}. Although less widely measured, oxygen consumption is arguably the most informative of these parameters, in that measurement allows a direct and specific assessment of the functioning of the electron transport chain (ETC), the cornerstone of oxidative phosphorylation and cellular metabolism. The rate of oxygen consumption can be modulated by inhibition of the ETC or the F_1/F_0 ATP synthase, or through the uncoupling of oxidative phosphorylation either by xenobiotics, uncoupling proteins or the opening of the mitochondrial permeability transition pore¹⁷. Oxygen consumption is also affected by the supply of reducing equivalents and is therefore sensitive to the inhibition of either the citric acid cycle or the β -oxidation of fatty acids. The supply of reducing equivalents can also be limited by altered outer mitochondrial membrane permeability¹⁸. Other factors, such as transporter inhibition, calcium-mediated alterations of regulatory mechanisms and cardiolipin damage, can also contribute to altered oxygen consumption. Such dysfunction will induce either an increase in oxygen consumption (i.e., uncoupling) or a decrease (inhibition). A skilled mitochondrial biologist will be able to connect the observed results to a possible mechanism(s).

Despite the specific and informative nature of oxygen consumption as an indicator of mitochondrial and cellular function, it is not a widely measured parameter. This is largely due to the fact that the traditional polarographic method¹⁹ has stringent measurement format requirements and very limited sample throughput. Quenched-fluorescence oxygen sensing offers an alternative method, which can address these limitations. The fluorescence-based approach allows direct, non-chemical quantification of oxygen levels, through the use of oxygen-sensitive materials based on long-decay fluorescent or phosphorescent dyes²⁰. The emission of such materials (solid-state sensors or soluble probes) is reversibly quenched by molecular oxygen via a collisional mechanism. A known analytical relationship between measured fluorescent signal and oxygen concentration allows the quantification of dissolved oxygen concentration and/or the monitoring of concentration changes over time. The principles of quenched fluorescence oxygen sensing are extensively reviewed in the literature^{20–22} and are summarized in **Figure 1a**.

The contact-less nature and high-throughput capabilities of fluorescence-based oxygen sensing make it particularly suited to biological applications. Using solid-state sensors, microtitre plates that are capable of detecting biological oxygen consumption have been developed^{23–27}. These systems do, however, exhibit a number of limitations. The solid-state nature limits measurement platform flexibility and restricts measurement to local concentrations at the base of the well. Slow sensor response times, short emission lifetimes and/or toxicity due to intrinsic singlet oxygen generation can also be problematic. The development of phosphorescent oxygen probes^{28,29}, which can be dispensed directly into test samples, has overcome many of these limitations and has increased the convenience and performance of such measurements, allowing the use of standard microtitre plates, as well as other more specialized formats^{30,31}. The probe used in this protocol (Mito-Xpress) has two intense absorbance bands with maxima at 380 nm and 535 nm that are suitable for excitation, and emits in the red

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spectral region with maximum at 650 nm. Its long-decay emission in the microsecond range facilitates the use of time-resolved fluorescence detection, which improves assay performance, selectivity and resistance to optical interferences. This probe chemistry and detection methodology provide a high degree of measurement flexibility and may be applied to a variety of biological tasks^{21,32}, including the assessment of drug-induced mitochondrial toxicity, in which a high level of validation has been achieved¹⁶. The probes provide researchers with a powerful tool for the analysis of oxygen-dependent biological processes.

Here, we describe the standard format and protocol developed for medium- and high-throughput analysis of mitochondrial oxygen consumption using a dispensable long-decay phosphorescent oxygen probe, standard microtitre plates and time-resolved fluorescence detection. In contrast to fully sealed low-throughput formats, samples in microplate wells are exposed to ambient oxygen, which can diffuse back into the sample through the plastic body of the microplate and at the air-liquid interface³³, thereby destroying oxygen gradients and compromising assay performance. The addition of a layer of mineral oil to each sample (Fig. 1b) limits back diffusion (i.e., a flux of oxygen from ambient air into the sample driven by concentration gradients), thus improving assay sensitivity and reproducibility^{23,28,33}. Furthermore, as both probe signal and biological oxygen consumption are temperature dependent, sample temperature must be carefully controlled. During the assay, depletion of dissolved oxygen in the sample is reflected by an increase in probe fluorescence above the basal (air-saturated) level. The signal stabilizes when equilibrium is reached between the rates of oxygen consumption and

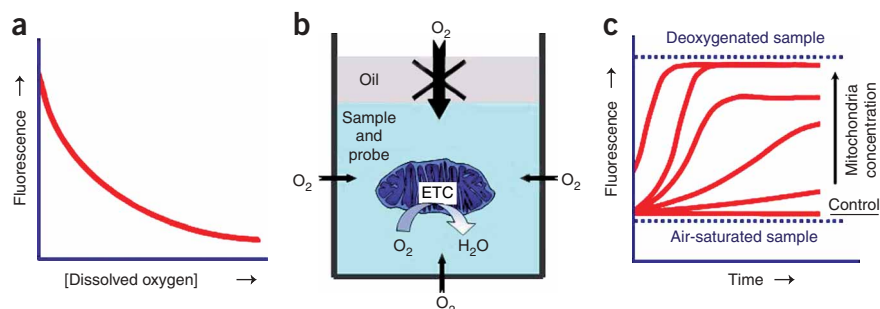


Figure 1 | Diagrammatic representation of measurement principles. Probe fluorescence is quenched by molecular oxygen via a non-chemical (collisional) mechanism, whereby increasing concentrations of dissolved oxygen reduce probe signal (a). This relationship is described by the Stern-Volmer equation $[I(O_2) = I_0/(1+K_{sv}[O_2])]$, where $I(O_2)$ is the intensity at a given concentration of oxygen, I_0 is the intensity in the absence of oxygen, K_{sv} is the Stern-Volmer quenching constant and $[O_2]$ is the concentration of dissolved oxygen⁴⁰. Measuring probe fluorescence therefore allows the quantification of dissolved oxygen, with changes in probe signal reflecting changes in oxygen concentration within the sample²⁰. The principles of quenched-fluorescence oxygen sensing are reviewed extensively in the literature^{21,22}. When measuring oxygen consumption in a microtitre plate, two processes are taking place (b). Dissolved oxygen is being consumed by the activity of the electron transport chain, while the oxygen gradients formed drive the back diffusion of ambient oxygen at the liquid-air interface and through the polystyrene body of the microtitre plate³³. Successful analysis requires a consumption rate sufficient to produce a measurable signal change. A sealing layer of mineral oil limits this back diffusion, thereby increasing assay sensitivity. Without mitochondrial protein (control) the concentration of dissolved oxygen within the well remains unchanged and probe signal remains stable (c). Mitochondrial protein consumes oxygen, which is seen as an increase in probe signal over time. The signal saturates when all oxygen is depleted. Time delay between the addition of enzyme and commencement of monitoring should be considered (left, i.e. steepest profile in c).

back diffusion (Fig. 1c). These fluorescence profiles may be used to extract quantitative information on oxygen-dependent processes.

This measurement methodology allows the assessment of mitochondrial function using conventional instrumentation, thereby combining the high degree of information provided by oxygen consumption analysis with the simplicity, throughput and scaling up capabilities of standard microtitre plate assays. All key steps, including mitochondrial isolation, basic assay optimization, and subsequent data processing are described in detail. This protocol may also be adapted for a variety of related applications, including enzymatic-, cell- and animal-based bioassays^{16,31,34–36}.

MATERIALS

REAGENTS

▲ **CRITICAL** All chemicals need to be of the highest purity available and solutions need to be prepared using ultra-pure water (e.g. Milli-Q, Millipore or HPLC grade).

- Sprague-Dawley rats or equivalent strain (Charles River) **! CAUTION** All animal experiments are to be performed in accordance with relevant authorities' guidelines and regulations. (see ref. 37). ▲ **CRITICAL** Change of species, animal strain, tissue or treatment will require re-optimization.
- MitoXpress oxygen-sensitive probe (Luxcel Biosciences, cat. no. A65N-1)
- BCA kit for protein determination (Fischer Scientific, cat. no. P123225)
- Heavy mineral oil (VWR, cat. no. IC15013880; or Luxcel Biosciences, cat. no. MO-50)
- D-(+)-glucose (Sigma, cat. no. D-8270)
- Glucose oxidase (*Asp. niger*; Sigma, cat. no. 49181)
- KCl (Sigma, cat. no. P9333)
- K_2HPO_4 (Sigma, cat. no. P3786)
- $MgCl_2$ (Sigma, cat. no. M2670)
- HEPES (Sigma, cat. no. H7523)
- MOPS (3-(N-morpholino)propanesulfonic acid; Sigma, cat. no. M1254)
- Mannitol (Sigma, cat. no. M9546)

- Sucrose (Sigma, cat. no. S7903)
- EGTA (ethylene glycol-bis (β-amino-ethylether)-N,N,N',N'-tetra-acetic acid tetrasodium; Sigma, cat. no. E8145)
- BSA, fatty acid free (Sigma, cat. no. A0281)
- ADP (adenosine 5'-diphosphate monopotassium salt dihydrate; Sigma, cat. no. A5285)
- Glutamate (L-glutamic acid monosodium salt hydrate; Sigma, cat. no. G1626)
- Malate (L-(-)-malic acid; Sigma, cat. no. M6413)
- Succinate (sodium succinate dibasic hexahydrate; Sigma, cat. no. S2378)
- DMSO (dimethylsulfoxide; Sigma, cat. no. D8418)
- FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Sigma, cat. no. C2920)
- Ice

EQUIPMENT

- ▲ **CRITICAL** All glass equipment, cylinders, spatulas, etc., need to be washed in ultra-pure water to avoid possible contamination with detergents and trace metals that can adversely affect mitochondrial function.
- Glass tissue homogenizer with Teflon pestle (100 ml)
- Glass beakers

- Glass stirring rods
 - Plastic funnel
 - Centrifuge tubes (50 ml)
 - Power drill
 - Cheesecloth
 - Refrigerated centrifuge
 - Cuvette spectrophotometer or absorbance plate reader with appropriate filter for protein determination (540–580 nm)
 - Standard 96-well microplates or disposable plastic cuvettes (e.g., Sarstedt, Corning, Nunc)
 - Standard 96-well PCR plate (e.g., Sarstedt, Corning, Nunc)
 - Black body clear bottom 96-well plates (Costar 3631 or equivalent)
 - Automated pipettes: Gilson P10, P100, P1000
 - 8- or 12-channel, 10 μ l and 100 μ l pipettes (optional)
 - Eppendorf syringe dispenser with 2.5 ml plastic syringes
 - Multi-Blok heater (Barnstead/LabLine)
 - Water bath, 30 °C (for warming solutions)
 - Time-resolved fluorescent plate reader. Recommended reader families include Safire (TECAN), ArcDia (Luxcel Biosciences) and Victor (Perkin Elmer).
- ▲ **CRITICAL** When using other instruments and/or customized filters, ensure that they provide sufficient sensitivity to probe in terms of signal-to-blank ratio. Readers should have: (i) a red-sensitive photomultiplier tube; (ii) filters compatible with the probe; (iii) temperature-controlled microplate

PROCEDURE

Mitochondrial preparation ● TIMING 2–3 h

- 1| House male Sprague-Dawley rats (average weight 150–180 g) in pairs in a controlled environment with constant temperature (21±2 °C) and a 12-h light/dark cycle. Food and water is provided *ad libitum*.
 - 2| Isolate mitochondria from rat liver using a modified procedure of Lapidus *et al.*^{16,38}. Euthanize animals with an overdose of carbon dioxide. Cervical dislocation can be used assuming the appropriate approvals have been obtained.
- ▲ **CRITICAL STEP** Avoid anaesthetics as they can have adverse effects on mitochondrial quality.
- 3| Excise liver rapidly and place it in a glass beaker into ice-cold isolation buffer I.
 - 4| Mince 3 g of liver very finely with scissors and wash several times in isolation buffer I until the homogenate is blood free.
- ! **CAUTION** For isolation of mitochondria from other tissue (e.g., skeletal and cardiac muscle mitochondria) different procedures may apply³⁹.
- 5| Add five volumes of isolation buffer I and homogenize the tissue using a smooth glass grinder with a Teflon pestle driven by a power drill on low speed (6–8 passes).
 - 6| Adjust the volume of the homogenate to eight volumes (generally ~24 ml) with isolation buffer I and centrifuge at 700g for 10 min at 4 °C.
 - 7| Filter through two layers of cheesecloth on a funnel and re-centrifuge for 10 min at 14,000g at 4 °C to precipitate the mitochondrial fraction.
 - 8| Discard the resultant supernatant, wash the mitochondrial pellet by re-suspending it in 20 ml of isolation buffer I using a glass stirring rod and re-centrifuge at 10,000g for 10 min at 4 °C.
 - 9| Repeat washing of mitochondria (Step 8), but using isolation buffer II.
 - 10| Re-suspend the mitochondria in 0.7 ml of isolation buffer II and keep on ice all the time. Determine protein concentration photometrically, using the BCA kit, manufacturer's protocol and measurement on absorbance reader (at 562 nm).
- ▲ **CRITICAL STEP** Performance of mitochondrial preparations that contain less than 30 mg ml⁻¹ of protein diminishes rapidly
- **PAUSE POINT** Preparation can be stored for up to 4–6 h on ice.

Instrument Setup ● TIMING < 30 min

- 11| Warm up fluorescent plate reader to 30 °C. Prepare the kinetic measurement protocol to read the desired wells of the plate at 0.5–1.5 min intervals over 30–60 min. The appropriate settings for various common instruments are given in **Table 1**.

Analysis of mitochondrial oxygen consumption ● TIMING 1 h per plate

- 12| Reconstitute MitoXpress probe (supplied dry in a plastic 2 ml vial) in 1 ml of respiration buffer to produce probe stock solution. Dilute probe stock to 10 ml with respiration buffer to produce working dilution of the probe. Warm this solution to 30 °C on a water bath.

compartment; and (iv) kinetic analysis software. Although less desirable, prompt fluorescence plate readers, such as SpectraMax Gemini (Molecular Devices), can also be used. Instrument performance can be verified using the following controls: i) 200 μ l sample without probe (blank); ii) 200 μ l aerated sample with probe (normoxic control); iii) 200 μ l deoxygenated sample with probe. Deoxygenation may be achieved using glucose/glucose oxidase system²⁸.

REAGENT SETUP

▲ **CRITICAL** To adjust pH of all the buffers, use HCl and KOH. Do not use NaOH. Solutions should be prepared with ultra-pure water and stored in pre-washed glassware. All substrate stocks can be prepared in advance and stored in aliquots at –80 °C for several weeks. However, buffers should be prepared fresh weekly. BSA should be added on the day of experiment.

Isolation buffer I 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, 0.5% w/w BSA, pH 7.4.

Isolation buffer II 210 mM mannitol, 70 mM sucrose, 10 mM MgCl₂, 5 mM K₂HPO₄, 10 mM MOPS, 1 mM EGTA, pH 7.4.

Respiration buffer 250 mM sucrose, 15 mM KCl, 1 mM EGTA, 5 mM MgCl₂, 30 mM K₂HPO₄, pH 7.4.

Glutamate/malate stock solution 0.5 M sodium glutamate, 0.5 M malate in H₂O, pH 7.4 (substrates for NADH-ubiquinone oxidoreductase).

Succinate stock solution 1 M succinate in H₂O, pH 7.4 (substrate for succinate-ubiquinone oxidoreductase).

ADP stock solution 100 mM ADP in H₂O.

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TABLE 1 | Details of recommended instruments and measurement settings.

	Safire/Genios Pro (Tecan)	ArcDia (Arctic Diagnostics)	Victor/Envision (PerkinElmer)	SpectraMax Gemini (Molecular Devices)
Light source	Xe-flashlamp	532 nm chip laser	Xe-flashlamp	Xe-flashlamp
Optical configuration	Filter based, bottom reading	Filter based, top reading	Filter based, top reading	Monochromator based, top/bottom reading
Measurement mode	TRF	TRF	TRF	Prompt fluorescence mode
Excitation, nm	380 ± 20 nm	532 nm	340 ± 40 nm	380 nm
Emission, nm	650 ± 20 nm	650 ± 20 nm	642 ± 10 nm	650 nm
Delay time	30 μs	20 μs	30 μs	n/a
Gate time	100 μs	100 μs	100 μs	n/a

TRF, time-resolved fluorescence.

▲ CRITICAL STEP Standard probe package is for one 96-well plate (or ~100 assay points). For a smaller or larger number of assays adjust the volumes accordingly to produce the same working dilution of the probe. Probe stock solution can be stored for several days in the dark at 4 °C. Diluted probe should be used on the same day.

13| At this point, solutions can be prepared to measure the basal respiration rate (option A) and/or the ADP-stimulated respiration rate (option B).

(A) Basal respiration rate (state 2)

(i) For basal respiration, mix 150 μl of either succinate or glutamate/malate stock solution with 1.35 ml of respiration buffer. Warm to 30 °C on a water bath.

(B) ADP-stimulated respiration rate (state 3)

(i) For ADP-stimulated respiration, mix 150 μl of either succinate or glutamate/malate stock and 100 μl ADP stock with 1.25 ml of respiration buffer. Warm to 30 °C on a water bath.

14| Prepare 10 ml of mineral oil and warm to 30 °C on a water bath.

15| Prepare a six point dilution series of mitochondrial preparation in respiration buffer in 1.5 ml total volume for each concentration. Starting concentrations recommended for different substrates and respiration states are:

Glutamate/malate:	1.5 mg ml ⁻¹
Glutamate/malate/ADP:	1.0 mg ml ⁻¹
Succinate:	1.0 mg ml ⁻¹
Succinate/ADP:	0.5 mg ml ⁻¹

▲ CRITICAL STEP Ensure that the 1:4 (v/v) dilution factor in the microwells is accounted for (see Steps 17–19).

16| Take a black 96-well plate and place it on a plate heater equilibrated to 30 °C. Recommended plate map for this experiment is given in **Figure 2a**. Mitochondrial protein concentrations are given in mg ml⁻¹ in the first well of each series, each concentration is run in quadruplicate (A–D or E–H). The top half of the plate assesses state 2 respiration (without ATP) while the bottom half assesses state 3 respiration (with ATP). The left half of the plate assesses glutamate/malate-driven respiration while the right half assesses succinate-driven respiration.

17| Using automatic or multi-channel pipettes, add to each well 100 μl of respiration buffer containing MitoXpress probe (described in Step 12).

18| Add 50 μl of mitochondrial stock dilutions giving the desired final concentration of mitochondria.

19| Add 50 μl of substrate solution giving a final concentration of 25 mM for succinate or 12.5/12.5 mM for glutamate/malate. For state 3 analysis, substrate solution also needs to contain ADP at final concentration of 1.65 mM.

20| Using a syringe dispenser, quickly add 100 μl of pre-warmed heavy mineral oil to each well.

21| Insert the microplate into the fluorescent plate reader pre-set as described above and commence measurements.

▲ CRITICAL STEP To minimize oxygen depletion in samples prior to the measurement, plate preparation time should be kept to a minimum (<10 min for Steps 17–21).

22| When the measurement cycle is complete, remove the plate from the instrument and save measured data to file.

Figure 2 | Recommended plate maps. Initial assay optimization (a): different substrate buffers are colour-coded and marked, mitochondrial protein concentrations are given in mg ml⁻¹ in the first well of each quadruplicate series. Screening compounds C1–C46 at a single dose (b): columns 7–12 are a replicate of columns 1–6, positive controls (FCCP) are in wells G6 and G12, untreated controls (DMSO) in H6 and H12. The generation of dose-response curves for compounds C1–C11 (c): two-fold serial dilutions with one compound per column and one well per concentration (100.0, 50.0, 25.0, 12.5, 6.3, 3.2, 1.6, 0.8 nmol mg⁻¹ protein in wells A–H, respectively).

a	1	2	3	4	5	6	7	8	9	10	11	12
A	1.5	1.0	0.5	0.25	0.125	0	1.0	0.5	0.25	0.125	0.06	0
B	Glutamate/malate-driven respiration state 2 (No ADP)						Succinate-driven respiration state 2 (No ADP)					
C	Glutamate/malate-driven respiration state 2 (No ADP)						Succinate-driven respiration state 2 (No ADP)					
D	Glutamate/malate-driven respiration state 2 (No ADP)						Succinate-driven respiration state 2 (No ADP)					
E	1.0	0.5	0.25	0.125	0.06	0	0.5	0.25	0.125	0.06	0.03	0
F	Glutamate/malate-driven respiration state 3 (ADP added)						Succinate-driven respiration state 3 (ADP added)					
G	Glutamate/malate-driven respiration state 3 (ADP added)						Succinate-driven respiration state 3 (ADP added)					
H	Glutamate/malate-driven respiration state 3 (ADP added)						Succinate-driven respiration state 3 (ADP added)					

b	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C9	C17	C25	C33	C41	C1	C9	C17	C25	C33	C41
B	C2	C10	C18	C26	C34	C42	C2	C10	C18	C26	C34	C42
C	C3	C11	C19	C27	C35	C43	C3	C11	C19	C27	C35	C43
D	C4	C12	C20	C28	C36	C44	C4	C12	C20	C28	C36	C44
E	C5	C13	C21	C29	C37	C45	C5	C13	C21	C29	C37	C45
F	C6	C14	C22	C30	C38	C46	C6	C14	C22	C30	C38	C46
G	C7	C15	C23	C31	C39	PCCP	C7	C15	C23	C31	C39	PCCP
H	C8	C16	C24	C32	C40	DMSO	C8	C16	C24	C32	C40	DMSO

c	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
B	DMSO	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	DMSO	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
D	DMSO	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	DMSO	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
F	DMSO	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	DMSO	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
H	DMSO	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11

23| Plot time profiles for different enzyme concentrations for each respiration media.

24| From these profiles, select the optimal concentrations of mitochondria for further use in compound screening. The concentration that produces appropriate signal change to allow analysis of both inhibition and uncoupling is usually selected as optimal. This optimal concentration will depend on the medium used (the substrate and availability of ADP). To assure functionality of mitochondrial preparations, respiratory control rates (RCR) can be assessed; determination of RCR values is described in Steps 39–43.

Compound screening ● **TIMING 1 h per plate**

25| Prepare (if required) respiration buffer with MitoXpress probe as described in Step 12. Experimental procedure for compound screening is similar to the measurement of enzyme activity (see previous section), but is performed at fixed (optimal) concentration of mitochondria and known concentrations of compounds.

26| Prepare stock solutions of compounds in DMSO in a separate 96-well plate according to the plate map given in **Figure 2b**. To reduce the volumes required, a 96-well PCR plate can be used.

▲ **CRITICAL STEP** Account for the dilution factor in the microwells (in this case 1:200; see Steps 32–34).

27| At this stage, compound stocks need to be diluted either for initial (option A) or detailed analysis (option B). Dilute compound stocks with respiration buffer to produce known concentrations as follows:

(A) For the initial screening (yes/no effect) assay is carried out at a single concentration (e.g. 100 nmol mg⁻¹ protein), as per plate map given in **Figure 2b**. Each compound C1–C49 is analysed in duplicate (columns 1–6 and 7–12, colour-coded). Positive controls (FCCP) are in wells G6, G12 and untreated controls (DMSO) in H6, H12.

(B) For the detailed analysis of ‘hits’ and IC50 generation, prepare for each compound C1–C11 a series of two-fold dilutions, as per plate map given in **Figure 2c**: one well per concentration in rows A to H, starting from 100 nmol mg⁻¹ protein in well A (concentration range can be adjusted to specific screening paradigms).

▲ **CRITICAL STEP** As drug concentrations are usually expressed in nmol mg⁻¹ mitochondrial protein, prepare different dilutions depending on the chosen working concentration of mitochondria. The final DMSO content (in assay wells) should not exceed 0.5% v/v.

28| For state 2 respiration, mix 600 µl of either succinate or glutamate/malate stock solutions with 5.4 ml of respiration buffer. For state 3 respiration mix 600 µl of substrate stock (succinate or glutamate/malate) and 400 µl of ADP stock with 5.0 ml of respiration buffer. Pre-warm reagents to 30 °C.

29| Prepare 6 ml of stock mitochondria at optimal working dilution. The optimal dilution(s) is/are determined from Step 24.

▲ **CRITICAL STEP** If > 50 compounds are to be screened, mitochondrial preparations need to be up-scaled. Because quality decays after 4–6 hours, two daily preparations may be necessary.

30| Take a black 96-well plate and place it on a plate heater equilibrated to 30 °C.

31| Using an appropriate automatic or multi-channel pipette, add to each well 100 µl of respiration buffer with MitoXpress probe.

32| Add 1 µl of compound at desired concentration (see Steps 27A and 27B) (**Fig. 2b,c**), according to the appropriate plate map.

! **CAUTION** Consult the technical specification of the pipette to ensure that it is capable of accurately dispensing 1 µl.

BOX 1 | ANCILLARY DETAILS

Data-processing algorithm

When analysing the oxygen consumption of isolated mitochondria using the MitoXpress probe, the following conditions apply: a classical Stern-Volmer quenching mechanism²⁰; zero order kinetics with respect to oxygen (i.e. enzyme saturation at all oxygen concentrations); and first order kinetics with respect to enzyme concentration. The following analytical relationship therefore applies:

$$\frac{I(t_0)}{I(t) - I(t_0)} = \frac{1 + K_{sv} * [O_2(t_0)]}{K_{sv} * k_2 * [E] * t}$$

where $I(t_0)$ and $I(t)$ represent initial fluorescence signal and the signal at time t , K_{sv} represents the Stern-Volmer quenching constant, $[O_2(t_0)]$ represents the initial, air saturated, dissolved oxygen concentration, while k_2 and $[E]$ represent the enzyme catalytic constant and concentration respectively. This relationship allows simple linearization of the non-linear raw fluorescence profiles, achieved using double reciprocal plots. This in turn facilitates analysis of altered levels of enzymatic activity.

Interferences

All fluorescence-based assays are susceptible to certain interferences. Complex optically active samples tend to interfere with measured signal via processes such as light scattering, sample autofluorescence and inner filter effects, thereby affecting assay results. The oxygen consumption assay outlined here is based on the measurement of relative, kinetic changes in probe fluorescence. In conjunction with time-resolved fluorescence detection, this secures high signal-to-blank ratios and easily detectable responses to oxygen depletion. These features provide a relatively broad assay window and resistance to interferences.

Interference may occur when a compound physically interacts with the probe, affecting its fluorescent signal (quenching or enhancement) and/or sensitivity to oxygen. This manifests itself as a dose-dependent change in probe initial and/or maximal signal. In most cases, these effects can be compensated for by normalizing fluorescence profiles. This is achieved by dividing all subsequent readings by the initial intensity reading, thereby compensating for the interference. In those rare cases in which the compound causes large changes in probe signal or alters the response to oxygen, additional controls and more complex oxygen-sensing approaches or consultation with the probe supplier may be required.

Adaptation to 384-well plates

The throughput of the assay can be further increased by adapting it to 384-well plates. In this case all the volumes are reduced appropriately, while the final concentrations of the probe, substrates and compounds remain the same. Working concentration of mitochondria needs to be optimized for this type of microplate, as assay sensitivity is dependent on sample size and geometry. 384-well format requires higher level of automation, such as robotic liquid handling, to retain the necessary short preparation times for the microplate (< 10 min).

Alternative applications

This protocol can also be adapted for respirometric analysis of whole cells^{34,36}, as well as for microbiological³⁵ and environmental³¹ studies.

- 33| Add 50 µl of mitochondria at optimal dilution to each well.
- 34| Add 50 µl of substrate stock solution to each well.
- 35| Using a syringe dispenser, quickly add 100 µl of heavy mineral oil to each well.
- 36| Insert the microplate into the fluorescent plate reader pre-set as described above and commence measurements. When completed, save data to file.
- 37| Repeat Steps 25–36 with the next set of compounds on a new plate or perform data analysis as described in Steps 43–45.

Data analysis and calculations

Analysis of mitochondrial oxygen consumption ● TIMING 0.5 h per plate

- 38| Export plate reader data into Excel spreadsheet (if required): fluorescence intensity versus time for each sample.
- 39| Examine individual profiles and select enzyme concentration(s) that produces reliably measurable signal changes to allow analysis of both inhibition and uncoupling.
- 40| Linearize fluorescence-time profiles for enzyme concentrations selected in Step 24, using the following co-ordinate scale (more details on data processing algorithm are given in **Box 1**):
Abscissa, Y: $I(t_0)/(I(t) - I(t_0))$ - where $I(t_0)$ and $I(t)$ represent fluorescence intensity signals at the start and at time t of monitoring, respectively; Ordinate, X: $1/t$, min^{-1} ; exclude zero time points and regions of signal saturation, i.e. long monitoring times.

- 41| Apply linear regression analysis to the transformed profiles and determine the slope and correlation coefficient (quality control of the fits) for each of the transformed profiles.

▲ CRITICAL STEP If a significant baseline drift is seen on the original profiles (i.e., at zero enzyme concentration), it is recommended that the baseline be subtracted from all profiles prior to the transformation. This usually improves the linearity of transformed plots.

42| Take the reciprocal ratio of the two calculated slopes (i.e., with and without ADP) for each individual substrate, thereby obtaining the state 3/state 2 ratio(s).

Compound screening ● TIMING 0.5–2 h per plate, may vary

43| Repeat Steps 38–41 with the corresponding data set. (See **Box 1** for information regarding compound interference.)

44| Plot or tabulate the inversed slope values normalized for the initial value (i.e., without the compound) versus compound concentration. This is carried out for inhibitors (option A) or uncouplers (option B) in different ways.

(A) For inhibitors:

(i) Apply sigmoidal fits to this dependence or plot it to determine IC₅₀ values.

(B) For uncouplers:

(i) Express the data as percentage of uncoupling relative to the uncoupling by FCCP, which is achieved at optimal concentration giving the maximal oxygen consumption.

45| Group the compounds based on their effect on mitochondria and rank order based on IC₅₀ values.

● TIMING

Day 1: Preparation (4–8 hours depending on number of compounds to be tested)

Prepare isolation buffer I and isolation buffer II for several isolations, respiration buffer and other solutions for measurement experiments. Prepare all drug stock solutions to be tested during the week and transfer them into 96-well plates.

Day 2: Assay setup

8.00–10.30	Mitochondrial isolation, protein determination, pre-warming of reagents and equipment. Make appropriate dilutions of drug stocks if IC ₅₀ determinations are desired.
10.30–11.30	1 st plate setup (different mitochondrial concentrations, media), measurement.
11.30–12.00	Data analysis, determination of working concentration.
12.00–13.00	2 nd plate setup (inhibitors, IC ₅₀ curves), measurement.
13:00–14:00	Lunch break.
14.00–16.00	Data analysis.

Day 3: Compound screening

8.00–10.30	Mitochondrial isolation, protein determination, pre-warming of reagents and equipment. Make appropriate dilutions of drug stocks if IC ₅₀ determinations are desired.
10.30–12.30	Setup and measurement of plates 1–4 (30 min per plate).
12.30–14.30	Lunch break.
14.30–15.00	New mitochondrial isolation, protein determination, pre-warming of reagents.
15.00–17.00	Setup and measurement of plates 5–8 (30 min per plate).

Day 4: Data analysis

Export and calculate all data from Day 3.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Possible cause	Solution
Mitochondrial preparation: Poor protein yield	Poor homogenization	Assure good fit of pestel and homogenizer, homogenize 6–10 times with medium speed until solution is pink and no debris is seen in the homogenate
	Non-optimal conditions for tissue enzymatic digestion (if used)	Optimize enzymatic digestion
	Upscaling sometimes results in lower yields	Optimize or revert to small scale preparation

TABLE 2 | Troubleshooting table (continued).

Problem	Possible cause	Solution
Lower than expected activity or poor RCR values	Animals are too old	Use younger rats (weight < 200g). Protocols might need to be adapted for older animals
	Contaminated glassware may lead to uncoupling	Pre-wash all glassware and other equipment used
	Homogenization or digestion are too harsh	Optimize conditions
Activity diminishes faster than in 4–6 h	Low temperature not maintained properly during isolation	Perform all steps on ice or in cold room
	Mitochondria stored at < 30mg ml ⁻¹ ; too much handling	Store mitochondria at > 30mg ml ⁻¹ and on ice at all times; minimize handling
All above	Lack of experience with mitochondrial preparations	Training in laboratories skilled in isolation procedures might be beneficial
Data output and processing:		
Signals indistinguishable from blanks	Incompatible instrument or incorrect instrument settings	Check instrument suitability and setup and run proper controls without mitochondria (probe/no probe)
Signals detectable, but signal changes too small	Instrument performance is poor (low signal/blank ratio)	Check the instrument and run proper controls
	Too little protein was used or mitochondria are not respiring	Use more protein. Make sure that mitochondria are fully functional
	Significant depletion of dissolved oxygen in samples prior to the start of monitoring	Optimize assay conditions: concentration of mitochondrial protein, plate preparation time
Signal increase upon ADP addition is small	Poor coupling of mitochondria	Make sure that mitochondria are fully functional
Rapid signal saturation	Long plate preparation times	Reduce plate preparation time to < 10 min
	Mitochondria concentration is too high	Optimize protein concentration
	Low measurement frequency	Increase frequency of measurements
Initial intensity is inconsistent	Long plate preparation times	Reduce plate preparation time to < 10 min. Use plate heater during plate preparation
	Interference by test compound (e.g., autofluorescence, optical effects, interaction with the probe)	Use time-resolved fluorescence mode. Compensate for interferences as recommended (see Box 1) or contact probe manufacturer
There is a drop in signal over the initial minutes	Plate temperature equilibration; baseline drift	Use plate heater during plate preparation. Pre-warm all solutions
Insufficient data points for generation of dose-response data	Some compound concentrations are outside measurable range	Adjust the range and concentrations
Compound causes both increase and decrease in consumption rate depending on concentration	Some compounds can be uncouplers and inhibitors depending on the concentration used	Evaluate mechanism quantitatively. Compare to other compounds
Linearization of profiles is poor	Significant signal drift (baseline)	Correct all profiles for baseline drift, prior to the linearization

ANTICIPATED RESULTS

Analysis of mitochondrial oxygen consumption

Due to possible batch-to-batch variability of mitochondrial preparations and/or variations in source tissue, quality control of the mitochondrial preparation, optimization of the working concentration and evaluation of assay performance are required prior to the assessment of effector action.

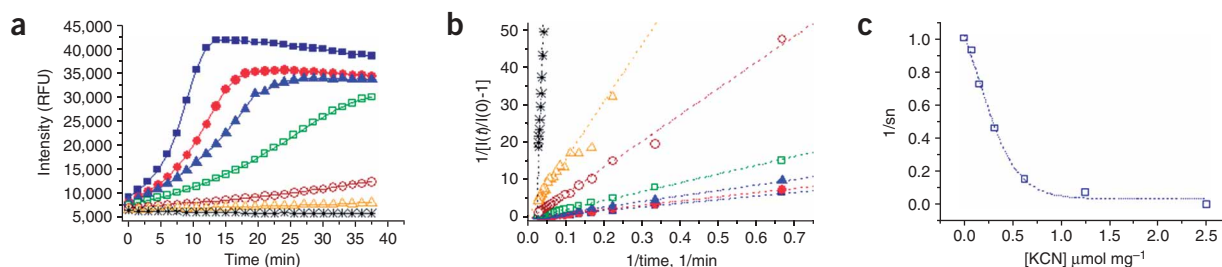


Figure 3 | Sample data output for inhibition analysis showing intensity profiles (a), transformed linearized profiles (b) and inversed normalized slopes (c) showing the effect of increasing KCN concentrations on glutamate/malate-driven ADP-activated respiration. Concentrations in $\mu\text{mol mg}^{-1}$ are as follows: 2.5 (\star), 1.25 (Δ), 0.625 (\circ), 0.312 (\square), 0.156 (\blacktriangle), 0.078 (\bullet) and 0 (\blacksquare). Linear fits are applied in b (see data in Table 3) and used for dose-response analysis (c).

Oxygen consumption rates are low under basal conditions (state 2; substrate only) and increase significantly in the presence of ADP (state 3). The ratio of ADP-stimulated to basal respiration (state 3/state 2) will yield a respiratory control ratio (RCR), which is a measurement for functionality (coupling) of the mitochondria and is an indication of preparation quality. Full coupling of mitochondrial preparations has to be assured prior to compound screening. Compare calculated RCR values with literature data, previous experiments or control data sets¹⁶. RCRs will differ for different substrates and are tissue dependent. As a guideline, rat liver mitochondria in general show RCRs of 3.0 or greater for succinate-driven respiration and 5.0 or greater for glutamate/malate-driven respiration. Different substrates can be used in screening paradigms for mechanistic purposes. Preparations can also be optimized for using ascorbate/TMPD (*N,N,N',N'*-tetramethyl-p-phenylenediamine) as substrates for ubiquinol-ferricytochrome-c-oxidoreductase.

For further drug testing, the optimal protein concentrations for each analyzed state and substrate needs to be determined. In our hands, the optimal protein concentrations for mitochondria from Sprague-Dawley rat liver are as follows¹⁶:

- Succinate-driven basal respiration: 0.5 mg ml^{-1}
- Succinate-driven ADP-activated respiration: $0.125\text{--}0.25 \text{ mg ml}^{-1}$
- Glutamate/malate-driven basal respiration: 1.0 mg ml^{-1}
- Glutamate/malate-driven ADP-activated respiration: 0.25 mg ml^{-1}

Assay performance is reflected by values of coefficient of variation (CV) for intra- and inter-assay variations. If CV values are less than 15% (normally they are at $\sim 10\%$), one can proceed to compound screening. Once mitochondrial preparations are seen to be reproducible and the assay is well established ($n = 3\text{--}5$), the optimization step also becomes unnecessary.

Compound screening

Mitochondrial toxicity, as measured by oxygen consumption, can be a result of either uncoupling (seen as an increase in oxygen consumption) or inhibition (seen as a decrease in oxygen consumption). Uncoupling is best evaluated during basal respiration (state 2), as effects on ADP-stimulated respiration (state 3) can easily be misinterpreted¹⁶. Inhibition is most easily detected during ADP-stimulated analysis (state 3).

For qualitative assessments of uncoupling or inhibition, straightforward analysis of raw fluorescence profiles is often sufficient. Figure 3a illustrates the raw data output of such an analysis. The effect of the ETC inhibitor potassium cyanide (KCN) on mitochondrial oxygen uptake is evidenced by the reduced rate of fluorescent signal increase at elevated KCN concentrations. Such data allow preliminary detection of inhibition or uncoupling and the generation of rudimentary dose-response information. For more quantitative data, these fluorescence time traces need to be converted to oxygen consumption activities, as outlined in the protocol (also see Box 1). The suggested transformation (result is shown in Fig. 3b) overcomes the fact that the relationship between probe fluorescence intensity and oxygen concentration is nonlinear²⁰, thereby allowing quantitative data analysis to be carried out by linear regression. In the case of compound screening it also eliminates the need of calibrating the system

TABLE 3 | Summary of data processing results for KCN.

[Drug] nmol mg^{-1}	Linear regression function	R^2	Inversed normalized slope
0	$Y = 9.6742X - 0.2185$	0.969	1.000
0.008	$Y = 11.239X - 0.2099$	0.990	0.861
0.016	$Y = 14.792X - 0.2954$	0.996	0.654
0.031	$Y = 23.703X - 0.3362$	0.998	0.408
0.063	$Y = 70.551X - 0.7661$	0.994	0.137
0.125	$Y = 154.28X + 0.0149$	0.961	0.063
0.25	$Y = 3059.5X - 70.003$	0.998	0.003

(probe and detector) with oxygen standards. The data obtained from such analysis is presented in **Table 3**. The linear regression function associated with each drug concentration is presented with the associated R^2 value. The slope values obtained from the linear regression function are inversed and normalized with respect to the untreated sample. These values can then be used for dose-response analysis (**Fig. 3c**) and/or compound ranking. Results can then be compared to reference literature or in-house data. The relatively high throughput of this measurement approach also allows for application in structure-activity relationship (SAR) investigations.

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