

Chapter 2

Evaluation of Respiration with Clark Type Electrode in Isolated Mitochondria and Permeabilized Animal Cells

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Abstract

In many studies, the evaluation of mitochondrial function is critical to understand how disease conditions or xenobiotics alter mitochondrial function. One of the classic end-points that can be assessed is oxygen consumption, which can be performed under controlled, yet artificial conditions. Oxygen is the terminal acceptor in the mitochondrial respiratory chain, namely at an enzyme called cytochrome oxidase, which produces water in the process and pumps protons from the matrix to the intermembrane space. Several techniques are available to measure oxygen consumption, including polarography with oxygen electrodes or fluorescent/luminescent probes. The present chapter will deal with the determination of mitochondrial oxygen consumption by means of the Clark-type electrode, which has been widely used in the literature and that still remains to be the most reliable technique. We focus our technical description in the measurement of oxygen consumption by isolated mitochondrial fractions and by permeabilized cells.

Key words: Mitochondria, Permeabilized cells, Clark type electrode, Cellular respiration, ADP/O ratio, Respiratory control ratio

1. Introduction

Aerobic eukaryotic cells present very developed intracellular machinery aimed at energy production, the mitochondrion, where O_2 is the terminal electron acceptor of a series of electron transfers through proteins comprising the so-called respiratory chain (1).

The diagram in Fig. 1 represents the mitochondrial respiratory chain, where electrons flow from NADH (oxidized in complex I) or succinate (complex II) to O_2 . The different complexes contain several co-factors and prosthetic groups. Complex I contains FMN and 22–24 iron–sulfur (Fe–S) proteins in 5–7 clusters. Complex II contains FAD, 7–8 Fe–S proteins in three clusters and cytochrome b_{560} .

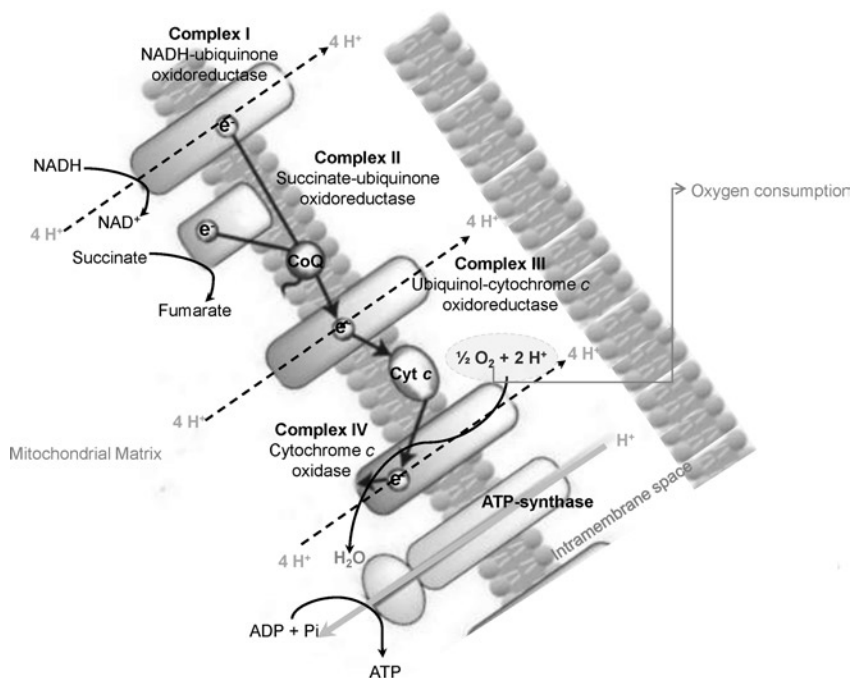


Fig. 1. Mitochondrial respiratory chain showing the process of oxidative phosphorylation.

Complex III contains cytochrome *b* (the ultimate electron acceptor in this complex), cytochrome *c*₁, and one Fe-S protein. Complex IV contains cytochrome *a*, cytochrome *a*₃, and two copper ions. It is proposed that when two electrons pass through complex I, four protons (H⁺) are pumped into the intramembrane space of the mitochondrion. In the same manner, as each pair of electrons flows through complex III, four protons are pumped into the intramembrane space. Electrons are then used to reduce O₂ to H₂O, on cytochrome *c* oxidase. The protons in intramembrane space return to the mitochondrial matrix down their concentration gradient by passing through the ATP synthase, also called complex V, which synthesizes ATP at the expense of the transmembrane electric potential generated by the proton imbalance between the two sides of the membrane (2). The process of ATP synthesis by mitochondria is termed oxidative phosphorylation (OXPHOS), which describes the coupling between substrate oxidation and ADP phosphorylation.

When OXPHOS and electron transfer system (ETS) in particular, needs to be evaluated, an approach to monitor oxygen dynamics is a possibility, especially when coupled to proper substrates and inhibitors of the electron transfer and ADP phosphorylation process (see Note 1).

Historically, the electrochemical reduction of oxygen was discovered by Heinrich Danneel and Walter Nernst in 1897 (revised by Severinghaus and Astrup (3)). In the 1940s, the technology was tested in biological tissues to follow oxygen supply or availability by the implantation of platinum electrodes (4), in an amperometric approach. The major problem of the direct contact of electrodes with biological preparations was contamination, diminishing the electrochemical response; one example was the contamination of the platinum electrode by compounds such as cyanide and iodide (5). The fundamental innovation introduced by L. Clark (3) was the presence of a permeable membrane, which allows the transport of ions but restricts the contact of higher molecular weight substances (e.g. proteins) with the electrode surface (6). Nevertheless, it is important to know the properties of the chosen oxygen permeable membrane, paying special attention to the electrochemical delays, and the possibility of organic degradation or physico-chemical interactions with some inhibitors such as antimycin A or oligomycin, among others (5), that could still be present from one experiment to the next.

It is thus necessary to obtain information from the current concentration relationship, thus maintaining a desired selectivity. The chemical transformations that take place at the electrodes after the passage of electrical current are ruled by Faraday's law and by the current-voltage equation.

In vivo cell respiration, considering organ and whole body organization, is regulated by intracellular non-saturating ADP levels during normal states of activity (7) and increases when submitted to a specific stimulus. One reason to use mitochondria in intact or permeabilized cells is that mitochondria interact with the cytoskeleton network, being grouped in functional clusters in close contact with other cell organelles and structures, probably an essential feature for the correct function of the whole mitochondrial bioenergetic apparatus (8). Cells permeabilization may be established by different methodologies, but the most popular is the one that considers the presence of amphipathic substances.

Detergents (such as digitonin and saponin) have been used for selective membrane permeabilization, but their usage conditions must be carefully controlled. By treating cells with specific detergents, it is possible to induce membrane permeabilization by promoting loss of lipid content, although without losing important intracellular proteins; subsequently it will be easier to follow the oxygen consumption in the presence of different substrates, nucleotides, and respiratory inhibitors.

Cell permeabilization by electroporation is also technically possible. Electrically permeabilized cells were used to allow the uptake of exogenously added ATP, which is essential for receptor-mediated activation of the respiratory burst. For example, blood cells as neutrophils, are ATP-depleted prior to permeabilization (9).

Differences between intact cell respiration and substrate oxidation in digitonin-permeabilized cells can be polarographically measured with a Clark oxygen electrode in a micro-jacketed chamber (10, 11), although quenched-fluorescence oxygen sensing can be also used in intact cells (12).

In the next sections of the present book chapter, some representative procedures to prepare permeabilized cell and isolate mitochondria are demonstrated, as well some hypothetical schemes for the assessment of oxygen respiration.

2. Materials

2.1. Cell Culture/ Preparation and Permeabilization

2.1.1. Electroporabilized Cells: Neutrophils (Adapted from ref. 9)

1. Ficoll 400 and dextran T-500 (Pharmacia LKB Biotechnology Inc.).
2. RPMI-1640, AMP-PNP, ATP, EGTA, GDP, GTP, NADPH, glucose, 2-deoxy-d-glucose, Coomassie Blue, analytical grade salts (Sigma).
3. Bicarbonate-free medium RPMI-1640 (buffered to 7.3, with 25 mM NaHEPES). Sterilize by autoclaving.
4. Permeabilization medium: 140 mM KCl, 1 mM MgCl_2 , 1 mM EGTA, 10 mM KHEPES (pH 7.0), and CaCl_2 enough to reach a final concentration of 100 nM, 1 mM ATP (except for experiments without ATP), and 10 mM glucose (except for ATP-depletion experiments, where is replaced by 2-deoxy-d-glucose). Store it on ice ($\pm 4^\circ\text{C}$).
5. Reaction medium for oxygen consumption assays: 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM MgCl_2 , 1 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM NaHEPES (pH 7.3).
6. HEPES-RPMI-1640 medium and all cell buffers must be at 37°C previously to usage.
7. All reagents (salts, sucrose, etc.) with analytical grade, from Sigma (St. Louis, MO).

2.1.2. Saponin- Permeabilization: Saponin-Skinned Muscle Fibers (Adapted from ref. 8)

1. Isolation/resuspension medium: 10 mM Ca-EGTA (0.1 μM free Ca^{2+}), 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT (dithiothreitol), 6.56 mM MgCl_2 , 5.77 mM ATP, and 15 mM phosphocreatine (pH 7.1). Store on ice.
2. Saponin (Sigma S-2149) and other reagents (salts, sucrose, etc.) with analytical grade, from Sigma (St. Louis, MO).

2.1.3. Digitonin- Permeabilization: Lymphocytes (Adapted from ref. 11)

1. Histopaque 10771 and other reagents (salts, etc.) with analytical grade, from Sigma (St. Louis, MO).
2. Phosphate buffer: 137 mM NaCl, 2 mM KCl, 1 mM KH_2PO_4 , and 10 mM Na_2HPO_4 (pH 7.4).

3. Digitonin (Sigma-Aldrich D-141) and other reagents (salts, sucrose, etc.) with analytical grade, from Sigma (St. Louis, MO).

2.2. Isolation of Mitochondrial Fraction

1. Brain isolation buffer (BIB): 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, and 1 mg/mL bovine serum albumin (fatty acid free) (pH 7.4).
2. Brain resuspension buffer (BRB): 225 mM mannitol, 75 mM sucrose, and 5 mM HEPES (pH 7.4).
3. Liver and heart isolation buffer (LIB and HIB): 250 mM sucrose, 10 mM HEPES, 0.5 mM EGTA, and for liver isolation supplement medium with 1 mg/mL bovine serum albumin (fatty acid free) (pH 7.4).
4. Liver and heart resuspension buffer (LRB and HRB): 250 mM sucrose and 10 mM HEPES (pH 7.4).
5. Protease (subtilisin fraction VIII) and other reagents (salts, sucrose, etc.) with analytical grade, from Sigma (St. Louis, MO).

2.3. Oxygen Electrode

2.3.1. Oxygen Electrodes and Polarographic Systems

Commercially available, being the most common, the following: Oroboros Oxygraph (Physica respirometer, Paar Physica, Oroboros, Austria); Hansatech Instruments Limited (Norfolk, UK); Yellow Springs Instrument Company (YSI Inc., Ohio, USA, the one we use in our laboratory); Gilson Medical Electronics (Middleton, WI); Mitocell micro respirometer (Strathkelvin Instruments Limited, Scotland). See specifications with manufactures.

2.3.2. Oxygen Permeable Membranes

We considered preferentially PTFE films [poly(tetrafluoroethylene), e.g. Teflon] with the reference YSI 5776, Oxygen Probe Service Kit (Yellow Springs, OH, USA).

2.4. Oxygen Consumption Assays: Permeabilized Animal Cells

1. Neutrophils respiration medium: 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM NaHEPES (pH 7.3).
2. Saponin-skinned muscle fibers respiration medium: 110 mM mannitol, 60 mM KCl, 10 mM KH_2PO_4 , 5 mM $MgCl_2$, 60 mM Tris-HCl, and 0.5 mM Na_2EDTA (pH 7.4).
3. Lymphocytes respiration medium: 300 mM mannitol, 10 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$, and 1 mg/mL bovine serum albumin (fatty acid free) (pH 7.4).

2.5. Oxygen Consumption Assays: Isolated Mitochondria

1. Brain mitochondria respiration medium: 100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4 , 5 mM HEPES, and 10 μM EGTA (pH 7.4).
2. Heart mitochondria respiration medium: 50 mM sucrose, 100 mM KCl, 1 mM KH_2PO_4 , 10 mM Tris, and 10 μM EGTA (pH 7.4).

3. Liver mitochondria respiration medium: 135 mM sucrose, 65 mM KCl, 5 mM KH_2PO_4 , 5 mM HEPES, and 2.5 mM MgCl_2 (pH 7.4).

It must be stressed that other reaction media are possible, using different compositions and if, required, with different osmolarities, although typically media with 250–300 mOsm are used.

3. Methods

3.1. Cell Culture and Permeabilization

3.1.1. Electroporated Cells: Neutrophils (Adapted from ref. 9)

1. Isolate neutrophils from fresh heparinized human blood by dextran sedimentation followed by Ficoll–Hypaque gradient centrifugation.
2. Add NH_4Cl for red cell lysis, centrifuge again, and wash with HEPES-buffered RPMI.
3. Count cells (using ZM Coulter Counter) and resuspend them at 10^7 cell/mL in HEPES-buffered RPMI [for ATP-depleted cells, cell (10^7 /mL) incubation at 37°C , during 5 min, in the specific buffer (with 2-deoxy-d-glucose, instead of glucose), prior to permeabilization].
4. Electroporation: Sediment cells (with ATP or ATP-depleted) by centrifugation and resuspend in ice-cold permeabilization solution at 10^7 cells/mL. Add 800 μL cell suspension/cuvette in a Bio-Rad Pulser (although others can be used) and apply two electric discharges (pulses – charges of 5 kV/cm from a 25- μF capacitor) as described by the manufacturer. Sediment cells by centrifugation in 1.5 mL conic tubes (Eppendorf 5415 microcentrifuge), between pulses, and resuspend in ice-cold permeabilization solution (at 10^7 cells/mL).
5. Electroporated cells are ready to be used by picking cells to a final density of 2×10^6 cell/mL or can be stored on ice up to 15 min.

3.1.2. Saponin-Permeabilization: Saponin-Skinned Muscle Fibers (Adapted from ref. 8)

1. Muscle fibers should undergo mechanical separation. Under a microscope, dissect small bundle fibers (submerged on isolation/resuspension medium ice-cold) at one end and separate down to the muscle mid belly (using fine jewellers forceps).
2. Place muscle bundles in ice-cold isolation medium (8–15 wet weight/mL), add 50 $\mu\text{g}/\text{mL}$ saponin, and gently mix the suspension during 30 min, at 4°C .
3. Wash muscle fibers with resuspension medium to remove all saponin.
4. Measure protein by Lowry et al. (13) method, using a 5- μL aliquot. Consider 0.5 mg/mL for oxygen consumption assays.

5. Store on ice (muscle fibers in resuspension medium) until start respiration analysis in specific reaction medium.

3.1.3. Digitonin-

Permeabilization:

Lymphocytes (Adapted from ref. 11)

1. Isolate human lymphocytes introducing 5 mL heparinized blood in a tube filled with a solution containing polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/mL (HISTOPAQUE® 10771-Sigma) (the proportion between Histopaque and blood should be 1/3 and 2/3, respectively).
2. Centrifuge (Eppendorf 5702 centrifuge) at $500 \times g$ for 30 min (20°C) until mononuclear cells form a distinct layer at the plasma-HISTOPAQUE® interface (the blood must remain on top, do not mix). Wash with phosphate-buffered saline (PBS), centrifuge, and resuspend. Repeat the procedure twice.
3. Resuspend in a final volume of 25 μL of the same buffer. Measure protein by the Lowry method (13), using a 5- μL aliquot. Consider 0.5 mg/mL for oxygen consumption assays.
4. Digitonin (1%) is added to lymphocytes just after introduction in the oxygen consumption chamber.

3.2. Isolation of Mitochondrial Fraction

Independently of cell mitochondrial origin, the first important procedure to achieve good and feasible results on oxygen respiration is the isolation of tightly coupled mitochondrial fractions. Several factors have to be taken into account, including the composition and temperature of all the solutions used.

3.3. Mitochondrial Isolation from Brain, Heart, and Liver (from Wistar Rats)

3.3.1. Brain Mitochondria
(Adapted from ref. 14)

1. After being anesthetized using different possible processes, rats should be sacrificed accordingly with the ethical proceedings established by the “*Guide for the Care and Use of Laboratory Animals*”, published by the US National Institutes of Health (NIH Publication No. 85–23, revised in 1996).
2. Decapitate rat (reserve body for other organ extraction) and remove rapidly the cerebellum. Wash and mince it (using sharp scissors) and later homogenize (in a small glass Potter-Elvehjem) at 4°C in 10 mL of brain isolation buffer (BIB) containing 5 mg of bacterial protease.
3. Brain homogenate is brought to 30 mL, and centrifuge $2,000 \times g$ for 3 min (Sorvall RC6 plus centrifuge; SS-34 rotor).
4. Resuspend pellet, including the fluffy synaptosomal layer, in 10 mL BIB containing 0.02% digitonin and centrifuge at $12,000 \times g$ for 8 min.
5. Resuspend brown mitochondrial pellet without the synaptosomal layer in 10 mL BRB and centrifuge at $12,000 \times g$ for 10 min.
6. Resuspend mitochondrial pellet in 300 μL BRB. Store mitochondria on ice until assays start.

7. Determine mitochondrial protein by biuret method (20) calibrated with bovine serum albumin (BSA). Consider 0.8 mg/mL for oxygen consumption assays, although other protein concentrations can be used.

3.3.2. Heart Mitochondria

(Adapted from ref. 15)

1. Open rat chest with scissors and remove the heart.
2. Place heart in isolation buffer (HIB) and cut slowly the organ in little pieces with cold scissors.
3. Wash heart pieces as many times as needed with HIB to remove all blood.
4. Homogenize heart pieces in a Potter-Elvehjem with 20 mL HIB complemented with 35 μ L protease (subtilisin fraction VIII or Nagarse). Be careful to maintain temperature under 4°C.
5. The homogenization takes place with 3 or 4 homogenizations with a refrigerated piston (preferably). The suspension is then left under incubation during 1 min on ice. After that period of time, homogenize 2–3 more times.
6. Place heart homogenate in centrifuge tubes and fill up with HIB.
7. The tubes are centrifuged at 12,000 $\times g$ during 10 min (Sorvall RC6 plus centrifuge; SS-34 rotor).
8. The supernatant is discharged and the pellet is freed after addition of HIB. Transfer to a smaller glass manual Potter-Elvehjem homogenizer, and promote gentle homogenization.
9. Centrifuge at 2,000 $\times g$ during 10 min. Pour supernatant to new refrigerated centrifuge tubes, and fill up with HIB.
10. Centrifuge supernatant at 12,000 $\times g$ during 10 min.
11. Discharge the new supernatant, and gently homogenise pellet with a smooth paintbrush, adding heart resuspension buffer (HRB). Place homogenate in centrifuge tubes and fill up with HRB.
12. Centrifuge at 12,000 $\times g$ during 10 min. Discharge supernatant and resuspend isolated mitochondria in 200–300 μ L in HRB. Store mitochondria on ice until start oxygen respiration assays.
13. Determine mitochondrial protein by biuret method (20) calibrated with BSA. Consider 0.5 mg/mL for oxygen consumption assays, although different mitochondrial protein concentrations can be used, according to the respiratory activity of the preparation.

3.3.3. Liver Mitochondria

(Adapted from ref. 4)

1. Open rat abdominal cavity with a scissor and remove liver.
2. Place liver in isolation buffer (LIB) and cut slowly in little pieces with a scissor.
3. Wash liver pieces as many times as needed with LIB to remove all blood.

4. Homogenize liver pieces in a Potter-Elvehjem with 60 mL LIB. Be careful to maintain temperature under 4°C.
5. Place liver homogenate in centrifuge tubes and fill up with LIB.
6. The tubes are centrifuged at $2,000 \times g$ during 10 min (Sorvall RC6 plus centrifuge; SS-34 rotor). Pour supernatant to new refrigerated centrifuge tubes, and fill up with LIB. Avoid contamination with debris from pellet.
7. Centrifuge supernatant at $12,000 \times g$ during 10 min.
8. Discharge the new supernatant, and gently homogenize pellet with a smooth paintbrush, adding liver resuspension buffer (LRB). Place homogenate in centrifuge tubes and fill up with HRB.
9. Repeat steps 7 and 8.
10. Centrifuge at $12,000 \times g$ during 10 min. Discharge supernatant and resuspend isolated mitochondria in 200–300 μ L in LRB. Store mitochondria on ice until start oxygen respiration assays.
11. Determine mitochondrial protein by biuret method (20) calibrated with BSA. Consider 1.5 mg/mL for oxygen consumption assays, although alternatives in mitochondrial protein content can be considered.

3.4. Oxygen Electrode Preparation and Maintenance

The Clark type electrode is, as it is expected from the name, an electrode that measures oxygen on a catalytic platinum surface. The classical Clark type is basically composed of two electrodes (cathode and anode). The electrical signal arises from the current developed on the cathode. The voltage supply unit (associated with a galvanometer) feeds the system with electrons, and when oxygen diffuses through an oxygen permeable membrane (commonly Teflon-based) to the platinum electrode, the cathode is reduced. The current is proportional to the oxygen tension in the solution (16) (see Note 2) where mitochondria or cells are placed. The silver ions combine with chloride ions in solution, precipitating silver chloride over the silver electrode, accordingly with the overall equation $4\text{Ag(s)} + 4\text{Cl}^- + \text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 4\text{AgCl} \cdot 2\text{H}_2\text{O}$ (17, 18). The electric signal can be analogically recorded using a flatbed recorder, or the electrode output current can pass through an analog-to-digital converter and later be analysed through a signal transducer coupled to a personnel computer (as exemplified in ref. 19) (Fig. 2).

3.5. Respiratory Parameters

Two important criteria used to assess the integrity or quality of a determined mitochondrial fraction after isolation or after incubation with test compounds are the respiratory control ratio (RCR) and the ADP/O.

The RCR is a measure of the coupling between substrate oxidation and phosphorylation and basically informs the researcher

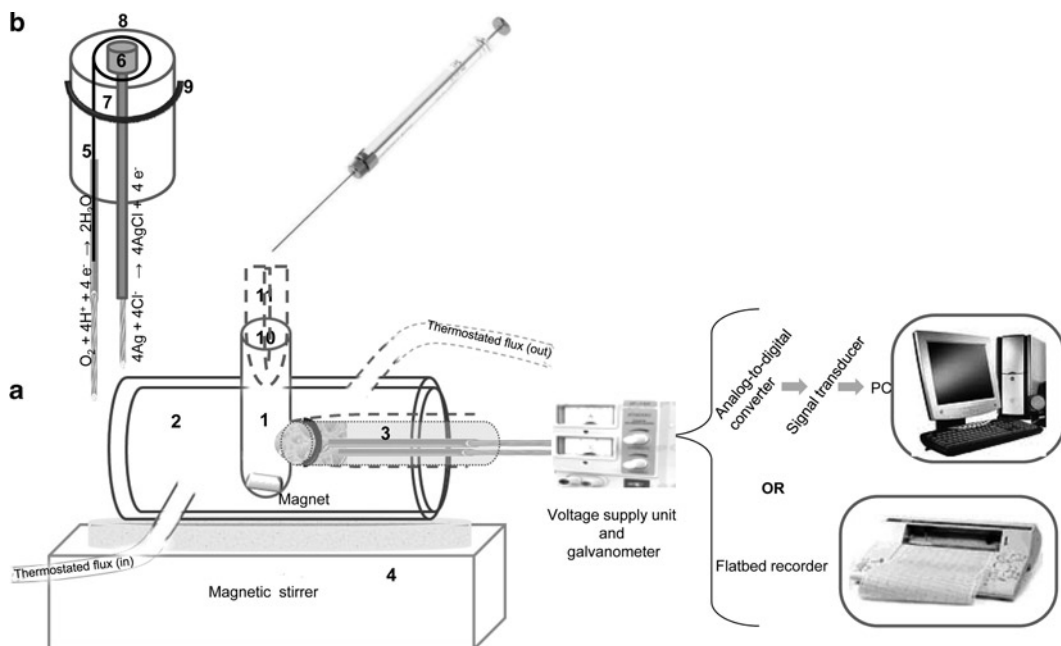


Fig. 2. General representation of the Clark-type electrode and experimental setup. (a) The biological preparations (mitochondrial or cell suspensions) are introduced in a well-defined volume of media (depending of the preparations), inside a thermostated incubation chamber (1) (with water jacket, 2) with an oxygen electrode inserted (3) and magnetic stirrer coupled (4). The electrode determines O_2 concentration in aqueous solutions over a period of time. (b) Oxygen electrode *inset* (from ref. 3) The electrode itself is located inside the chamber, in horizontal position (or depending the general apparatus design). The platinum cathode (5) is located surrounding a rod-like center anode (6) made by silver (reference Ag/AgCl electrode). These two electrodes are connected with each other by a thin layer of electrolyte (50% saturated KCl-solution, 7). Directly placed on top of the rod, there is an oxygen-permeable Teflon membrane (8), that is tight fitted by a rubber ring (9). The O_2 recordings can be done in open or closed chamber mode. In the last case, the reciprocal air-solution O_2 diffusion is avoided which allows for better determination of the respiratory rates. Experimental additions of solutions, substrates, mitochondria, and substrates/inhibitors are done through the top of the chamber (10) (open mode) or through the small hole (11) inside a stopper, respectively.

how intact or how coupled mitochondria are. The initial step to obtain RCR is to independently calculate state 3 and state 4 respiration rates. State 3 respiration is triggered in a suspension of isolated mitochondria by the addition of ADP. The increase in respiration denotes the use of the proton-motive force for the synthesis of ATP, being restored by the augmented proton pumping activity of the respiratory chain. When all ADP is phosphorylated into ATP by the action of the ATP-synthase, the respiration returns to or close to the initial pre-ADP addition values. The new respiratory state is now termed state 4. Some authors describe other respiratory states including state 2, which is the mitochondrial respiration in the presence of substrate only (pre-ADP addition), while others even establish a respiratory state 1, when mitochondria are consuming oxygen by oxidizing internal substrates only.

Regardless of the respiratory state being measured, units are usually expressed as $\text{nmol } O_2/\text{min}/\text{mg protein}$ or $\text{natom O}/\text{min}/\text{mg protein}$.

There are several important values to take into account when calculating the rates of oxygen consumption. By the units involved, it is clear that the exact time in which an x amount of oxygen is consumed is critical. Also, the amount of mitochondrial protein used in the experiment must be well known. There are several precise protein quantification methods including Bradford and Biuret methods, among others (20, 21). For cells, the oxygen consumption may be rationalized for the number of cells considered in oxygen reaction chamber, and data present in function of nmol O_2 /min/cells number (cell number may be quantified, depending from the biological samples) or when considering the total protein, we can use the method described by Lowry et al. (13).

Some authors may actually normalize oxygen consumption rates to the activity of citrate synthase, to normalize for mitochondrial content (22, 23) (see Note 3). If oxygen consumption is being measured in a flatbed recorder, one important parameter that should be taken into account is the scale of the paper, which will determine which distance in the paper will correspond to 100% oxygen saturation in water or in saline (several tables can be consulted for the values at the desired temperature (see Notes 4 and 5)). Also, paper recording velocity, which is adjustable, must be recorded even before the assays start. In this regard, it is advised to reach a compromise between a slow paper velocity, which would allow for a better measurement of the rates or a faster paper velocity, which turns the measurement of the faster rates harder but allows to spare the researcher the waste of long stretches of paper. After measuring state 3 and state 4 respiration rates, the RCR is easily calculated by performing the rate between state 3 and state 4. The final value should not present any units. There is a wide variety of tables showing values that are consistent with a good mitochondrial preparation (for example, see ref. 4). As an example, we routinely measure the RCR of isolated mitochondria in our laboratory just after the isolation or when investigating the effects of different xenobiotics. We usually have RCRs in the order of 6–8 for complex I substrates and 2–4 for complex II substrates (we have slightly lower values for heart vs. liver, most likely because of higher intrinsic ATPase activity in our heart mitochondrial fractions, which lead to a higher state 4 respiration). When using ascorbate plus N,N,N',N' -tetramethyl-*p*-phenylenediamine (TMPD) to directly feed complex IV via cytochrome c , the RCR value is around 1.5–2, which occurs due to the very fast respiration during state 4 respiration, since only one proton pump is working (complex IV or cytochrome c oxidase).

Damaged mitochondria usually have low RCRs, being 1, the theoretical minimum value. One test compound will decrease the RCR as it leads to increased state 4 respiration, decreased state 3 respiration or both.

The ADP/O has a completely different meaning. This parameter measures the efficiency of the mitochondrial phosphorylative system; in other words, the ADP/O index, which has no units, will give us a measure of how much oxygen the respiratory chain reduces to water per ADP phosphorylated. The index is calculated as the number of nmol of ADP added to the system divided by the number of nmols oxygen consumed during state 3 respiration. And this last point is critical, since a common mistake is to calculate the ADP/O as the amount of ADP added in nmol divided by the respiration rate during state 3. After measuring the absolute oxygen consumption and converting it into nmols, the calculation is now easy to do. One common strategy to calculate the amount of oxygen during state 3 respiration is to draw lines (as shown in Fig. 3) on top of the respiration curves during states 2, 3, and 4 and use the intersection points between state 2/3 and state 3/4 as a measure to calculate the absolute distance in the paper, parallel to the axis corresponding to oxygen concentration in the media.

There is a large controversy on the theoretical values for the ADP/O value (24), since the ADP/O is dependent on the coupling ratios of proton transport. New discoveries on the structure and activity of the ATP-synthase justify a new assessment of the established values, also because several other mechanisms account for the different ADP/O values found in the literature (24, 25). Under our conditions, we routinely have values around 2.4–3.1 for

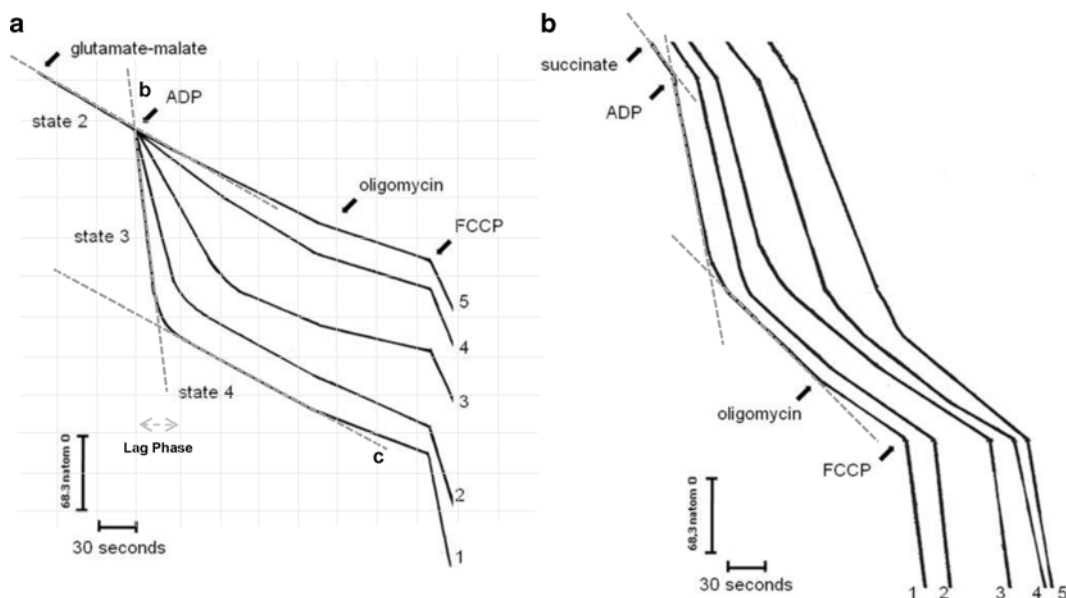


Fig. 3. Representative oxygen consumption recording (in a flatbed recorder) from mitochondrial respiration assays, using Clark-type electrode. Dashed lines (a, b, and c) were drawn (for plot 1, as an example) for ADP/O and RCR calculations (see text body). (a) Respiratory plots in the presence of glutamate/malate; (b) respiratory plots in the presence of succinate.

complex I substrates and 1.4–1.9 for complex II substrates. Also common during bench work is the appearance of ADP/O values much higher or lower than expected. The researcher must be aware that an incorrectly measured ADP stock solution will lead to an incorrect determination of ADP/O values (see Note 6). Finally, one common source of error is when the oxygen electrode used is not responding fast enough to the alterations in the media oxygen content. When this happens, the transition between each state is slow and does not have an abrupt profile, which will lead to the underestimation of the ADP/O value. A good and fast-responding oxygen electrode is thus essential for correct estimate of the index (see Note 7). Also, membranes need to have good O₂ permeability properties (see Note 8).

In oxygen consumption experiments, we can also consider other mitochondrial respiration states (7), such as “state FCCP” (uncoupled respiration, maximal activity of the respiratory chain) or “state oligomycin”, which is usually used to determine oxygen consumption with inhibited ATP synthase.

3.6. Oxygen Consumption Assays: Permeabilized Animal Cells

Oxygen consumption assessment implies the choice of the best-suited reaction chamber, accordingly with the biological preparation volumes.

For all types of presented cells we need to consider:

1. Check if temperature in reaction/O₂ consumption chamber is 37°C.
2. Calibrate O₂ scale using distilled water.
3. Introduce the desired volume of respiration buffer, and just place biological preparations after reaching a steady baseline on the chart.
4. In electroporabilized, digitonin-permeabilized or saponin-permeabilized cells, a schematic sequence of additions (using for example microsyringes) such as 10 mM pyruvate + 5 mM malate, 10 mM glutamate + 5 mM, 2 mM ascorbate + TMPD and 2.5 μM FCCP (see Note 1) and the addition of ADP (125–250 nM) after a substrate can be used to check OXPHOS function.

3.7. Oxygen Consumption Assays: Isolated Mitochondria

1. Define appropriate temperature, usually between 25 and 30°C.
2. Calibrate appropriate O₂ scale using distilled water (for example, a 1.5× scale can be appropriate in most cases to get a better signal resolution for isolated mitochondria).
3. Introduce the desired volume of respiration buffer, and just place biological preparations after reaching a steady baseline on the chart.
4. Add mitochondrial suspension with the desired protein concentration.

Sequential additions of mitochondrial substrates and inhibitors can allow characterizing different segments of the respiratory chain in one experiment only.

We can use a schematic sequence of additions such as 10 mM pyruvate + 5 mM malate followed by ADP (125–250 nmol) and later rotenone (2 μ M), followed by 5 mM succinate, ADP (same as before), with antimycin (2 μ M) later on. If still far from total chamber oxygen consumption, a subsequent addition can be performed with 2 mM ascorbate + TMPD, plus ADP. Another possibility is the addition of 1 μ M FCCP (see Note 1, Table 1) to uncouple respiration. The number of additions depends on the oxygen availability in the chamber and enough time should be elapsed between additions to warranty correct measurement of oxygen consumption rates.

Table 1
Most-used substrates and inhibitors during oxygen consumption assays

Name	Classification	Place of action
Antimycin A	Inhibitor	Complex III
Ascorbate	Substrate	Complex IV (by donating electrons to cytochrome <i>c</i>)
Atractyloside	Inhibitor	Adenine nucleotide translocator (no ADP/ATP exchange occurs)
FCCP	Protonophore and uncoupler of mitochondrial OXPHOS	Mitochondrial membrane (although proton shuttling activity can be facilitated by some mitochondrial proteins)
Fumarate	Substrate	Intermediate in Krebs cycle
Glutamate	Substrate	Complex I (converted into NADH in the matrix)
KCN	Inhibitor	Complex IV
Malate	Substrate	Complex I (converted into NADH in the matrix)
Malonate	Inhibitor	Inhibition of succinate oxidation
Myxothiazol	Inhibitor	Complex III
Oligomycin	Inhibitor	Complex V
Pyruvate	Substrate	Feeds Krebs cycle (via Acetyl-CoA)
Rotenone	Inhibitor	Complex I
Succinate	Substrate	Complex II
TMPD	Reducing co-substrate	By-passing electrons to Complex IV (via cytochrome <i>c</i>), as an artificial electron mediator

FCCP carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, *KCN* potassium cyanide, *TMPD* *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

4. Notes

1. During oxygen consumption, assays to follow mitochondrial or cell respiration must be considered particular substrates and/or inhibitors for individual respiratory complexes assessment (see Table 1).
2. Electric circuitry that controls oxygen electrode. One tension division circuit (inside galvanometer) generates an electrode potential difference of 0.71 V (between anode and cathode) which is proportional to the activity of water-dissolved O_2 solution (16).
3. By measuring the latent citrate synthase activity (calculating the difference between total citrate synthase activity and free citrate synthase activity) or citrate synthase ratio (latent citrate synthase activity/free citrate synthase activity), it is possible to infer not only the structural integrity of the mitochondrial preparation but also to correlate oxygen consumption with the quantity of the functional mitochondria or cells with functional mitochondria, instead of the total protein. Rationing oxygen consumption by citrate synthase content is time-consuming and thus rarely used.
4. Oxygen solubility differs according to the type of media we use. We can find many tabled values for distilled water and different saline buffers (26, 27) but our experience tells us that a good approach is to use standard oxygen-saturated deionised water for calibration.
5. The most correct step to calibrate the O_2 scale is by comparing the signal of air-saturated water with the zero level created by adding dithionite to the water in the reaction chamber (this starts a reaction that quickly consumes the dissolved oxygen) or flushing the cell with nitrogen (17). But when comparing oxygen consumption from specific conditions to a control, knowing or not, the precise values of dissolved O_2 will not have a meaningful impact on data analysis.
6. Spectrophotometric determination of ADP stock solutions should always be conducted before start experiments with a new prepared batch. ADP concentration can be determined by measuring the absorbance of the batch at 259 nm (molar extinction coefficient = $15.4 \times 10^3 / \text{M cm}$) (28).
7. To maintain the electrode in good conditions, it should be cleaned after use and before prolonged storage. Never allow the electrode to dry without the electrolyte in place, as crystallization of the electrolyte may occur and cause the platinum/epoxy resin seal to be breached and allow the deposition of electrolyte salt crystals around the cathode, leading to necessary

electrode replacement. The silver electrode (anode) must be cleaned to remove the excessive deposits of black oxides, which could cause deterioration. For that, we use a small cotton bud moistened with distilled water. Some commercial products can be used with the purpose of cleaning silver electrodes, but avoid any harsh abrasive substance.

Platinum cathode cleaning involves the usage of a non-corrosive polishing agent (as a soft white tooth paste, with small silica spheres in the composition) on top of a cotton bud moistened with few drops of distilled water. Circular movements in the polish platinum electrode surface can be performed to obtain a final shinny effect. Make sure that all electrical connections are preserved and completely dry (29).

8. Several different membrane materials can be used for the oxygen electrode including Teflon, polyethylene, and silicon rubber, among others. Thin hydrophilic polymers are usually not mechanically strong which may cause problems. It is possible to obtain some degree of selectivity by choosing the material of this membrane according to the conditions of application. The diffusion through such a structure is more complicated. For radial geometry, the steady-state current, I , is given by the mathematical expression:

$$I = \frac{4\pi F_0 D_s S_s P(r_1)}{D_s(r_1 - r_0) / D_m r_1 + S_m r_0 r_1}$$

D corresponds to the diffusion coefficient (in solution and in the membrane), r_0 is the outer radius of the membrane, and r_1 is the radius of the electrode. S represents oxygen solubility in solution and in the membrane (S_s and S_m , respectively). Another important parameter is the oxygen partial pressure on the membrane pressure, $P(r_1)$ (6).

Literature reports a wide range of membranes for Clark-type oxygen sensors. Jobst et al. (30) considered a membrane based on a thin-layer polymer, to prepare a planar oxygen sensor with a three-electrode configuration (platinum working and counter electrodes and Ag/AgCl reference electrode) that allows the regeneration of oxygen consume in the cathode. The electrolyte is based in a hydrogel layer, preventing the typical buffer degradation and self oxygen consuming behavior. But for mitochondrial preparations, the system is not the best suited to be coupled to the common oxygen recording chamber, and membrane preparation requires a complex experimental setup.

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References

1. Du G, Mouithys-Mickalad A, Sluse FE (1998) Generation of superoxide anion by mitochondria and impairment of their functions during anoxia and reoxygenation *in vitro*. *Free Rad Biol Med* 25(9):1066–1074
2. Stryer L (1999) *Biochemistry*, 4th edn. W. H. Freeman, New York
3. Severinghaus JW, And Astrup PB (1986) History of blood gas analysis. IV. Leland Clark's oxygen electrode. *J Clin Monit* 2(2):125–139
4. Pereira GC, Branco AF, Matos JA, Pereira SL, Parke D, Perkins EL, Serafim TL, Sardão VA, Santos MS, Moreno AJ, Holy J, Oliveira PJ (2007) Mitochondrially targeted effects of Berberine [natural yellow 18,5,6-dihydro-9,10-dimethoxybenzo(g)-1,3-benzodioxolo(5,6-a)quinolizinium] on K1735-M2 mouse melanoma cells: comparison with direct effects on isolated mitochondrial fractions. *J Pharmacol Exp Ther* 323(2):636–649
5. Estabrook RW (1967) Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods Enzymol* 10:41–47
6. Janata J (2009) *Principles of chemical sensors*, 2nd edn. Springer, New York
7. Respiratory states and coupling control ratios. In: *Mitochondrial pathways and respiratory control*, 2nd edn. Oroboros MiPnet Publications, Innsbruck
8. Wenchich L, Drahota Z, Honzík T, Hansíková, Tesařová M, Zeman J, Houštěk J (2003) Polarographic evaluation of mitochondrial enzymes activity in isolated mitochondria and in permeabilized human muscle cells with inherited mitochondrial defects. *Physiol Res* 52:781–788
9. Grinstein S, Furuya W, Lu DJ, Mills GB (1990) Vanadate stimulates oxygen consumption and tyrosine phosphorylation in electroporabilized human neutrophils. *J Biol Chem* 265:318–327
10. Conget I, Barrientos A, Manzanares JM, Casademont J, Viñas O, Barceló J, Nunes V, Gomis R, Cardellach F (1997) Respiratory chain activity and mitochondrial DNA content of nonpurified and purified pancreatic islet cells. *Metabolism* 46(9):984–987
11. Artuch R, Colomé C, Playán A, Alcaine MJ, Briones P, Montoya J, Vilaseca MA, Pineda M (2000) Oxygen consumption measurement in lymphocytes for the diagnosis of pediatric patients with oxidative phosphorylation diseases. *Clin Biochem* 33(6):481–485
12. Rolo AP, Palmeira CM, Cortopassi GA (2009) Biosensor plates detect mitochondrial physiological regulators and mutations *in vivo*. *Anal Biochem* 385(1):176–178
13. Lowry OH, Rosenbrough NJ, Far AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193(1):265–275
14. Moreira PI, Santos MS, Moreno AM, Seica R, Oliveira CR (2003) Increased vulnerability of brain mitochondria in diabetic (Goto-Kakizaki) rats with aging and amyloid- β exposure. *Diabetes* 52:1440–1456
15. Alves MJ, Oliveira PJ, Carvalho RA (2009) Mitochondrial preservation in celsior versus histidine buffer solution during cardiac ischemia and reperfusion. *Cardiovasc Toxicol* 9(4):185–193
16. Moreno AJM (1992) Estudo do efeito de Insecticidas de usos comum na bioenergética mitocondrial. Universidade de Coimbra, Tese de Doutoramento
17. Renger G, Hanssum B (2009) Oxygen detection in biological systems. *Photosynth Res* 102:487–498
18. Diepart C, Verrax J, Calderon PB, Feron O, Jordan BF (2010) Comparison of methods for measuring oxygen consumption in tumor cells *in vitro*. *Anal Biochem* 396:250–256
19. Kopustinskas A, Adaskevicius R, Krusinskas A, Kopustinskiene DM, Liobikas J, Toleikis A (2006) A user-friendly PC-based data acquisition and analysis system for respirometric investigations. *Comp Method Prog Biomed* 82:231–237
20. Gornall AG, Bardawill CS, David MM (1949) Determination of serum proteins by means of the biuret reaction. *J Biol Chem* 177:751–766

21. Bradford MM (1976) A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254
22. Bugger H, Chemnitz J, Doenst T (2006) Differential changes in respiratory capacity and ischemia tolerance of isolated mitochondria from atrophied and hypertrophied hearts. *Metab Clin Exp* 55:1097–1106
23. Figueiredo PA, Ferreira RM, Appell HJ, Duarte JA (2008) Age-induced morphological, biochemical, and functional alterations in isolated mitochondria from murine skeletal muscle. *J Gerontol A Biol Sci Med Sci* 63: 350–359
24. Hinkle PC (2005) P/O ratios of mitochondrial oxidative phosphorylation. *Biochim Biophys Acta* 1706(1–2):1–11
25. Lee CP, Gu Q, Xiong Y, Mitchell RA, Ernster L (1996) P/O ratios reassessed: mitochondrial P/O ratios consistently exceed 1.5 with succinate and 2.5 with NAD-linked substrates. *FASEB J* 10(2):345–350
26. Atkins PW (1998) *Physical chemistry*, 6th edn. Oxford University Press, New York
27. Rasmussen HN, Rasmussen UF (2003) Oxygen solubilities of media used in electrochemical respiration measurements. *Anal Biochem* 319:105–113
28. Young M (1967) On the interaction of adenosine diphosphate with myosin and its enzymatically active fragments. *J Biol Chem* 242(11):2790
29. Hansatech Instruments (2006) *Operations manual – setup, installation and maintenance. Electrode preparation and maintenance. Version 2.2.* Hansatech Instruments Ltd, Norfolk
30. Jobst G, Urban G, Jachimowicz A, Kohl F, Tilado O, Lettenbichler I, Nauer G (1993) Thin-film Clark-type oxygen sensor based on novel polymer membrane systems for *in vivo* and biosensor applications. *Biosens Bioelectron* 8(3–4):123–128

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