Video Article

High-resolution Respirometry to Assess Mitochondrial Function in Permeabilized and Intact Cells

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Abstract

A high-resolution oxygraph is a device for measuring cellular oxygen consumption in a closed-chamber system with very high resolution and sensitivity in biological samples (intact and permeabilized cells, tissues or isolated mitochondria). The high-resolution oxygraph device is equipped with two chambers and uses polarographic oxygen sensors to measure oxygen concentration and calculate oxygen consumption within each chamber. Oxygen consumption rates are calculated using software and expressed as picomoles per second per number of cells. Each high-resolution oxygraph chamber contains a stopper with injection ports, which makes it ideal for substrate-uncoupler-inhibitor titrations or detergent titration protocols for determining effective and optimum concentrations for plasma membrane permeabilization. The technique can be applied to measure respiration in a wide range of cell types and also provides information on mitochondrial quality and integrity, and maximal mitochondrial respiratory electron transport system capacity.

Video Link

The video component of this article can be found at https://www.jove.com/video/54985/

Introduction

Mitochondria fulfill important roles in cellular energy metabolism, especially by using oxygen to produce adenosine triphosphate (ATP). They are implicated in cell death and in several human diseases. Mitochondrial oxidative phosphorylation (OXPHOS) combines electron transport along the electron transport chain with oxygen consumption and ATP synthesis. The mitochondrial tricarboxylic acid (TCA) cycle is involved in the conversion of proteins, carbohydrates and fats into energy rich compounds as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). Electrons of the NADH and $FADH_2$ are then transferred to the respiratory electron transport chain protein complexes (I to IV) located in the inner mitochondrial membrane. In addition, two other redox pathways can transfer electrons to electron transport chain: i) mitochondrial electron-transferring flavoprotein (ETF) which is located on the matrix face of the inner mitochondrial membrane, and supplies electrons from fatty acid β-oxidation; and ii) mitochondrial glycerophosphate dehydrogenase which oxidizes glycerophosphate to dihydroxyacetone phosphate and feeds electrons to the mitochondrial electron transport chain. Complex IV (the ultimate electron acceptor) transfers the electrons to one oxygen molecule, converting oxygen to two molecules of water. Moving of the electrons from respiratory electron transport chain complex I to IV is coupled with proton flow from the mitochondrial matrix to the intermembrane space which establishes an electrochemical gradient across the mitochondrial inner membrane. Afterwards, mitochondrial complex V (ATP synthase) shuttles the hydrogen ions back into the mitochondrial matrix and synthesizes ATP molecules. OXPHOS function can be assessed *in vivo* and in *vitro* using various techniques and various mitochondrial respiration states can be obtained. In isolated mitochondria the following respiratory states can be measured: i) basal mitochondrial respiration (state 1), ii) oxygen consumption after the addition of specific substrates of the mitochondrial respiratory chain complexes (state 2), iii) maximal mitochondrial oxygen consumption after the addition of saturating concentrations of adenosine diphosphate (ADP) (state 3) and, iv) resting respiration after ADP consumption (converted to ATP) (state 4). In intact cells the following respiratory states can be measured: i) basal cellular oxygen consumption in the presence of endogenous substrates and ADP, ii) basal cellular oxygen consumption in the presence of oligomycin (oligomycin-insensitive respiration) and oligomycin-sensitive respiration (ATP turnover), iii) FCCP uncoupled respiration, and iv) non-mitochondrial respiration after the addition of antimycin A and rotenone. In permeabilized cells, specific substrates of the electron transport chain complexes and ADP can be added and maximal complex-dependent respiratory rates such as complex I-, II- and IV-dependent respiratory rates can be measured.

Measurements of cellular respiration provide important insights into mitochondrial respiratory capacity specific to complexes I-IV, mitochondrial integrity and energy metabolism[1,2,3]. One of the devices which enable measurements of mitochondrial oxygen consumption with high accuracy, resolution and sensitivity is the high-resolution oxygraph[4]. The high-resolution oxygraph device contains two chambers with injection ports and each chamber is equipped with a polarographic oxygen sensor. Cellular or isolated mitochondrial suspensions are stirred continuously in the respirometer. To assess mitochondrial function, substrates and inhibitors for mitochondrial complex activity can be added following standard protocols. Substrates and inhibitors can be titrated by injection into the chambers of the oxygraph, and oxygen consumption rates are calculated using software and expressed as picomoles per second per number of cells. High-resolution respirometry offers several advantages over

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traditional and conventional polarographic oxygen electrode devices including increased sensitivity and the ability to work with small numbers of biological samples such as intact or permeabilized cells. In addition, each device contains two chambers, and respiratory rates can be recorded simultaneously for comparisons of oxygen concentrations. The high-resolution oxygraph also has the advantage of reduced leakage of oxygen from the device chambers compared to traditional polarographic oxygen electrode devices. Another device recently developed to measure cellular oxygen consumption is the 96-well extracellular flux analyzer[5]. The extracellular flux analyzer is equipped with fluorescence instead of polarographic sensors. The advantages of the extracellular flux analyzer compared to the high-resolution oxygraph are i) it is a largely automated device, ii) it is possible to measure oxygen consumption in 24- and 96-well plates for high-throughput screening, therefore requiring lower amounts of biological samples, and iii) additional measurement of cellular glycolytic flux is possible. The disadvantages of the extracellular flux analyzer in comparison to the high-resolution oxygraph are i) the high costs of the device and of consumables such as fluorescent plates, which are non-reusable, and ii) only four compounds per assay/well are injectable, therefore the system is not feasible for substrate-uncoupler-inhibitor titration protocols.

In the present study, we use high-resolution respirometry to determine mitochondrial respiration. For cellular oxygen consumption experiments, the cells are permeabilized to allow the entry of exogenous ADP and oxidizable mitochondrial substrates for feeding electrons into complexes of the respiratory system. This approach allows the dissection of individual mitochondrial complexes respiratory capacities, which is a distinct advantage compared to intact cells (many substrates are cell-impermeant). However, cell membrane permeabilization will disrupt the barrier between the cytosol and extracellular space and medium (wash out of cytosolic solutes) and the composition of the intracellular space is equilibrated with the extracellular medium. One of the disadvantages of permeabilized cells over intact cells is that the mitochondrial outer membrane can be damaged if excessive amounts of detergent are employed during cell permeabilization. In intact cells, basal, coupled and uncoupled respiration of intact cells can be measured. This method evaluates oxygen consumption of intact cells without the addition of exogenous substrates and ADP, reproducing the respiratory function in the integrated cell and also providing information on maximal mitochondrial electron transport capacity[6,7]. One of the advantages of intact cells over permeabilized cells is that cellular environment is not disrupted and mitochondria are in contact with the whole components of the cells. In order to permeabilize the cellular plasma membrane, detergents such as digitonin have been used[8]. However, mitochondrial outer membrane integrity can be compromised if excessive amounts of digitonin are employed. To confirm that mitochondrial outer membrane integrity is not compromised in permeabilized cells, digitonin titration is performed to determine the optimal concentration for cellular permeabilization. For these experiments, cells are resuspended in respiration medium and digitonin concentration is titrated by respirometry in the presence of mitochondrial substrates and ADP, and respiration rates are measured. Respiration of intact, non-permeabilized cells is not stimulated in the presence of mitochondrial substrates and ADP. However, subsequent stepwise digitonin titration would yield gradual permeabilization of plasma membranes, and optimal digitonin concentration is obtained. This is shown by the increase of respiration up to full permeabilization. Mitochondrial quality and outer membrane integrity can be verified by adding exogenous cytochrome c[2,9]. Cytochrome c is a 12 kDa electron carrying protein of the mitochondrial electron transport chain [10,11,12]. It is localized in the mitochondrial intermembrane space, and is involved in oxygen consumption, carrying electrons from complex III to complex IV. Once the mitochondrial outer membrane is damaged, cytochrome c is released, and mitochondrial oxygen consumption is reduced. Upon addition of exogenous cytochrome c, any augmentation in mitochondrial respiration is indicative of a disrupted mitochondrial outer membrane.

In permeabilized cells, substrates and inhibitors of mitochondrial complex activity are added following various protocols[3,9]. For example in order to investigate mitochondrial complex-driven respiratory rates, the following protocol can be used. After permeabilization of the cells, first complex I is stimulated by the substrates malate and glutamate, which generate NADH as a substrate to the respiratory chain and provoke the activation of complex I. Afterwards, ADP is added for conversion to ATP (state 3, active complex I-dependent respiration). After a stable signal is reached, rotenone (mitochondrial complex I inhibitor) is administered to inhibit complex I. Rotenone is followed by succinate to $FADH_2$ and to activate complex II (state 3, active complex II-dependent respiration). In order to measure complex IV-dependent respiration, first complex III-dependent respiration is inhibited by adding antimycin A (mitochondrial complex III inhibitor). Afterwards, complex IV-dependent respiration is stimulated by administering ascorbate and tetramethylphenylendiamine (TMPD). TMPD can auto-oxidize in the respiration buffer, therefore the maximal complex IV-dependent respiration rate (State 3) is calculated by subtracting respiration rates before and after the addition of sodium azide, an inhibitor of mitochondrial complex IV. The respiration experiments can be carried out in two chambers of an oxygraph in parallel-one serving as a control (unstimulated cells), the other containing the stimulated cells. Obviously, the cells can be pre-treated in various ways, *e.g.*, with drugs affecting mitochondrial functions, before their oxygen consumption is measured in the oxygraph chamber. This protocol allows functional examination of the individual mitochondrial respiratory chain complexes. In addition, one can measure maximal ADP-stimulated respiration (state 3) of permeabilized cells, using exogenous fatty acid in the form of palmitate. In this protocol, concentrated stocks of sodium palmitate are conjugated with ultra fatty acid free bovine serum albumin (BSA) (6:1 molar ratio palmitate:BSA). Afterwards, cells first are permeabilized with digitonin and mitochondrial respiration is assessed by the addition of carnitine and palmitate followed by addition of ADP (state 3, maximal respiration). Then, oligomycin is added to mimic state 4 (state 4o) and respiratory control ratio (RCR value) is calculated as state 3/state 4o. β-oxidation promotes production of acetyl CoA (which enters in the TCA cycle) and generation of $FADH_2$ and NADH, the electrons of which are passed to the electron transport chain by electron-transferring flavoprotein and β-hydroxyacyl-CoA dehydrogenase. Mitochondria are at the center of fatty acid metabolism and described palmitate-BSA protocol can be used by researchers examining fatty acid oxidation. In intact cells, activators and inhibitors of mitochondrial complex activity are added following a different protocol[6,9]. For these experiments, first oxygen consumption of non-permeabilized cells in the absence of exogenous substrates is measured (phosphorylating respiration rate). Then, the non-phosphorylating respiration rate is measured after the addition of oligomycin, which is an inhibitor of mitochondrial ATP synthase. Afterwards, the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) is administrated at various concentrations and the maximal mitochondrial uncoupled respiratory rate is measured. Protonophores such as FCCP can induce an augmentation in proton permeability of the inner membrane, allowing passive movement of protons to dissipate the chemiosmotic gradient. An increase in proton permeability uncouples oxidative respiration (no ATP production) and induces an increase in oxygen consumption. Afterwards, rotenone and antimycin A are added to inhibit mitochondrial respiration, and non-mitochondrial respiration is subtracted from all other respiratory rates.

The oxygen consumption rates can be expressed as IO_2 [pmol x sec^{-1} x 10^{-6} cells] (oxygen flow per million cells) which is calculated by dividing volume-specific oxygen flux (in the closed oxygen chamber), JV,O_2 [pmol x sec^{-1} x ml^{-1}] by cell concentration in the cell chamber (number of cells per volume [10^6 cells·ml^1])[15]. Cell-mass specific oxygen flux, JO_2 [pmol x sec^{-1} x mg^{-1}], is flow per cell, IO_2 [pmol x sec^{-1} x 10^{-6} cells], divided by

mass per cell [mg·10^6 cells]; or volume-specific flux, JV, O_2 [pmol x sec^{-1} x ml^{-1}], divided by mass per volume [mg·ml^1]. JO_2 is the oxygen flux per cell protein, dry weight or cell volume.

In the present study using high-resolution respirometry, we describe protocols to determine i) optimum digitonin concentration for complete cellular plasma membrane permeabilization (digitonin titration assay), ii) mitochondrial outer membrane integrity using exogenous cytochrome c, iii) mitochondrial respiratory chain complexes I, II and IV maximal respiratory rates in digitonin-permeabilized HepG2 cells in the presence of exogenous ADP and mitochondrial respiratory chain substrates, and iv) basal, coupled and maximal uncoupled respiration (maximal electron transport capacity) of intact cells without the addition of exogenous substrates and ADP, reproducing the respiratory function in the integrated cell.

Protocol

1. Cell Culture

1. Culture human hepatoma HepG2 cells[6] in 25 cm^2 cell culture flasks in Dulbecco's Modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in an incubator (5% CO_2, 95% air) (seeding density: 1 x 10^6 cells per 25 cm^2 cell culture flask, incubation time in the 37 °C incubator: 48 hr, cell density at confluency: 4-5 x 10^6 cells per 25 cm^2 cell culture flask).
2. Perform the experiments when cells are 90% to 95% confluent.

2. High-resolution Respirometry Calibration of Polarographic Oxygen Sensors

1. Pipette 2.1 ml of respiration buffer into an oxygraph chamber and stir the buffer continuously using a magnetic stirring bar present in the chamber (700 rpm) at 37 °C for 1 hr until a stable oxygen flux signal of the polarographic oxygen sensor is obtained.
   NOTE: Polarographic oxygen electrodes within each oxygraph chamber measure oxygen concentration and calculate oxygen consumption (flux) within each chamber. The oxygen concentration and oxygen consumption rates (flux) are displayed real-time online in a computer using software for data acquisition and analysis.
2. Perform an air calibration of the polarographic oxygen sensor according to the manufacturer's protocols[14].
   NOTE: Calibration of polarographic oxygen sensors in respiration media and oxygen concentration in the media at air saturation experimental temperature is performed only once daily in the morning. Afterwards the media can be removed from the chambers and cells resuspended in a fresh respiration media are added to an oxygraph chamber and respiratory rates are measured. After the first experiment, the chamber can be washed and additional series of experiments can be performed in the same chamber without further calibrations.

3. Trypsinization of Adherent Cells, Counting Cells

1. On the day of the experiment, aspirate the DMEM from the 25 cm^2 cell culture flask.
2. Rinse the cell culture monolayer in the culture flask with 5 ml of phosphate buffered saline (PBS).
3. Pipette 0.5 ml of 25 mg/ml of trypsin solution (prewarmed to 37 °C in a 37 °C water bath) into the cell monolayer in the culture flask and incubate for 5 min at 37 °C in an incubator (5% CO_2, 95% air).
4. Pipette 5 ml of DMEM containing 10% FBS into the detached cells in the cell culture flask and suspend the cells by pipetting.
5. Transfer resuspended cells to a 15 ml centrifuge tube and centrifuge for 5 min at 350 x g at room temperature and decant the supernatant.
6. Resuspend the cell pellet in 1 ml of respiration buffer[13] (T**able 1**).
7. Count the cells using a cell counter and resuspend them in the respiration buffer to a final density of 1 x 10^6 cells/ml. Since cellular respiration rates will be normalized to cell number, count the cells with an accurate and precise cell counter.

4. High-resolution Respirometry

1. After air calibration (performed only once daily, steps 2.1-2.2), aspirate the respiration medium from a chamber of the oxygraph and add 2.1 ml of cell suspension (1 x 10^6 cells/ml) from step 3.7 to the chamber.
2. Close the oxygraph chamber by insertion of the stopper.
3. Stir the cells continuously using a magnetic stirring bar present in the chamber (700 rpm) at 37 °C and record cellular respiration for 5-10 min until a stable oxygen flux signal is achieved.
   NOTE: The oxygen concentration and oxygen flux signal are displayed real-time online in a computer using software for data acquisition and analysis[14].
4. Afterwards, inject substrates and inhibitors for mitochondrial respiration through the titanium injection ports of the stoppers using the following protocols.

5. Digitonin Titration in Intact Cells by Respirometry

1. Prepare an oxygraph chamber containing cell suspension (1 x 10^6/ml) following the procedure described in steps 4.1 to 4.3 of the protocol.
2. Inject 2 µl of 0.2 mM rotenone (0.2 µM) 'CAUTION' into the oxygraph chamber containing cell suspension through the titanium injection port of the chamber stopper using a syringe and record cellular respiration for 5-10 min until a stable oxygen flux signal is achieved.
   NOTE: All the injections in the following steps are performed through the titanium injection ports of stoppers using syringes. Addition of rotenone is optional (it prevents reverse electron flow) and can be omitted for digitonin titration experiments, see step 6.3.
3. Inject 20 µl of 1 M succinate (10 mM) into the oxygraph chamber and record cellular respiration for 5-10 min until a stable oxygen flux signal is achieved.

4. Inject 10 µl of 0.5 M ADP (2.5 mM) into the oxygraph chamber and record cellular respiration for 5-10 min until a stable oxygen flux signal is achieved.
5. Inject 2 µl of 2 mM digitonin (2 µM) into the oxygraph chamber and record cellular respiration for 2-5 min until a stable oxygen signal is achieved.
6. Again inject 2 µl of 2 mM digitonin into the oxygraph chamber and record cellular respiration for 2-5 min until a stable oxygen signal is achieved.
   NOTE: Stepwise addition of digitonin to the cells will induce an increase in cellular oxygen consumption and the oxygen flux signal will increase.
7. Continue injecting 2-4 µl of 2 mM digitonin stepwise (2-4 µM each step) into the chamber. After each step, record cellular respiration for 2-5 min until a stable oxygen flux signal is achieved.
   NOTE: Stop injecting digitonin when the oxygen flux signal reaches a maximal level and further injections of digitonin do not increase the respiration rate. The readers should determine optimal digitonin concentration in their laboratories using their own reagents and use obtained optimal digitonin concentration in the steps 6.2 and 7.2 of the following protocols for their experiments.

6. Evaluation of the Mitochondrial Outer Membrane Integrity: Cytochrome C

1. Prepare an oxygraph chamber containing cell suspension (1 x 10^6/ml) following the procedure described in steps 4.1 to 4.3 of the protocol.
2. Inject 2 µl of 8 mM digitonin (8 µM) into the oxygraph chamber containing the cell suspension (1 x 10^6/ml) and permeabilize the cells for 5 min.
3. Inject 20 µl of 1 M succinate (10 mM) into the oxygraph chamber and record cellular respiration for 5-10 min until a stable oxygen flux signal is achieved.
4. Inject 10 µl of 0.5 M ADP (2.5 mM) into the oxygraph chamber and record cellular respiration for 5-10 min until a stable oxygen flux signal is achieved.
   NOTE: Addition of ADP to the cells will stimulate complex I and induce an increase in oxygen consumption, and oxygen flux signal will increase and stabilize.
5. Inject 5 µl of 4 mM cytochrome c (10 µM) into the oxygraph chamber and record cellular respiration for 5-10 min until a stable oxygen flux signal is achieved.
6. Finally inject 1 µl of 4 mg/ml oligomycin (2 µg/ml) and record cellular respiration until a stable oxygen flux signal is achieved.

7. Maximal ADP-stimulated Respiration (State 3) of Permeabilized HepG2 Cells

1. Prepare an oxygraph chamber containing cell suspension (1 x 10^6/ml) following the procedure described in steps 4.1 to 4.3 of the protocol.
2. Inject 2 µl of 8 mM digitonin (8 µM) into the oxygraph chamber containing the cell suspension and permeabilize the cells for 5 min.
3. Inject 12.5 µl of 0.8 M of malate (5 mM) and 10 µl of 2 M of glutamate (10 mM) into the oxygraph chamber. Record cellular respiration until a stable oxygen flux signal is achieved.
4. Inject 10 µl of 0.5 M ADP (2.5 mM) into the oxygraph chamber and record cellular respiration until the oxygen flux signal increases and stabilizes.
   NOTE: Addition of ADP to the cells will induce an increase in oxygen consumption and the oxygen flux signal will increase.
5. Inject 2 µl of 0.2 mM rotenone (0.2 µM) 'CAUTION' into the oxygraph chamber and record cellular respiration until the oxygen flux signal decreases and stabilizes.
6. Afterwards, inject 20 µl of 1 M succinate (10 mM) into the oxygraph chamber and record cellular respiration until the oxygen flux signal increases and stabilizes.
7. Then, inject 2 µl of 5 mM antimycin A (5 µM) 'CAUTION' into the oxygraph chamber and record cellular respiration until the oxygen flux signal decreases and stabilizes.
8. Then inject 2.5 µl of 0.8 mM ascorbate (1 mM) and immediately after inject 2.5 µl of 0.2 mM TMPD (0.25 mM) into the oxygraph chamber and record cellular respiration until the oxygen flux signal increases and stabilizes.
9. Finally inject 10 µl of 1 M sodium azide (5 mM) 'CAUTION' into the oxygraph chamber and record cellular respiration until the oxygen flux signal decreases and stabilizes.

8. Oxygen Consumption of Intact Cells

1. Prepare an oxygraph chamber containing cell suspension (1 x 10^6/ml) following the procedure described in steps 4.1 to 4.3 of the protocol.
2. Inject 1 µl of 4 mg/ml oligomycin (2 µg/ml) into the oxygraph chamber containing cell suspension and record cellular respiration until a stable oxygen flux signal is achieved.
3. Afterwards inject 1 µl of 0.2 mM of FCCP (0.1 µM) 'CAUTION' into the oxygraph chamber and record cellular respiration until the oxygen flux signal increases and stabilizes.
4. Inject 3 µl of 0.2 mM of FCCP (0.4 µM) into the oxygraph chamber and record cellular respiration until the oxygen flux signal increases further and stabilizes.
5. Titrate FCCP in 0.1 to 0.3 µM steps by injecting 1-3 µl of 0.2 to 1 mM FCCP (0.1 to 2 µM final concentration in the chamber) into the oxygraph chamber until the oxygen flux signal reaches its maximal levels and no further increases and then starts declining.
   NOTE: Stop injecting FCCP when the oxygen signal reaches a maximal level and starts declining.
6. Then, inject 2 µl of 0.2 mM rotenone (0.2 µM) and 2 µl of 5 mM antimycin A (5 µM) into the chamber. Record respiration until the oxygen flux signal decreases and stabilizes.

Representative Results

Determination of Optimum Digitonin Concentration for Cellular Permeabilization: Digitonin Titration Experiment

Digitonin titration is performed to determine the optimal concentration for permeabilization of HepG2 cells. For these experiments, digitonin is titrated in intact cells in the presence of rotenone, succinate (mitochondrial complex II substrate) and a saturating amount of ADP (to induce complex II-dependent state 3), and respiratory rates are measured at baseline and after each titration (**Figures 1A** and **1B**). The result of this experiment shows that in the absence of digitonin, cellular respiration is very low and respiration of intact, non-permeabilized cells is not stimulated in the presence of mitochondrial substrate and ADP. However, upon stepwise addition of digitonin, the cellular plasma membrane is permeabilized and mitochondrial respiration (complex II-dependent state 3) increases up to full permeabilization when succinate and ADP enter the cells. The results show that permeabilization at a digitonin concentration of 8-12 µM is optimal for ADP-stimulated respiration of HepG2 cells. However, mitochondrial outer membrane integrity can be compromised if excessive amounts of digitonin are employed. As shown in **Figure 1**, excessive amounts of digitonin induced a reduction in complex II-dependent state 3 respiration indicating compromised mitochondrial outer membrane integrity.

In the present and following protocols, all the oxygen consumption rates are expressed as IO_2 [pmol x sec^{-1} x 10^{-6} cells] (oxygen flow per million cells) which is calculated by dividing volume-specific oxygen flux (in the closed oxygen chamber), JV,O_2 [pmol x sec^{-1} x ml^{-1}] by cell concentration in the cell chamber (number of cells per volume [10^6 cells x ml^{-1}])[15].

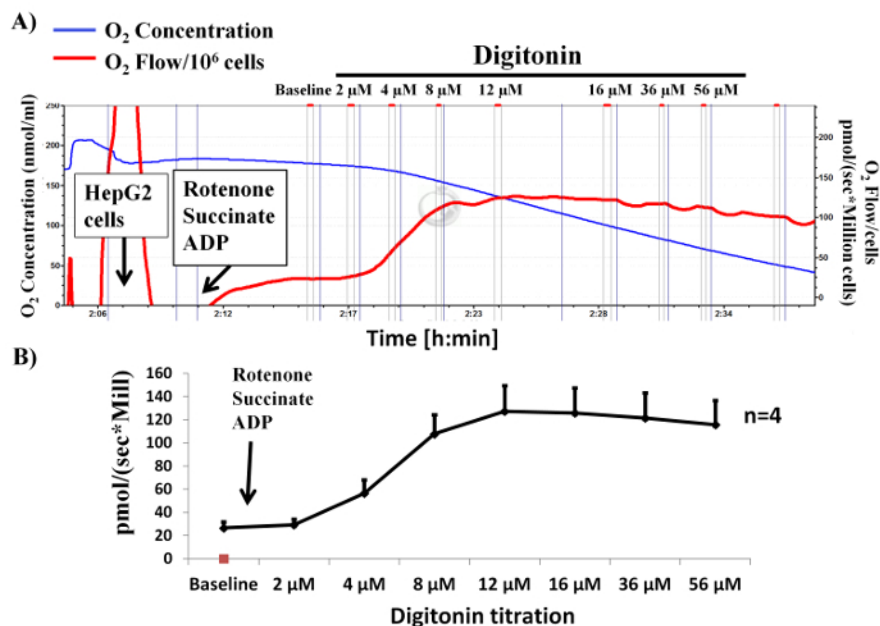

Figure 1: Digitonin titration to determine the optimal concentration for permeabilization of HepG2 cells. The blue line represents oxygen concentration; the red line represents the oxygen flow (slope of oxygen concentration). The oxygen concentration decreases over time as cells use the available oxygen. Oxygen consumption is expressed as pmol/(sec x number of cells). (**A**) Tracings from the high-resolution respirometry using digitonin, ADP and succinate as substrate for mitochondrial complex II-dependent respiration (1 experiment). (**B**) Mitochondrial respiration rates from 4 individual experiments presented as means ± SD. Please click here to view a larger version of this figure.

Evaluation of the Mitochondrial Outer Membrane Integrity Using an Optimal Digitonin Concentration

To evaluate the effect of optimal digitonin concentration (8 µM) on outer mitochondrial membrane integrity, cells are permeabilized with digitonin and mitochondrial outer membrane integrity is tested by measurement of mitochondrial respiration after the subsequent addition of succinate (mitochondrial complex II-dependent resting state 2 respiration in the absence of ADP), ADP (ADP stimulated complex II-dependent state 3 respiration) and cytochrome c (ADP stimulated complex II-dependent state 3 respiration in the presence of cytochrome c), followed by the addition of oligomycin (an inhibitor of ATP synthase) to mimic state 4. As shown in **Figure 2**, cytochrome c does not enhance complex II-dependent state 3 respiration of the cells treated with digitonin, indicating that there was no loss of cytochrome c from the mitochondrial outer membrane and mitochondrial integrity is preserved.

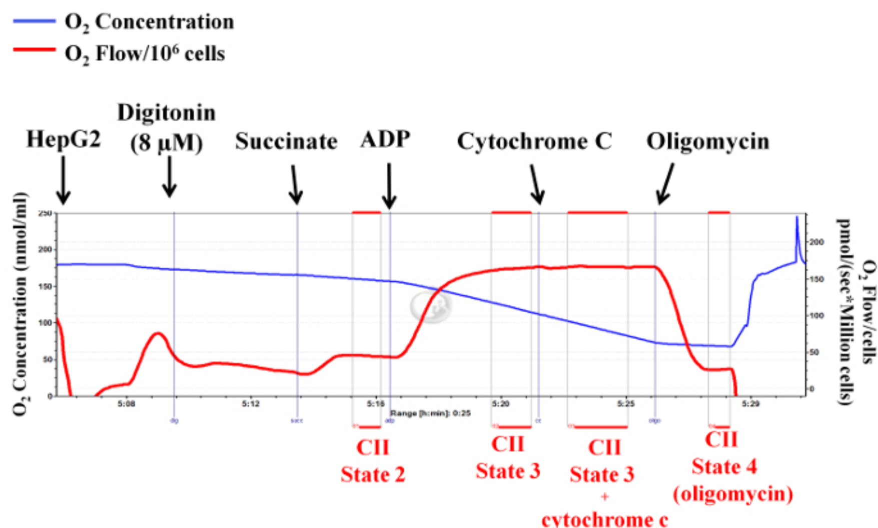

Figure 2: Cytochrome c does not enhance respiration of the cells treated with digitonin (8 µM). Representative respiratory traces for the cytochrome c test using high-resolution respirometry. Cells are permeabilized with 8 µM digitonin and mitochondrial outer membrane integrity is tested by measurement of respiratory rates after the subsequent addition of 10 mM succinate (state 2), 2.5 mM ADP (state 3) and 10 µM cytochrome c (state 3 in the presence cytochrome c), followed by the addition of 2 µg/ml oligomycin to mimic state 4. Respiratory rate is expressed as pmol/(sec x million cells). CII: complex II. Please click here to view a larger version of this figure.

Evaluation of the Mitochondrial Outer Membrane Integrity Using a High Concentration of Digitonin

To demonstrate that a very high concentration of digitonin can compromise mitochondrial outer membrane integrity, we performed an experiment using a high dose of digitonin. For this experiment, cells are permeabilized with 40 µM digitonin instead of 8 µM, and mitochondrial outer membrane integrity is tested by measurement of mitochondrial respiration after the subsequent addition of succinate (mitochondrial complex II-dependent resting state 2 respiration in the absence of ADP), ADP (ADP stimulated complex II-dependent state 3 respiration) and cytochrome c (ADP stimulated complex II-dependent state 3 respiration in the presence of cytochrome c), followed by the addition of oligomycin to mimic state 4 in the presence of oligomycin (**Figure 3**). The result of this experiment shows that cytochrome c enhances respiration of the cells treated with a high dose of digitonin, indicating a loss of cytochrome c from the mitochondrial outer membrane indicating compromised mitochondrial outer membrane integrity.

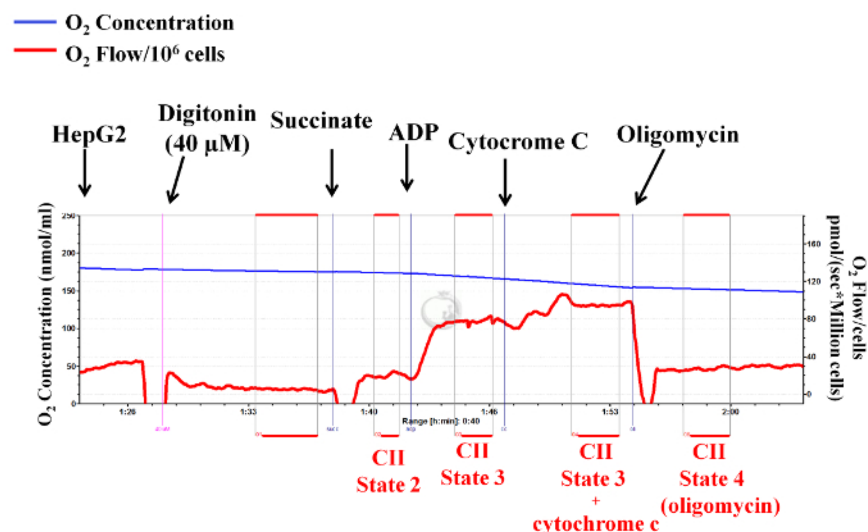

Figure 3: High dose of digitonin (40 µM) compromises mitochondrial outer membrane integrity. Tracings from the high-resolution respirometry using succinate as substrate. The blue line represents oxygen concentration; the red line represents the oxygen flow (slope of oxygen concentration). The oxygen concentration decreases over time as cells use the available oxygen. Oxygen consumption is expressed as pmol/(sec x number of cells). Subsequent addition of 40 µM digitonin, succinate (10 mM), ADP (2.5 mM), cytochrome c (10 µM) and oligomycin (2 µg/ml) is indicated. CII: complex II. Please click here to view a larger version of this figure.

Maximal ADP-stimulated Respiration (State 3) of Permeabilized HepG2 Cells, Using Excess Exogenous Substrates

Complex I-, II- and IV-dependent maximal ADP-stimulated respiration (state 3) of permeabilized HepG2 cells (1 x 10^6 cells/ml) is successfully measured using excess exogenous substrates (**Figure 4**). For this experiment, cells are permeabilized with digitonin and mitochondrial oxygen consumption rates are measured after the subsequent addition of substrates and inhibitors as described in **Figure 4**. The substrate- inhibitor titration protocol results show that cells are successfully permeabilized and the addition of exogenous substrates of mitochondrial complex activity induces an increase in mitochondrial respiratory chain complexes I, II and IV maximal respiratory rates (state 3).

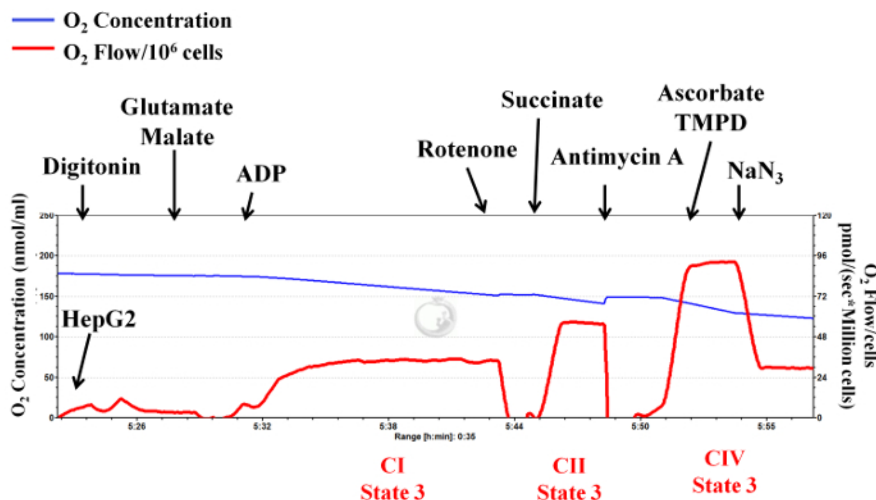

Figure 4: Successful measurement of maximal ADP-stimulated respiration (state 3) of permeabilized HepG2 cells. Respiratory rate is expressed as pmol/(sec x million cells). Cells are permeabilized with 8 µM digitonin and mitochondrial respiratory rates are measured after the subsequent addition of glutamate and malate (state 3, complex I), rotenone to inhibit complex I, succinate (state 3, complex II), antimycin A to inhibit complex III and ascorbate/TMPD and sodium azide to inhibit complex IV. Complex IV respiration (State 3) is interpreted by subtracting the oxygen consumption before and after addition of sodium azide. CI: complex I, CII: complex II, CIV: complex IV. Please click here to view a larger version of this figure.

Unsuccessful Measurement of Maximal ADP-stimulated Respiration (State 3) of Permeabilized HepG2 Cells

Complex I-, II- and IV-dependent maximal ADP-stimulated respiration (state 3) of permeabilized HepG2 cells (1 x 10^6 cells/ml) is not successfully measured using excess exogenous substrates (**Figure 5**). For this experiment, cells are permeabilized with digitonin and mitochondrial oxygen consumption rates are measured after the subsequent addition of substrates and inhibitors as described in **Figure 5**. As shown in **Figure 5**, the respiration rates are very low in permeabilized cells compared to results shown in **Figure 4**. A possible explanation for reduced respiratory levels is contamination of oxygraph chambers with mitochondrial inhibitors from previous experiments. Mitochondrial inhibitors such as antimycin and rotenone are soluble in ethanol and can stick to the oxygraph chambers and stoppers. Therefore oxygraph chambers and stoppers should be washed extensively after each experiment.

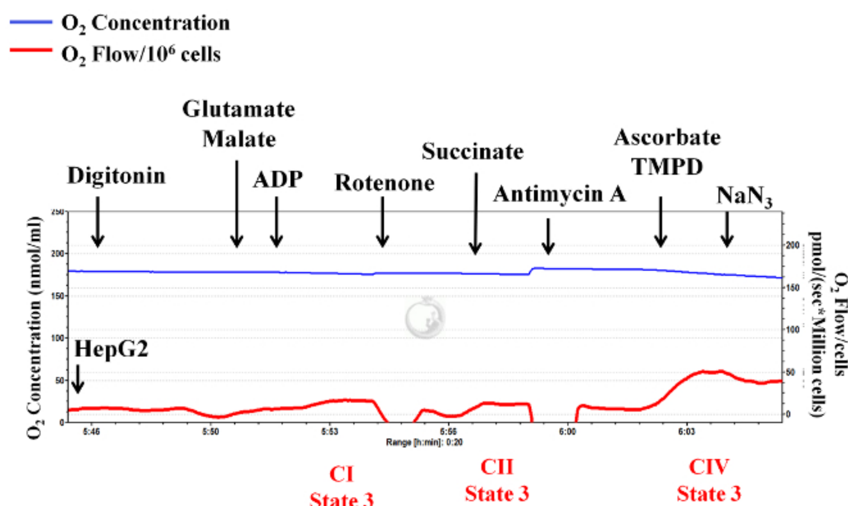

Figure 5: Unsuccessful measurement of maximal ADP-stimulated respiration (state 3) of permeabilized HepG2 cells. Representative respiratory traces of an unsuccessful experiment of complex I-, II- and IV-driven maximal ADP-stimulated respiration (state 3) of permeabilized HepG2 cells using high-resolution respirometry. Respiratory rate is expressed as pmol/(sec x million cells). Cells are permeabilized with 8 µM digitonin and mitochondrial respiratory rates are measured after the subsequent addition of glutamate and malate (state 3, complex I), rotenone to inhibit complex I, succinate (state 3, complex II), antimycin A to inhibit complex III and ascorbate/TMPD and sodium azide to inhibit complex IV. Complex IV respiration (State 3) is interpreted by subtracting the oxygen consumption before and after addition of sodium azide. Please click here to view a larger version of this figure.

Coupled and Uncoupled Respiration of Intact Cells

HepG2 cells' basal oxygen consumption is measured in the presence of oligomycin (oligomycin-insensitive respiration) and sequential addition of FCCP (**Figure 6**). Basal cellular respiration rate represents oxygen consumption of intact HepG2 cells in the presence of endogenous cellular substrates and can alter in response to cellular ATP demand. Oligomycin-insensitive respiration rate represents leak cellular respiration and oligomycin-sensitive respiration rate which represents cellular ATP turnover and is calculated by subtracting the oligomycin-insensitive respiration rate from basal endogenous respiration rate. The chemical uncoupler FCCP is sequentially added at different concentrations and maximal uncoupled respiration recorded. The maximal mitochondrial uncoupled respiration rate (maximal electron transport system capacity) for this experimental condition is obtained at 0.9-1.2 µM FCCP. The results show a biphasic activity of FCCP in intact HepG2 cells. Therefore, for each experimental condition, FCCP is titrated to obtain maximal uncoupled respiration rate. The uncoupled respiratory control ratio (uRCR) is calculated by dividing the FCCP uncoupled respiration rate by the respiration rate in the presence of oligomycin. Oligomycin-sensitive respiration (ATP turnover) is calculated by subtracting oligomycin-insensitive respiration rate from basal endogenous respiration. Coupling efficiency which represents the proportion of mitochondrial oxygen consumption used to synthesize ATP is calculated by dividing the oligomycin-sensitive respiration rate by the basal respiration rate. At the end of the experiment, to obtain non-mitochondrial respiration rate, mitochondrial electron transport chain inhibitors are added and non-mitochondrial respiration rate is subtracted from all results. For the experiment shown in **Figure 6**, the non-mitochondrial respiration rate is 26 pmol/(sec x Million cells), the basal respiration rate is 48 pmol/(sec x million cells), oligomycin-insensitive respiration rate 7 pmol/(sec x million cells), oligomycin-sensitive respiration (ATP turnover) 41 pmol/(sec x million cells) and coupling efficiency 0.85.

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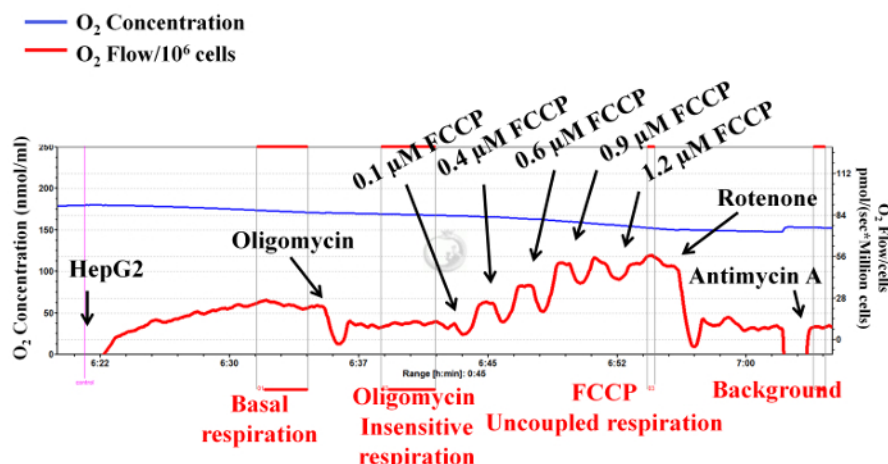

Figure 6: Coupled and uncoupled respiration of intact cells. Representative respiratory traces of HepG2 cells' basal oxygen consumption measured in the presence of oligomycin (oligomycin-insensitive respiration) and sequential addition of FCCP using high-resolution respirometry. Thereafter, once a stable signal is reached, respiration is inhibited with the addition of rotenone and antimycin A and the remaining background respiration (non-mitochondrial respiration) is subtracted from all results. Oxygen consumption is expressed as pmol/(sec x number of cells). Please click here to view a larger version of this figure.

Respiration buffer [13]	
Chemical	Concentration
Sucrose	110 mM
EGTA	0.5 mM
$MgCl_2$	3.0 mM
KCl	80 mM
K-lactobionate	60 mM
KH_2PO_4	10 mM
Taurine	20 mM
Hepes	20 mM
BSA	1.0 g/L
pH	7.1

Table 1: The composition of respiration buffer.

Discussion

The objective of the present protocol was to use high-resolution respirometry to measure mitochondrial respiratory chain complexes' (I-IV) respiratory rates, maximal mitochondrial electron transport system capacity and mitochondrial outer membrane integrity.

There are some critical steps within the present protocol. First, cellular oxygen consumption rates are usually normalized to the number of cells (pmol/[sec x number of cells]). Therefore, before monitoring cellular oxygen consumption, it is critical to use a device that enables accurate and reliable measurements of the number of cells. In the present protocol an automated cell counter has been used (step 3.7 of the protocol and table of materials). A second critical step, especially in permeabilized cells, is the choice of respiration buffer in which permeabilized cells are resuspended (step 3.6 of the protocol). Since cellular permeabilization can induce loss of intracellular ions, it is very crucial that the medium used during permeabilization is compatible with intracellular environment. Therefore, respiration buffer should contain a composition and osmolarity which reflects both the intracellular and the extracellular environment (**Table 1**)[13]. In addition, for optimal and effective cellular permeabilization, it is crucial to titrate digitonin concentration not only for each cell type but also for each cell density, to identify its optimal and lowest effective concentration. If digitonin concentration is too low, the cells will not be permeabilized. In contrast, exposure of the cells to large amounts of digitonin will damage the mitochondrial outer membrane. Therefore, another important point is to investigate mitochondrial outer membrane integrity. In order not to underestimate cellular respiratory capacity, it is also crucial that respirometry experiments are performed at a physiological temperature (37 °C). Another important point to consider is the use of saturating amounts of ADP, due to the diffusion limitation of ADP versus oxygen in permeabilized cells and sufficient substrate levels to obtain maximal respiratory flux. In some experiments in permeabilized cells, it may happen that addition of exogenous substrates and ADP does not lead to a considerable increase in the rate of respiration. This can be caused by several factors. For example, use of high concentrations of digitonin to permeabilize the cells may damage the mitochondrial outer membrane. Therefore, one should titrate digitonin to determine the optimal concentration for each cell type and density. Further, purity of digitonin is also very critical, and commercially available digitonin can vary significantly in purity and new batches of purchased digitonin should be titrated to determine their optimal concentrations for cellular permeabilization. In addition, there are several types of cellular

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plasma membrane pore-forming agents with different properties. For example, saponin is a milder detergent than digitonin, and depending on the cell type, an appropriate cell permeabilization reagent should be selected.

Most inhibitors of mitochondrial function used in respirometry assays, such as rotenone antimycin A, are soluble only in ethanol and stick to the oxygraph chambers and stoppers, inhibiting cellular respiration. To avoid this problem, oxygraph chambers and stoppers must be washed extensively with 95% ethanol after each assay. Another important point to consider is that for both intact and permeabilized cellular respiration assays, cell density, type and cell culture confluence may affect respiratory rates. For example, with HepG2 cells, we usually use a cell density of 1 to 2 million cells per chamber. Using very low cell density during respirometry can give rise to a very weak signal. We also observed that cell culture confluency may affect the respiratory rates. For example, when HepG2 cells are grown very confluent (more than 100%), they consume much less oxygen.

To obtain a stable and reproducible oxygen flux during experiments, another important point to consider is the maintenance of the high-resolution respirometry instrument—*i.e.*, regular exchange of polarographic oxygen sensor membranes and instrumental background corrections. For experiments with the uncoupler FCCP, it may be that addition of FCCP to the cells does not stimulate cellular respiration and maximal flux is not obtained, but instead cellular respiration is inhibited. Inhibition of cellular oxygen consumption upon addition of FCCP may indicate that FCCP concentration was not optimal and was too high. For these experiments, for each assay, it is essential to carefully titrate FCCP concentration to obtain maximal flux.

A limitation of high-resolution respirometry is that high-throughput screening, establishment of dose-response curves and time course experiments for limited amounts of biological samples are not feasible. For these assays, one can use other instruments, such as the extracellular flux analyzer. Other limitations are i) the device is not automated and requires the continuous presence of an operator, ii) it is time consuming, iii) it has only two chambers and only two assays can be run at a time, iv) maintenance of the instrument, such as changing the membranes and calibrations, requires lots of time and v) the chambers are not single use and may become contaminated with inhibitors. The advantages of the high resolution oxygraph over traditional polarographic oxygen electrode devices are i) higher sensitivity, ii) smaller numbers of biological samples required, iii) respiratory rates can be measured simultaneously in two chambers and iv) the device has reduced oxygen leak. Some advantages of the high-resolution oxygraph over the extracellular flux analyzer are i) lower costs of the device and consumables and ii) feasibility of substrate-uncoupler-inhibitor titration protocols.

Future applications or directions after mastering this technique are simultaneous measurements of cellular respiration, mitochondrial membrane potential, H_2O_2, ATP and calcium levels using optical sensors in the same chamber.

Disclosures

The authors have nothing to disclose.

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