Video Article

Respirometric Oxidative Phosphorylation Assessment in Saponin-permeabilized Cardiac Fibers

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Abstract

Investigation of mitochondrial function represents an important parameter of cardiac physiology as mitochondria are involved in energy metabolism, oxidative stress, apoptosis, aging, mitochondrial encephalomyopathies and drug toxicity. Given this, technologies to measure cardiac mitochondrial function are in demand. One technique that employs an integrative approach to measure mitochondrial function is respirometric oxidative phosphorylation (OXPHOS) analysis.

The principle of respirometric OXPHOS assessment is centered around measuring oxygen concentration utilizing a Clark electrode. As the permeabilized fiber bundle consumes oxygen, oxygen concentration in the closed chamber declines. Using selected substrate-inhibitor-uncoupler titration protocols, electrons are provided to specific sites of the electron transport chain, allowing evaluation of mitochondrial function. Prior to respirometric analysis of mitochondrial function, mechanical and chemical preparatory techniques are utilized to permeabilize the sarcolemma of muscle fibers. Chemical permeabilization employs saponin to selectively perforate the cell membrane while maintaining cellular architecture.

This paper thoroughly describes the steps involved in preparing saponin-skinned cardiac fibers for oxygen consumption measurements to evaluate mitochondrial OXPHOS. Additionally, troubleshooting advice as well as specific substrates, inhibitors and uncouplers that may be used to determine mitochondria function at specific sites of the electron transport chain are provided. Importantly, the described protocol may be easily applied to cardiac and skeletal tissue of various animal models and human samples.

Protocol

1. Reagent Preparation

1. The relaxation and preservation solution (RP Solution) is prepared as previously described with minor modifications¹. Briefly, the RP Solution consists of 2.77mM CaK₂EGTA, 7.23mM K₂EGTA, 20mM imidazole, 0.5mM dithiothreitol, 20mM taurine, 50mM K-MES, 6.56 MgCl₂, 5.7mM ATP, 14.3mM phosphocreatine, pH 7.1, adjusted at room temperature (RT). Filter the solution through a 0.45-µm filter to sterilize. Divide into 15 mL portions (Falcon polypropylene tubes) and store at -20 °C. See discussion for recipes.
2. The Mitochondrial Respirometry Solution (MiR05) is prepared as previously described² and contains 0.5mM EGTA, 3mM MgCl₂·6H₂O, 20mM taurine, 10mM KH₂PO₄, 20mM HEPES, 1g/L BSA, 60mM potassium-lactobionate, 110mM sucrose, pH 7.1, adjusted at 30°C. Filter the solution through a 0.45-µm filter to sterilize. Divide into 50 mL portions (Falcon polypropylene tubes) and store at -20 °C. See discussion for recipes. The oxygen solubility factor for MiR05 at 30°C and 37°C is 0.92³.

2. Tissue Preparation

A. Cardiac fibers mechanical preparation

1. Procedures were approved by the University of Calgary Animal Care and Use Committee and abide by the Canadian Association for Laboratory Animal Science guidelines for experimentation.
2. Cervical dislocation to immobilize the mouse is the preferred method. Alternatively, intraperitoneal (IP) injection of ketamine and xylazine (80 and 10mg/kg, respectively) or 0.5mg/kg of sodium pentobarbital may be administered. Note: Sodium pentobarbital is a reversible inhibitor of NADH dehydrogenase (complex I)⁴.
3. Remove the heart and place it in a petri-dish containing the RP solution on ice (Figure 1A). Carefully remove any connective tissue and/or fat using a dissection microscope.
4. Cut the heart in half along the septum. Excise 10-25mg wet weight (ww) of tissue by cutting from the endocardium surface of the left ventricle (Figure 1B). Ensure incision is along the fiber orientation to minimize mechanical damage to myocardium.
5. Place the dissected, subsample of tissue in a separate petri-dish containing the RP solution on ice. Using a dissection microscope cut the heart into longitudinal strips along fiber orientation with a diameter of 1-1.5mm.
6. Shorten the strips with a scalpel to lengths of 2-4mm (Figure 1C).
7. Using extreme care and sharp forceps mechanically separate fiber bundles. Final fiber bundles should contain a maximum of 6-8 fibers, connected by small areas of contact, weighing no more than 5mg ww. Cardiac fibers of 1-3mg ww are recommended. Visually, the cardiac tissue should change from original red to a pale pink coloring (Figure 1D).

B. Cardiac fiber bundles chemical preparation

1. Place a 12-well plate on ice.
2. Rinse the well(s) with RP solution to minimize calcium contamination⁵.

3. Transfer the mechanically prepared fiber bundles to a well containing 3mL ice-cold RP solution with 50ug/ mL saponin. Note: The concentration of saponin does not depend on the amount of cardiac muscle present in the solution.
4. Incubate for 20min with mild stirring on ice.

C. Cardiac Fiber Bundle Washing

1. Rinse a new well with MiR05 to minimize calcium contamination⁵.
2. Transfer the cardiac bundles to the new well containing 10mL ice-cold MiR05. Incubate for 10min with mild stirring on ice.
3. Repeat (1-2) 2-3 times with fresh MiR05. The repetitive washing steps are to ensure the removal of saponin, ATP, ADP and any remaining substrates from the fiber bundles.

D. Wet weight determination

1. Directly prior to respiratory analysis, wet weight is obtained by blotting the individual fiber bundles (1-3mg ww) on an absorbent surface (filter paper) using forceps and holding the fiber to the surface for 5s or until all moisture is wicked away.
2. Using another absorbent surface, remove excess liquid from forceps.
3. Tare the scale and place the fiber bundle on a plastic weigh boat for mass measurement.
4. Transfer the cardiac fiber bundle to a new well with ice-cold MiR05. The fiber bundle is ready for oxygen consumption assessment.

3. Respirometric OXPHOS Analysis

A. Respirometric Equipment

1. The laboratory utilizes and recommends a two chamber titration-injection oxygraph (Oroboros Oxygraph2-k, Oroboros Instruments). The Oxygraph 2-k offers high resolution respirometry in large part by utilizing instrumental hardware and software (DatLab) that minimizes instrumental background that contribute to oxygen consumption artifacts.
2. Calibration of the oxygraph is an essential step to minimize confounding effects of instrumental oxygen consumption. Calibration varies slightly depending on the oxygraph. Refer to oxygraph user manual for specific procedures.

B. Representative Quality Control/Technique Validation Protocol

1. Ensure MiR05 has been added to oxygraph chambers housing the polarographic oxygen sensors (POS) and give adequate time for air saturation and equilibration of the MiR05 at 37°C prior to putting the murine cardiac fibers into the oxygraph chambers.
2. The cardiac fiber samples are placed in the stirred MiR05 of the oxygraph chambers. Note: Ensure the stir bars are PVDF- or PEEK-coated stirrer bars (6mm diameter).
3. Using an oxygen-filled syringe, increase the oxygen concentration of the oxygraph chambers to 250-550uM. It should be noted that oxygen concentration is limiting for permeabilized fiber preparations at even 50% above air saturation⁶. Allow 5-10min for oxygen concentration stabilization.
4. Using a 25μL Hamilton microsyringe, add 10μL of 2M glutamate and 5μL of 800mM malate to obtain a final concentration of 10mM and 2mM, respectively. These titrations allow for the determination of basal complex I-supported respiration (State 2; absence of ADP). Oxygen flux should stabilize within approximately 5min of titration. Proper preparation should provide very reproducible state 2 oxygen consumption.
5. Following 2-5min of stable oxygen consumption, add 20μL of 500mM ADP for a final concentration of 5mM (saturating), using a 25μL Hamilton microsyringe, for maximal (state 3) mitochondrial respiration through complex I. Few studies use this high of a concentration of ADP, however, it is important to note that greater than 90% saturation is only reached at concentrations above 5mM⁷.
6. Following 2-5min of stable oxygen consumption, add 5μL of 4mM cytochrome c to obtain a final concentration of 10μM, using a 10μL Hamilton microsyringe, for quality control analysis of outer mitochondrial membrane (OMM) integrity.
7. Following 2-5min of stable oxygen consumption, add 1μL of 4mg/ mL oligomycin, using a 10μL Hamilton microsyringe, to inhibit ATP synthase. This titration step will offer validation of inner mitochondria membrane intactness.
8. Following the respirometric OXPHOS assessment. The sample is retained for dry weight or mitochondrial marker determination.
9. Remove MiR05 from the glass chambers of the oxygraph. Wash the chambers at least three times with distilled water (ddH₂O).
10. Wash the chambers at least three times with 100% ethanol (EtOH) to remove EtOH-soluble inhibitors such as oligomycin.
11. Wash the chambers with 70% EtOH three times with the final 70% EtOH wash lasting 30min to sterilize the oxygraph chambers.
12. Prior to addition of MiR05 for new experimental titration protocol ensure the chambers containing the POS are washed at least five times with ddH₂O.
13. The validation titrations may be performed as an individual protocol (Figure 2) or the titrations may be included into a well-planned titration protocol depending on the experimental and diagnostic aims of the respirometry studies.

4. Representative Results:

Oxygen consumption in properly prepared murine cardiac fibers is evaluated by the quality control protocol as shown in Figure 2. Figures 3-5 provide commonly encountered examples of incorrectly prepared cardiac fibers. The respiratory control ratio (RCR) represents an important index in respirometry. This parameter indicates the coupling between oxygen consumption and oxidative phosphorylation. In permeabilized fiber preparations, the RCR is the rate of respiration in state 3 relative to state 2 or alternatively state 3 over state 4 (induced by oligomycin and/or atractyloside, ATR). Furthermore, RCR can be used as a quality assurance marker and can identify changes in coupling resulting from experimental or pathological interventions⁵. Well-coupled permeabilized murine cardiac preparations yield an RCR between 3-6 depending on the incubation solution utilized^{1, 8, 9}.

The titration of cytochrome c is used as a validation of proper tissue preparation. Cytochrome c is a protein located in the intermembrane space at the mitochondrial inner membrane¹⁰. When the outer membrane of mitochondria is intact, the endogenous cytochrome c remains in the intermembrane space and the titration of exogenous cytochrome c has a negligible effect on respiration (Figure 2). If the outer membrane of mitochondria is damaged, the endogenous cytochrome c can be released from the intermembrane space and will inhibit respiration until exogenous cytochrome c addition (Figure 3). Proper preparations should experience only a slight elevation in oxygen flux following cytochrome c addition in the 5-15% range⁵. Additionally, this experimental titration allows for the assessment of the pathological or experimental stressor's influence on mitochondrial outer membrane intactness. If a cytochrome c effect is experienced, ensure care during mechanical preparation of the tissue and/or reduce saponin concentration.

The addition of oligomycin and/or ATR is used to assess alterations in leak respiration; oxygen consumption not contributing to ADP phosphorylation¹¹. Additionally, this titration step may be used as a validation of proper sample preparation. Control or wild-type fibers should be sensitive to this addition and experience a significant reduction in oxygen flux. A low RCR and reduced sensitivity to oligomycin and/or ATR resulting in a relatively elevated oxygen flux indicates damage to the inner mitochondrial membrane during preparation (Figure 4). Damage is likely induced during mechanical separation of the cardiac fibers as the inner mitochondrial membrane is less susceptible to insult by saponin relative to the outer mitochondrial membrane. However, both mechanical and chemical preparation may have to be adjusted accordingly to avoid improperly prepared cardiac fibers^{5, 12}.

The saponin-skinned fiber bundles from mice should not remain in the RP solution for more than 6h or the MiR05 solution for greater than 2h. Lack of response to substrate-inhibitor-uncoupler titrations as seen in Figure 5 may be indicative of prolonged incubation periods. Subsequent efforts should minimize periods between animal sacrifice and oxygen consumption measurements.

Figure 1. Mechanical preparation of murine cardiac fiber bundles. A. The entire heart immediately following dissection. B. A 10-25mg sample of the anterior left ventricle. C. Cardiac tissue separated into 1mm diameter and 2-4mm length strips. D. Final cardiac fiber bundles ready for chemical permeabilization with saponin.

Figure 2. Representative results of proper tissue preparation utilizing polarographic assessment. The state 2 oxygen flux is supported by complex I substrates glutamate and malate (M/G) and is significantly stimulated following the addition of ADP (state 3). No stimulatory effect of exogenous cytochrome c addition indicates the outer mitochondrial membrane is intact. Sensitivity of oxygen consumption to oligomycin suggests the inner mitochondrial membrane integrity is intact. Oxygen concentration in a 2 mL closed chamber is identified by the blue line. Oxygen consumption of the cardiac tissue sample in a 2 mL closed chamber is represented by the red line. Malate and glutamate, M/G; Adenosine Diphosphate, ADP; Cytochrome c, Cyto c; Oligomycin, o.

Figure 3. Cytochrome c effect validation test utilizing polarographic assessment. The state 2 oxygen flux is supported by complex I substrates glutamate and malate (M/G) and is significantly stimulated following the addition of ADP (state 3). Stimulatory effect of exogenous cytochrome c addition indicates the outer mitochondrial membrane integrity is compromised. Oxygen concentration in a 2 mL closed chamber is identified by the blue line. Oxygen consumption of the cardiac tissue sample in a 2 mL closed chamber is represented by the red line. Malate and glutamate, M/G; Adenosine Diphosphate, ADP; Cytochrome c, Cyto c.

Figure 4. Inner mitochondrial membrane integrity validation test utilizing polarographic assessment. The state 2 oxygen flux is supported by complex I substrates glutamate and malate (M/G) and a relatively weak stimulation of oxygen consumption following the addition of ADP (state 3). Poor sensitivity of oxygen consumption to oligomycin suggests the inner mitochondrial membrane is damaged. Oxygen concentration in a 2 mL closed chamber is identified by the blue line. Oxygen consumption of the cardiac tissue sample in a 2 mL closed chamber is represented by the red line. Malate and glutamate, M/G; Adenosine Diphosphate, ADP Oligomycin, o.

Figure 5. Polarographic assessment following prolonged incubation in MiR05. Oxygen consumption is insensitive to exogenous addition of glutamate and malate (M/G) and oxygen consumption following the addition of ADP (state 3) is reduced. There is no stimulatory effect of exogenous cytochrome c addition and insensitivity of oxygen consumption to oligomycin is experienced. Lack of response to additions to extramitochondrial incubation solution suggests mitochondrial functional stability is compromised. Oxygen concentration in a 2 mL closed chamber is identified by the blue line. Oxygen consumption of the cardiac tissue sample in a 2 mL closed chamber is represented by the red line. Malate and glutamate, M/G; Adenosine Diphosphate, ADP; Cytochrome c, Cyto c.

1. Notes: Reagent Preparation

Preparation of K₂EGTA 100mM Stock Solution

Name of the reagent	Final Concentration (mM)	g/100 mL H ₂ O
Ethylene glycol-bis-(2-aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid (EGTA)	100	3.805
Potassium Hydroxide (KOH)	200	1.15

Note: Adjust pH to 7.4 at room temperature.

Preparation of Ca₂EGTA 100mM Stock Solution

Name of the reagent	Final Concentration (mM)	g/100 mL H ₂ O	Comments (optional)
Ethylene glycol-bis-(2-aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid (EGTA)	100	3.805	Heat to 80°C and stir mildly.
Calcium Carbonate (CaCO ₃)	100	1.001	The calcium concentration must be precise as calcium regulates the function of various organelles including mitochondria. Ensure complete solubilization of all CaCO ₃ . The final solution must be completely transparent. CaCO ₃ is initially mixed with EGTA and some water to activate the formation of carbonic acid and CO ₂ evaporation. This reaction may be accelerated by heating up to 80 °C.
Potassium Hydroxide (KOH)	200	1.15	Neutralize with KOH after evaporation of CO ₂ is completed.

Note: Adjust pH to 7.4 at room temperature.

Preparation of Relaxation and Preservation Solution (RP Solution)¹

Reagent	Final Concentration (mM)	Per litre	Comments
K ₂ EGTA	7.23	72.3 mL	
CaK ₂ EGTA	2.77	27.7 mL	
Imidazole	20	1.36g	
Dithiothreitol	0.5		
Taurine	20	2.52g	
Adenosine 5'-triphosphate disodium salt hydrate (ATP)	5.7	3.14g	
Phosphocreatine (PCr)	14.3	4.0g	
Magnesium chloride (MgCl ₂)	6.56	0.624g	
K-MES	50	14.0g	

Note: Adjust pH to 7.1 at room temperature

Preparation of Mitochondrial Respirometry Solution (MiR05 Solution)²

Reagent	Final Concentration (mM)	Per litre	Comments (optional)
Ethylene glycol-bis-(2-aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid (EGTA)	0.5	0.190g	Used as a chelator of calcium
Magnesium chloride hexahydrate (MgCl ₂ ·6H ₂ O)	3.0	0.610g	The quality of fiber preparation cannot be tested without Mg ²⁺ .
Taurine	20.0	2.502g	Taurine is a membrane stabilizer and antioxidant. 20mM is the intracellular concentration present in the heart.
Potassium phosphate monobasic (KH ₂ PO ₄)	10.0	1.361g	
HEPES	20.0	4.77g	
Potassium-lactobionate	60.0	120 mL of 0.5 M K-lactobionate stock	0.5M K-lactobionate Stock: Add 35.83 g lactobionic acid to 100 mL H ₂ O and pH to 7.0 at RT. Adjust volume to 200 mL with ddH ₂ O. Used to replicate the high intracellular K ⁺ concentration. Previously KCl was used, however, the high Cl ⁻ inhibits mitochondrial creatine kinase function.
Sucrose	110.0	37.65g	Used as a ROS scavenger.
Bovine Serum Albumin (BSA)	1g/L	1g	Used as a membrane stabilizer, antioxidant, and chelator of calcium and free fatty acids.

Note: Adjust pH to 7.1 at 30°C

3. Notes: Respirometric OXPHOS Analysis

B. Select Substrates, uncouplers and inhibitors

List of Selected Substrates for Mitochondrial Respirometry Analysis

Substrate	[Stock] [mM]	Preparation	Volume per 2 mL	[Final] [mM]	Comments
Adenosine 5'-diphosphate monopotassium salt dihydrate (ADP)	500 mM	246mg/ mL ddH ₂ O. Adjust pH to 7.1 at RT. Store at -80°C in 250 µL aliquots.	20ul	5mM	To maintain constant Mg ²⁺ during respirometry experiments add 0.6 mol MgCl ₂ /mol ADP.
Ascorbate	800 mM	0.1584g/ mL ddH ₂ O. Store at -20°C in 200 µL aliquots. Light sensitive	5 µL	2mM	Acts as substrate when used in parallel with TMPD. Must correct for oxygen flux for auto-oxidation.
Cytochrome C	4mM	50mg/ mL ddH ₂ O. Store at -20°C in 250 µL aliquots.	5 µL	10u M	
Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP)	0.1m M	0.254mg/10 mL 100% EtOH. Store in glass vials at -20°C in 500 µL aliquots.	Steps of 1 µL		Acts as an uncoupler. Determines maximal electron transport capacity and any limitation of electron transport by phosphorylation system.
Glutamate	2M	0.3742g/ mL ddH ₂ O. Adjust pH to 7.1 at RT. Store at -20°C in 250 µL aliquots.	10ul	10m M	Acts as a substrate for NADH dehydrogenase (complex I).
Malate	800 mM	0.1073g/ mL ddH ₂ O. Adjust pH to 7.1 at RT. Store at -20°C in 250 µL aliquots.	5ul	2mM	Acts as a substrate for NADH dehydrogenase (complex I). Cannot support respiration alone.
Pyruvate	1M	11mg/0.1 mL ddH ₂ O. Prepare fresh.	5 µL	2.5m M	Acts as a substrate for NADH dehydrogenase (complex I).
Succinate	1000 mM	1.3505g/5 mL ddH ₂ O. Adjust pH to 7.1 at RT. Store at -20°C in 250 µL aliquots.	20ul	10m M	Acts as a substrate for succinate dehydrogenase (complex II).
N,N,N',N'-Tetramethyl-p-phenylenediamine Dihydrochloride (TMPD)	200 mM	47.1mg/ mL ddH ₂ O. Add 0.8M ascorbate to final concentration of 10mM to prevent auto-oxidation. Store at -20°C in 200 µL aliquots.	5 µL	0.5m M	Autooxidation of stock solution evident by appearance of blue coloring. Acts as substrate when used in parallel with TMPD. Must correct for oxygen flux for auto-oxidation.

List of Selected Inhibitors for Mitochondrial Respirometry Analysis

Substrate	[Stock] [mM]	Preparation	Volume per 2 mL Chamber	[Final] [mM]	Comments
Antimycin A	5mM	27.4mg/10 mL 100% EtOH. Store at -20°C in 250 µL aliquots.	1 µL	2.5µ M	Inhibitor of coenzyme Q : cytochrome c oxidoreductase (Complex III)
Atractyloside	50m M	40mg/ mL ddH ₂ O. Store at -20°C in 250 µL aliquots.	30 µL	0.75 mM	Inhibitor of ATP Synthase.
Oligomycin	4mg/ mL	4mg/ mL 100% EtOH. Store at -20°C in 200 µL aliquots.	1 µL		Inhibitor of ATP synthase.
Potassium cyanide	1M	65.1mg/ mL ddH ₂ O. Prepare fresh. Adjust pH to 7.1 at RT	1 µL	1mM	Inhibitor of cytochrome c oxidase (complex IV). Utilize following TMPD and Ascorbate titration to access autooxidation.
Rotenone	0.1m M	0.39mg/10 mL 100% EtOH. Store at -20°C in 250 µL aliquots. Light sensitive.	1 µL	0.05µ M	Inhibitor of NADH dehydrogenase (complex I). Higher concentrations may be required, however, to reduce rotenone retention in chamber begin as outlined.

Discussion

The saponin-permeabilized cardiac fiber technique offers a unique compromise between *in vitro* and *in vivo* assessment of mitochondrial OXPHOS oxygen consumption. Advantages of this technique include increased physiological relevance in comparison to isolated mitochondria as cellular architecture is preserved. While the plasma membrane is degraded, intracellular membrane structures including mitochondria^{12, 14}, sarcoplasmic reticulum¹⁴, myofilaments and the cytoskeleton^{1, 17} remain intact. Moreover, the interactions between mitochondria and cytoskeleton^{1, 17} are also unaltered. Mitochondria in permeabilized fibers show increased stability. Rodent fiber preparations can be stored in ice-cold preservation solutions for 6h and human fiber samples show stability for up to 24h^{18,2}. Mitochondria experience rapid equilibration with the incubation solution. This allows direct control over site-specific analysis of the electron transport chain in response to added substrates, inhibitors and uncouplers^{1, 5}. Additionally, this *in situ* technique requires only a few milligrams of tissue.

Limitations of the saponin-skinned fiber technique cannot be ignored. Cardiac mitochondria are heterogeneous, consisting of two subpopulations; subsarcolemmal and interfibrillar. *In situ* mitochondrial respirometry assesses the total mitochondrial population without the ability to distinguish between the subpopulations⁵. Additionally, various cellular metabolites and cytosolic factors regulate mitochondrial function. These cytosolic components of the cardiac fibers are lost during the permeabilization process resulting in the inability to assess mitochondrial at the exact *in vivo* environment⁵.

Reporting issues are an important concern. Oxygen consumption measurements may be expressed per wet weight or dry weight, however, mitochondrial density is a major factor in respiratory flux heterogeneity when expressed per tissue mass⁷. It is important to understand that interpretation of respiratory rates and changes are greatly influenced by the choice of reference state. For direct comparisons of respirometric OXPHOS results in permeabilized fibers, rates must be expressed relative to a common reference marker such as mitochondrial DNA, citrate synthase activity, cytochrome c oxidase activity, and/or cytochrome aa3 content¹⁹.

In summary, the permeabilized fiber preparation used in conjunction with respirometry analysis allows for the assessment of integrative function of cardiac mitochondria. Various pathological states and genetic models have utilized this technique. These include the evaluation of drug-induced toxicity, aging, diabetes, congestive heart failure, ischemic injury and oxidative stress on mitochondrial physiology^{5, 20}. Several excellent methodological reviews and manuscripts of the saponin-permeabilized fiber technique and polarographic oxygen consumption assessment have been previously published^{1, 5, 14, 20} and are highly recommended.

Disclosures

No conflicts of interest declared.

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