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Assessing Mitochondrial Bioenergetics in Isolated Mitochondria from Mouse Heart Tissues Using Oroboros 2k-Oxygraph

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Abstract

In this chapter, we describe detailed protocols for measuring high-resolution respirometry on mitochondria extracted from adult whole mouse heart using the Oroboros 2k-Oxygraph system. The method provides detailed procedures for the preparation of mitochondria and measurement of high-resolution respirometry in response to various respiration inhibitions. The method described in this chapter could discern the different respiration rate on mitochondria extracted from two spatially distinct mitochondrial subpopulations, subsarcolemmal mitochondria (SSM) and intermyofibrillar mitochondria (IFM). These approaches can easily be translated to other cells and tissues.

Keywords

Mitochondrial oxygen consumption; Subsarcolemmal mitochondria (SSM); Intermyofibrillar mitochondria (IFM); Mouse heart; Mitochondria extraction; Oxygraph

1 Introduction

The mitochondrion is a complex cellular organelle governing multiple metabolic processes including fatty acid oxidation, Krebs cycle, and oxidative phosphorylation (OXPHOS) [1], providing nearly 90% of cellular ATP demand. Mitochondria are an essential part of the normal cellular function, particularly in their role in oxidizing carbon substrates to meet cellular energy needs. Hydrolysis of ATP to ADP releases free energy to drive energy-requiring processes such as cross-bridge cycling, maintenance of membrane potentials,

sarcoplasmic reticulum calcium uptake, signal transduction, membrane transport, and protein synthesis. Cardiomyocytes have the highest concentrations of mitochondria of any cell types. Because of the high mitochondrial ATP demands of the heart, relatively subtle defects in the mitochondrial ATP-generating apparatus, oxidative phosphorylation, can particularly affect cardiac function [2]. Mitochondrial defects in many critical functions also occur in the development of heart failure [3, 4].

There are two spatially distinct subpopulations of mitochondria in cardiac and skeletal muscles, interfibrillar (IFM, located between the myofibrils) and subsarcolemmal (SSM, found along the perimeter of the cell) [5]. Comparing to SSM, IFM are more susceptible to damages related to aging, diabetes, and heart failure [6–8]. However, the differences between the two populations in heart failure are not entirely clear.

Mitochondrial respirometry in a closed chamber is derived from the rate of oxygen concentration, which declines as the biological sample consumes oxygen. Oxygen electrodes (such as the Clark electrode) have been used to measure oxygen concentration and have led to an intimate understanding of mitochondrial respiratory function. Although the oxygen electrode remains an inexpensive and useful tool for the evaluation of mitochondrial respiration, the notable limitations include unstable signals and relatively high instrumental background noise.

Oroboros 2k respirometry systems incorporate a closed, airtight reaction chambers, the glass and low O₂ permeable polymers comprising all components, which minimizes O₂ back-diffusion and overestimations of respiration, and the sensors capable of detecting changes in O₂ with minimal noise. Oroboros 2k-oxygraph maximize respirometric sensitivity and precision with the minimal O₂ leak and highly sensitive electrodes. Datlab software advances in flux derivations of changes in chamber O₂ also permit real-time reporting of respiratory kinetics, which improves data analyses over other systems requiring visual assessments of steady-state kinetics. Moreover, the unavoidable background O₂ consumption by the electrode itself, combined with varying degrees of O₂ back-diffusion into the oxygraphic chamber, constitute potential sources of systematic error. In high-resolution systems, global instrumental background O₂ consumption, which increases positively as a function of PO₂, must be corrected for accurate determinations of absolute O₂ consumption [9].

In this chapter, using isolated mitochondria as examples of materials, we describe the detailed protocols for measuring mitochondrial oxygen consumption rates with Oroboros 2k-Oxygraph. Specifically, we measured and compared oxygen consumption rates on the two spatially distinct mitochondrial subpopulations from heart: subsarcolemmal mitochondria (SSM) and intermyofibrillar mitochondria (IFM). The protocol can easily be translated to other cells and tissues.

2 Material

2.1 Oroboro Oxygraph-2k System

The Oxygraph-2k (O2k, OROBOROS INSTRUMENTS, Austria) for High-resolution respirometry is a closed two-chamber respirometer for high-resolution respirometry [10, 11]. The O2k is a barometric pressure transducer enables automatic oxygen calibrations implemented in the DatLab software. Mitochondrial energy metabolism can be tested comprehensively in real time by titrating various substrates, uncouplers, inhibitors, and other substances during the experiment (Fig. 1).

2.2 Medium and Reagents

Isolation medium for mouse heart SS mitochondria (Buffer A): 250 mM sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, pH adjusted to 7.4 with HCl at room temperature (RT).

2.2.1 Mitochondrial Isolation Medium—Isolation medium for mouse heart IF mitochondria (Buffer B): 100 mM KCl, 50 mM MOPS, 5.0 mM MgSO₄, 1.0 mM EGTA, 1.0 mM ATP, 2 mg/ml BSA, pH adjusted to 7.4 with 10 N KOH at room temperature (RT).

2.2.2 Mito5 Mitochondrial Respiration Medium—3 mM MgCl₂, 60 mM K⁺-lactobionate, 20 mM Taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM D-sucrose, 0.5 mM EGTA, pH adjusted to 7.1 with KOH at RT and 1 g/L BSA, essentially fatty acid-free.

2.2.3 Substrates, Uncoupler, and Inhibitors

1. 2 M pyruvate stock solution in double-distilled water (ddH₂O), prepares fresh daily.
2. 400 mM malate stock solution in ddH₂O, adjust to pH 7.0 with 10 N KOH at RT, store at −20 °C.
3. 500 mM ADP stock solution in ddH₂O, store at −20 °C (*see* Note 1).
4. 2 M glutamate stock solution in ddH₂O, adjust to pH 7.0 with 5 N KOH at RT, store at −20 °C.
5. 1 M succinate stock solution in ddH₂O, adjust to pH 7.0 with 1 N HCl at RT, store at −20 °C.
6. 4 mg/ml oligomycin stock solution in absolute ethanol, store at −20 °C.
7. 1 mM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) stock solution in absolute ethanol, store at −20 °C.
8. 0.394 g/ml rotenone stock solution in absolute ethanol, store at −20 °C. Light sensitive.
9. 5 mM antimycin A stock solution in absolute ethanol, store at −20 °C.
10. 260 mg/ml azide stock solution in ddH₂O, aliquot of 500 µl in each tube, store at −20 °C.

2.3 Animal

Male C57BL/6 mice (16–20 g body weight) were purchased from the Experimental Animal Center of Wuhan University (Wuhan, China).

3 Methods

3.1 Isolation of SSM from Mouse Heart

1. Dissected the heart from the mouse chest, blood and main vasculature were dismissed, and heart tissue placed immediately in ice-cold buffer A (*see* Note 2).
2. Transfer heart to a precooled glass 35 mm dish (5 ml) and wash twice with ice-cold buffer A. (*see* Note 3).
3. Chop the heart tissue into 1 mm³ pieces using blade and forceps without squeezing, tearing, and ripping (Fig. 2B) (*see* Note 4).
4. Suspended the chopped heart tissue in 2 ml buffer A (Fig. 2C).
5. Transfer to a precooled Potter-Elvehjem Teflon-glass homogenizer (Fig. 2A) and homogenize the tissue with four to six strokes at 1000 g in 2 ml buffer A (Fig. 2D) (*see* Note 5).
6. Transfer the homogenate to an ice-cold 2 ml tube, then centrifuge at $1000 \times g$ for 10 min at 4 °C (Fig. 2E).
7. Transfer the supernatant into a new ice-cold 2 ml tube and centrifuge at $8000 \times g$ for 5 min at 4 °C (Fig. 2F).
8. Discard the supernatant and wash mitochondrial pellet with 1.5 ml ice-cold buffer A and centrifuge at $8000 \times g$ for 5 min at 4 °C.
9. Resuspend mitochondria in a small volume of buffer A (100–150 µl/heart) (Fig. 2G) (*see* Note 6).
10. Store isolated mitochondria on ice until use, and mitochondrial protein concentration was determined by the Lowry method (Solarbio Science & Technology Co., Ltd, Cat# PC0030) (*see* Note 7).

3.2 Isolation of IFM from Mouse Heart

1. Following the SSM mitochondria isolation **steps** as **1–6** in Subheading 3.1 above.
2. Resuspend pellet with 5 mg/g [heart weight] trypsin dissolved in 1 ml Buffer A.
3. Incubate on a shaker at 200 rpm/min for 10 min on ice.
4. Add 3 ml Buffer B to dilute trypsin effect and stop the shaker.
5. Homogenize on ice with Potter-Elvehjem Teflon-glass homogenizer until no massive deposits are seen with two up-and-down strokes.
6. Transfer the homogenate to an ice-cold 2 ml tube and centrifuge at $7500 \times g$ for 10 min at 4 °C.

7. Discard the supernatant, resuspend the pellet in 2 ml buffer A, and then centrifuge at $580 \times g$ for 7 min at 4 °C.
8. Transfer the supernatant into an ice-cold 1.5 ml tube, then discard pellet.
9. Centrifuge the supernatant at $3000 \times g$ for 7 min at 4 °C, discard supernatant. The resulting pellet is IFM.
10. Resuspend IFM mitochondria in a small volume of buffer A (~100–150 µl/heart).
11. Store isolated mitochondria on ice until use, and mitochondrial protein concentration is determined using the Lowry method.

3.3 Assessment of Mitochondrial Respiration (Fig. 3)

1. Prepare Oxygraph-2k chambers. Rinse chamber A and B three times with ddH₂O, then add 2.5 ml of Miro5 buffer to each chamber for R1 (air-saturated calibration) (*see Note 8*).
2. Let the system stabilize for 15 min to get a stable oxygen readout (*see Note 9*).
3. Add 200 µg of freshly isolated mitochondria into the chambers.
4. Add 5 mM pyruvate, 5 mM malate, and 10 mM glutamate to obtain CI-linked LEAK respiration (*see Note 10*).
5. Add 1 mM ADP to induce CI-linked OXPHOS.
6. Add 10 mM succinate to observe CI + II-linked OXPHOS.
7. Add 2 µg/ml oligomycin to inhibit ATP synthase and measure LEAK respiration in the presence of adenylates.
8. Titrate FCCP to obtain maximum noncoupled respiration fueled by complex II (*see Note 11*).
9. Add 0.5 µM rotenone to inhibit complex I and measure C II-linked OXPHOS.
10. Add 2.5 µM antimycin A to inhibit C III.
11. Wash the Oxygraph-2k lids and chambers three times with 75% ethanol to ensure that all of the inhibitors are completely removed, then rinse chamber three times with ddH₂O.

3.4 Mitochondrial Respiration of SSM and IFM Subpopulations

To investigate the mitochondrial respiration of SSM and IFM, we freshly isolated mitochondria SSM and IFM subpopulations from mice and measured real-time oxygen consumption on these mitochondria in response to specific substrates and inhibitors using an Oroboros Oxygraph SUIIT protocol (Fig. 3). Routine mitochondrial respiration was established by the concomitant addition of pyruvate (5 mM) and malate (5 mM), followed by ADP (1 mM), to measure the oxidative phosphorylation capacity of complex I (OXPHOS CI), driven by the NADH-related substrates. The SSM and IFM mitochondria showed no difference in Routine mitochondrial respiration (Fig. 4A). We then measured coupling respiration by adding a saturating concentration of ADP to assess maximal oxidative

phosphorylation (OXPHOS CI + CII). The coupling respiration in SSM was higher greater than that of IFM (Fig. 4B). LEAK CI + CII respiration measured by adding oligomycin, and IFM has lower respiration than SSM (Fig. 4C). We further analyzed the respiratory control ratios (RCR) to evaluate the OXPHOS efficiency. Consistently, the SSM RCR was significantly higher than IFM RCR (Fig. 4D). These results demonstrate that cardiac SSM respiration is greater than IFM in mice.

The method described here provides the step-by-step procedure for running a basic respirometry measurement. Additional parameters of mitochondrial respiration can be obtained by changing the composition of inhibitors, uncouplers and other chemical compounds depending on the specific research question.

4 Notes

1. It is crucial to keep the concentration of Mg^{2+} constant during respiration measurement mix ADP with $MgCl_2$ (0.6 mol/mol ADP).
2. The use of anesthetics should be avoided because they affect mitochondrial functions.
3. After the heart is removed, the residual blood or blood clots should be quickly washed with precooled Buffer A. The blood must be removed as much as possible. Otherwise the final mitochondrial purity is affected.
4. The heart should be chopped into pieces as small as possible.
5. The whole process must be operated on ice to avoid accelerated mitochondrial impairment. After the mitochondrial pellet is obtained, the respiratory measurement should be performed as soon as possible.
6. The mitochondria cannot be homogenate excessively. If the isolated mitochondrial sediment is pale brown, it is considered that the mitochondria are not good for respiration assay. The high quality isolated mitochondria should be with dark brown color.
7. About 4–5 mg of cardiac mitochondria can be extracted from a male mouse with 25 g of body weight.
8. The pH of Mito5 buffer should be adjusted to 7.4 precisely with KOH. It is crucial to have precise pH for mitochondrial respiration measurement.
9. Assessment of mitochondrial respiration is carried out at 37 °C in 2 ml chamber, but the temperature can also be adjusted to 30 °C for prolonged mitochondrial activity.
10. Pyruvate must be freshly prepared.
11. CCCP can replace FCCP at a stock concentration of 1 mM.

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Fig. 1.

Substrates and inhibitors of mitochondrial respiratory chain. Malate and pyruvate drive the TCA cycle to generate NADH, which travels through the electron transport chain (complex I, III, and IV) and release electrons to drive F_oF_1 -ATP synthesis (F_oF_1). Succinate is oxidized through complex II to generate $FADH_2$, which also help transport electrons. Ascorbate plus TMPD, which can directly provide electrons through complex IV to supported ATP synthesis. Rotenone inhibits Complex I, antimycin A inhibits Complex III, azide inhibits electron flow from complex IV to oxygen, and oligomycin inhibits F_oF_1 -ATP synthase activity

Fig. 2. Isolation steps for mouse heart mitochondria. **(a)** Potter-Elvehjem Teflon-glass homogenizer. **(b–g)** Images of key steps for mitochondrial isolation. Detailed steps are shown in Subheading 3.1

Fig. 3.

Representative respiration traces in the CI + II-linked OXPHOS state by Oroboros 2k-Oxygraph. Substrate and coupling states (Purple) are shown by horizontal axis. Blue curve indicates O₂ concentration in O₂k chamber, oxygen fluxes per chamber volume are depicted (red curve) for O₂k chamber operated simultaneously. The upper horizontal bar denotes the respiratory states (Routine, OXPHOS, Leak and electron transport system (ETS state), CI: complex I, CII: complex II

Fig. 4. Mitochondrial respiration of SSM and IFM subpopulations. (A) Basal mitochondrial respiration capacities (Routine), (B) Maximal phosphorylating respiration capacity via convergent input through complexes I and II (OXPHOS CI + CII). (C) Inhibition of the phosphorylation system by oligomycin (LEAK CI + CII). (D) Respiratory control ratio (RCR)