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Isolation of Mitochondria from Mouse Skeletal Muscle for Respirometric Assays

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Abstract

Most of the cell's energy is obtained through the degradation of glucose, fatty acids, and amino acids by different pathways that converge on the mitochondrial oxidative phosphorylation (OXPHOS) system, which is regulated in response to cellular demands. The lipid molecule Coenzyme Q (CoQ) is essential in this process by transferring electrons to complex III in the electron transport chain (ETC) through constant oxidation/reduction cycles. Mitochondria status and, ultimately, cellular health can be assessed by measuring ETC oxygen consumption using respirometric assays. These studies are typically performed in established or primary cell lines that have been cultured for several days. In both cases, the respiration parameters obtained may have deviated from normal physiological conditions in any given organ or tissue.

Additionally, the intrinsic characteristics of cultured single fibers isolated from skeletal muscle impede this type of analysis. This paper presents an updated and detailed protocol for the analysis of respiration in freshly isolated mitochondria from mouse skeletal muscle. We also provide solutions to potential problems that could arise at any step of the process. The method presented here could be applied to compare oxygen consumption rates in diverse transgenic mouse models and study the mitochondrial response to drug treatments or other factors such as aging or sex. This is a feasible method to respond to crucial questions about mitochondrial bioenergetics metabolism and regulation.

Introduction

Mitochondria are the primary metabolic organelles in the cell[1]. These specialized membrane-enclosed organelles use nutrient molecules to produce energy in the form of adenosine triphosphate (ATP) by OXPHOS. This process relies on the transfer of electrons from donor molecules in a series of redox reactions in the ETC[2]. CoQ is the only redox-active lipid

that is endogenously produced in all cellular membranes and circulating lipoproteins that shows antioxidant function[3]. It is an essential component of the ETC, transferring electrons from NADH-dependent complex I and $FADH_2$-dependent complex II to complex III, although many other reductases can drive the reduction of mitochondrial CoQ to ubiquinol as a mandatory step in multiple cellular metabolic pathways[4,5].

Throughout the process, an electrochemical proton gradient is created across the mitochondrial inner membrane, which is transformed into biologically active energy by the ATP synthase complex V[2]. Consequently, mitochondrial dysfunction leads to a myriad of pathological conditions mainly affecting tissues with high-energy requirements- the brain, heart, and skeletal muscle[6,7]. Therefore, it is fundamental to develop methods to accurately analyze mitochondrial bioenergetics to investigate its role in health and disease, particularly in highly energetic tissues such as skeletal muscles.

The Clark-type oxygen electrode has been classically used in the study of mitochondrial respiration[8]. However, this system has been progressively displaced by higher-resolution technologies, with microplate-based oxygen consumption technologies such as Agilent Seahorse XF analyzers being especially popular[9]. In the skeletal muscle field, these studies are typically conducted in cultured cells, mainly in the C2C12 immortalized mouse myoblast cell line or primary cultures derived from satellite cells[10,11]. However, these studies do not fully recapitulate the situation *in vivo*, especially when investigating mitochondrial biology and function at the tissue level upon specific insults, nongenetic interventions, or genetic manipulations.

Furthermore, respiration assays in cells are more complex due to additional factors, including extra-mitochondrial demand of ATP and assay substrates or signaling events, which could mislead the interpretation of the results. Alternatively, it is also possible to use single or bundles of freshly isolated myofibers from muscles. However, the isolation method is technically challenging and only feasible for a few muscle types. In this case, flexor digitorum brevis (FDB) and extensor digitorum longus (EDL) muscles are mainly used[10,12,13], although a few reports describe the use of other muscle types as well[14,15].

Bioenergetic profiling of skeletal muscle sections has also been reported[16]. The major advantage of this method is that intact muscles can be studied (the authors show that slicing through fibers does not disturb results when compared with isolated myofibers). However, mitochondrial access to substrates and assay inhibitors is limited, and thus, only a few parameters can be measured[16]. Finally, isolated mitochondria can be likewise employed[9,17,18,19]. In this case, mitochondria lose their cytosolic environment, which could affect their function. In contrast, this method guarantees access to substrates and inhibitors, enables the analysis of a plethora of sample types, and typically requires less material.

This paper describes a method to perform the bioenergetic profiling of isolated mitochondria from mouse skeletal muscle using microplate-based respirometric assays (**Figure 1**). In particular, three protocols are detailed: the Coupling Assay, CA to assess the degree of coupling between the ETC and the OXPHOS machinery; the Electron Flow Assay, EFA to measure the activity of the individual ETC complexes; and the BOX assay to determine mitochondrial β-oxidation capacity. Notably, only small amounts of samples are required compared with conventional respirometry methods. The isolation protocol used here has been modified from the method published elsewhere[18].

Protocol

Mouse housing and tissue collection were performed using protocols approved by the Universidad Pablo de Olavide Ethics Committee (Sevilla, Spain; protocols 24/04/2018/056 and 12/03/2021/033) in accordance with Spanish Royal Decree 53/2013, European Directive 2010/63/EU, and other relevant guidelines.

1. Preparation of stocks, buffers, and reagents for the respiration assays

1. Prepare the following stock solutions, which can be stored at the indicated temperature for months. Use ultrapure H_2O in all cases.
    1. Dissolve phosphate-buffered saline (PBS) tablets in H_2O (1 tablet per 200 mL of H_2O) to prepare 1x PBS. Autoclave the solution and store it at room temperature (RT).
    2. Dissolve 2 g of NaOH pellets in 50 mL of H_2O to prepare 1 M NaOH.
    3. Prepare 0.1 M, 1 M, 5 M, and 10 M KOH stocks for pH calibration.
    4. Add 14.612 g of EDTA to 70 mL of H_2O. Add NaOH pellets until the pH reaches 8.0 so that EDTA fully dissolves. Add H_2O *quantum satis* (QS) to 100 mL. Autoclave the resulting 0.5 M EDTA (pH 8) solution and store it at RT.
    5. Add 19 g EGTA to 70 mL of H_2O. Add NaOH pellets until the pH reaches 8.0 to allow the EGTA to dissolve fully. Add H_2O QS to 100 mL. Autoclave the resulting 0.5 M EGTA (pH 8) solution and store it at RT.
    6. Add 2 mL of 0.5 M EDTA to 98 mL of PBS. Store the resulting 10 mM EDTA/PBS solution at RT.
    7. Dissolve 5.958 g of HEPES in 40 mL H_2O. Adjust the pH to 7.2 with KOH and QS to 50 mL with H_2O. Filter the resulting 0.5 M HEPES buffer through a 0.45 µm mesh and store it at RT.
    8. Dissolve 3.402 g of KH_2PO_4 in up to 50 mL of H_2O. Filter the resulting 0.5 M KH_2PO_4 solution through a 0.45 µm mesh and store it at RT.
    9. Dissolve 9.521 g of anhydrous $MgCl_2$ in up to 100 mL of H_2O. Autoclave the resulting 1 M $MgCl_2$ solution and store it at RT.
    NOTE: As this is an exothermic reaction, proceed with caution and dissolve the $MgCl_2$ on ice.
2. Prepare substrate and inhibitor stock solutions (see **Table 1**). Aliquot and store them at -20 °C. Avoid freeze-thaw cycles. As an exception, always prepare pyruvic acid immediately before use.
    1. Prepare in ultrapure H_2O: 0.5 M succinate, 0.5 M pyruvic acid, 0.5 M malic acid, 100 mM ADP, 1 M ascorbic acid, and 50 mM *N,N,N′,N′*-tetramethyl-para-phenylene-diamine (TMPD) (protect from light).
    2. Prepare in 100% dimethyl sulfoxide (DMSO): 50 mM palmitoyl-L-carnitine, 4 mM rotenone, 20 mM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 4 mM oligomycin, and 5 mM Antimycin A.
    NOTE: Do not exceed 0.1% final DMSO concentration in the microplate wells. Therefore, prepare highly concentrated stock solutions to stay below this limit.

3. Prepare the buffers for mitochondria isolation and protein quantification freshly on the day of the experiment. Use ultrapure H_2O in all cases and keep all buffers on ice unless otherwise stated.

   NOTE: To save time, all reagents can be weighed and kept in the appropriate containers (e.g., 15 mL tubes) at the right temperature on the previous day.

   1. To prepare 10% free fatty acids (FFA) BSA, thoroughly dissolve 150 mg of FFA BSA in 1.5 mL of H_2O by inversion/rotatory wheel. Do not vortex to avoid foaming.
   2. To prepare 1x Bradford reagent, dilute the 5x commercial stock solution with H_2O and keep it in the dark at RT.
   3. To prepare 8x Mitochondria Buffer (MB), dissolve 4.112 g of sucrose and 763 mg of HEPES in 15 mL of H_2O. Adjust the pH to 7.2, add 1.6 mL of 10% FFA BSA and QS to 20 mL with H_2O.
   4. To prepare 20 mL of Isolation Buffer 1 (IB1) (4 mL used per sample), dissolve 400 µL of 0.5 M EDTA, 784 mg of D-mannitol, and 2.5 mL of 8x MB in 15 mL of H_2O. Adjust the pH to 7.2 and QS with H_2O.
   5. To prepare 5 mL of Isolation Buffer 2 (IB2) (500 µL used per sample), dissolve 30 µL of 0.5 M EGTA, 196 mg of D-mannitol, and 625 µL of 8x MB in 4 mL of H_2O. Adjust the pH to 7.2 and QS with H_2O.
   6. To prepare 5 mL of Resuspension Buffer (RB) (200 µL used per sample), dissolve 120 mg of sucrose, 191.3 mg of D-mannitol, 50 µL of 0.5 M HEPES, and 10 µL of 0.5 M EGTA in 4 mL of H_2O. Adjust the pH to 7.2 and QS with H_2O.
   7. To prepare 2x Mitochondrial Assay Solution-1 (MAS-1), dissolve 1.199 g of sucrose, 2.8 g of mannitol, 1 mL of 0.5 M KH_2PO_4, 250 µL of 1 M $MgCl_2$, 200 µL of 0.5 M HEPES, and 100 µL of 0.5 M EGTA in 20 mL of H_2O. Adjust the pH to 7.2 and QS to 25 mL with H_2O. Keep in ice for short periods. For longer periods, keep it at 4 °C to avoid precipitation.
   8. To prepare 1x Coupling Assay medium (CAM), prepare two different MAS-1-based buffers: 1) CAM +BSA for the CA assay proper, and 2) CAM-BSA for preparing the assay inhibitors. Always keep the pyruvate:malate ratio at 10:1.

      NOTE: As BSA can clog the injection ports, dilute the inhibitors in BSA-free CAM.

      1. To prepare CAM+BSA, dilute 300 µL of 0.5 M pyruvic acid, 30 µL of 0.5 M malic acid, and 7.5 mL of 2x MAS-1 in 6 mL of H_2O. Adjust the pH to 7.2. Add 300 µL of 10% FFA BSA and QS to 15 mL with H_2O.
      2. To prepare CAM-BSA, dilute 120 µL of 0.5 M pyruvic acid, 12 µL of 0.5 M malic acid, and 3 mL of 2x MAS-1 in 2 mL of H_2O. Adjust the pH to 7.2 and QS to 6 mL with H_2O.
   9. To prepare 1x Electron Flow Assay medium (EFAM), prepare both BSA-containing and BSA-free EFAM buffers.
      1. To prepare EFAM+BSA, dilute 300 µL of 0.5 M pyruvic acid, 60 µL of 0.5 M malic acid, 3 µL of 20 mM FCCP, and 7.5 mL of 2x MAS-1 in 6 mL of H_2O. Adjust the pH to 7.2. Add 300 µL of 10% FFA BSA and QS to 15 mL with H_2O.

2. To prepare EFAM-BSA, dilute 120 µL of 0.5 M pyruvic acid, 24 µL of 0.5 M malic acid, 1.2 µL of 20 mM FCCP, and 3 mL of 2x MAS-1 in 2 mL of H_2O. Adjust the pH to 7.2 and QS to 6 mL with H_2O.

10. To prepare 1x β-oxidation medium (BOXM), prepare BSA-containing and BSA-free BOXM solutions.
    1. To prepare BOXM+BSA, dilute 12 µL of 50 mM palmitoyl-L-carnitine, 30 µL of 0.5 M malic acid, and 7.5 mL of 2x MAS-1 in 7 mL of H_2O. Adjust the pH to 7.2. Add 300 µL of 10% FFA BSA and QS to 15 mL with H_2O.
    2. To prepare BOXM-BSA, dilute 4.8 µL of 50 mM palmitoyl-L-carnitine, 12 µL of 0.5 M malic acid, and 3 mL of 2x MAS-1 in 2 mL of H_2O. Adjust the pH to 7.2 and QS to 6 mL with H_2O.

2. Muscle dissection, homogenization, and mitochondrial isolation

1. Place all materials and buffers on ice. Ensure all materials are ice-cold during the entire procedure to protect mitochondria from damage.
2. Place three 50 mL beakers per sample on ice, and add the following solutions: 10 mL of PBS in beaker 1, 10 mL of 10 mM EDTA/PBS in beaker 2, and 4 mL of IB1 in beaker 3.
3. Euthanize the mouse by cervical dislocation. Avoid CO_2 euthanasia as skeletal muscles could become hypoxic, interfering with respiration analyses.
4. Spray the right hindlimb with 70% ethanol to prevent fur from shedding, and tape limb to the cork dissection board. Tape the left forelimb as well.
5. Make an incision with a sterile disposable scalpel through the skin from knee to toe.
6. Grip the skin with toothed forceps at ankle level and cut it with fine scissors around the ankle.
7. Ease the skin away from the underlying musculature with fine-tip tweezers in one hand while pulling it up with toothed forceps in the other hand.
8. Dissect all skeletal muscles from ankle to knee (**Figure 2**).
    1. Remove all connective tissue over the tibialis anterior (TA) muscle to facilitate muscle extraction using fine tweezers and scissors.
    2. Find the four distal tendons of the EDL muscle and section them close to their insertions in the toes. Locate the TA distal tendon and cut it close to its insertion, always below the ankle.
    3. Carefully pull the TA and EDL tendons above the ankle to liberate the loose ends.
    4. Grip the loose ends of the tendons and ease the muscles away from the rest of the musculature and bones by pulling them up. Use fine tweezers or scissors to facilitate the process. Proceed with care to avoid myofiber contraction.
    5. Cut the proximal tendon of the EDL and the TA muscle as close as possible to the kneecap.
    6. Turn the mouse upside down to proceed with gastrocnemius (GA) and soleus muscle extraction.
    7. Grip the pocket formed between the biceps femoris and the GA with toothed forceps. Use fine scissors to separate these muscles and visualize the proximal GA tendon.

8. Grip the Achilles tendon with fine forceps and carefully cut it with fine scissors. Release the GA and soleus muscles from the underlying bone by pulling them up through their tendons. Cut the proximal GA tendon liberating it together with the underlying soleus.
9. Carefully dissect the remaining muscles from tendon to tendon following the same procedure until only bones remain.
10. Re-pin the mouse in the initial position to dissect the quadriceps muscle.
11. Discard the adipose tissue over the quadriceps at the proximal side using toothed forceps and fine scissors.
12. Insert fine tweezers between the quadriceps and the femur and move them in both directions along the femur axis to separate the muscle from the bone.
13. Grip the distal quadriceps muscle tendons with toothed forceps and cut the tendon with fine scissors as close as possible to the kneecap.
14. Pull the quadriceps up and liberate it at the proximal insertion with fine scissors.

9. Repeat steps 4-8 from this section with the left hindlimb.
10. Rinse all muscles in Beaker 1 first and then in Beaker 2.
11. Transfer all muscles to Beaker 3 and finely mince all muscles with sharp scissors on ice.
12. Transfer the suspension to a C Tube (purple lid), always keeping it in ice.
13. Tightly close the C Tube and attach it upside down onto the sleeve of the homogenizer. Make sure that the sample material is in the area of the rotator/stator. Select the 1-min program called **m_mito_tissue_01**.
14. Divide the homogenate into two 2 mL prechilled microcentrifuge tubes and centrifuge at 700 × *g* for 10 min at 4 °C in a tabletop centrifuge.
15. Transfer the supernatants to new 2 mL prechilled tubes, carefully avoiding fat and non-homogenized tissue. Store the pellets at -80 °C for fragmentation purity determination (fraction N) (**Figure 3**).
16. Centrifuge the supernatants at 10,500 × *g* for 10 min at 4 °C.
17. Transfer the supernatants to new 2 mL prechilled tubes and label them as Supernatant Number 1 (SN1). Store them at -80 °C for fragmentation purity determination (**Figure 3**).
18. Resuspend and combine both pellets in a total volume of 500 µL of IB2 in ice.
19. Centrifuge at 10,500 × *g* for 10 min at 4°C.
20. Transfer the supernatant to a new 2 mL prechilled tube and label it as Supernatant Number 2 (SN2). Store it at -80 °C for fragmentation purity determination (**Figure 3**).
21. Resuspend the final mitochondrial pellet in 200 µL of RB. Quickly set aside 10 µL for protein quantification and immediately add 10 µL of 10% FFA BSA to the remaining mitochondrial suspension to prevent damage.
22. Determine protein concentration using the Bradford assay.
    1. For the standard curve, prepare 30 µL of serial dilutions of known concentration of a protein in RB buffer. For example, use 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, and 0 mg/mL of BSA.

2. Prepare 1:3 and 1:6 serial dilutions of the mitochondrial samples in RB buffer.
3. In a 96-well flat-bottom plate, first load 2.5 µL of sample/standard per well. Next, add 10 µL of 1 M NaOH to each sample and, finally, 200 µL of 1x Bradford reagent. Mix well, avoiding air bubbles. Check visually that the color of the samples is within the calibration line. Always perform the analysis in triplicate.
4. Incubate the plates for 5 min at RT in the dark, and read the absorbance at 595 nm in a microplate spectrophotometer.
5. Calculate the standard curve by plotting the absorbance values (y-axis) versus the corresponding protein concentration of the dilutions prepared in step 22.1 in this section (x-axis).
6. Calculate the total amount of protein in the mitochondrial samples by extrapolation with the standard curve.

3. Preparation of the microplate-based respirometric assays

1. Hydrate the respirometric assay sensor at least 12 h before the experiment with 1 mL of calibration buffer per well. Incubate at 37 °C (no CO_2).

   NOTE: Hydrated sensors can be used for up to 72 h. The cartridge should be handled carefully during this step: if anything touches the sensors, measurement sensibility could be affected.
2. Prechill the preparative centrifuge with the swinging bucket microplate rotor and the corresponding microplate adapters at 4 °C.
3. Turn on the computer, open the analysis software, and select the desired protocol.

   NOTE: A click is heard when the software connects to the oxygen consumption-measuring instrument. The heating sensor should be green and at 37 °C before the experiment starts.
4. Prepare the inhibitor solutions from the stocks indicated in section 1, step 2 according to the assay to be performed. Prepare enough volume for each solution. See **Table 1** and **Table 2** for reference.
5. Add the appropriate volume of each inhibitor in each port, insert the cartridge into the oxygen consumption-measuring instrument, and start calibration.

   NOTE: Adding inhibitor to the cartridge and inserting the cartridge into the instrument takes15-20 min. Ensure the correct cartridge components are introduced. The cartridge lid and hydro booster could be discarded while sensor cartridge and utility place are needed.
6. Centrifuge the concentrated mitochondrial suspension of section 2, step 21 at 10,500 × *g* for 10 min at 4 °C.
7. Resuspend the mitochondrial pellet in 100 µL of 1x CAM +BSA, 1x EFAM+BSA, or 1x BOXM+BSA, depending on the protocol to be performed.
8. Further dilute the concentrated mitochondrial sample to a final 0.2 µg/µL concentration in the corresponding assay medium.
9. Seed 50 µL of the suspension (total 10 µg of protein) per well in a prechilled 24-well microplate on ice. Do not add mitochondria in the background correction wells; only add the corresponding assay medium. Store the remaining mitochondrial suspension at -80 °C for fragmentation purity determination (**Figure 3**).

10. Spin the microplate in the prechilled preparative centrifuge at 2,000 × *g* for 20 min at 4 °C. Counterweight to balance accordingly.
11. Warm the remaining assay medium at 37 °C during microplate centrifugation.
12. After centrifugation, leave the microplate on the bench for 5 min to equilibrate. Add 450 µL of warm assay medium for a final volume of 500 µL per well at RT. Do this slowly and carefully, adding the medium to the wall of the wells to avoid detaching mitochondria.
13. Place the microplate immediately in the oxygen consumption-measuring instrument without the lid, and start the protocol (**Table 3**). Ensure that the first step is a 10 min incubation to allow the microplate to warm.
14. Once the experiment is finished, remove the cartridge and microplate, switch off the instrument, and start the analysis.

4. Analysis of the results

1. In the case of the CA and BOX assays, perform the following analyses:
    1. Record nonmitochondrial O_2 consumption, which corresponds to the mean of the values obtained after Antimycin A and rotenone injection.
    NOTE: Antimycin A and rotenone are complex III and I inhibitors, respectively.
    2. Calculate basal respiration by subtracting nonmitochondrial O_2 consumption from basal values (measurement points 1 and 2).
    3. Subtract nonmitochondrial O_2 consumption from the values after the injection of the complex V substrate ADP (injection A) to determine mitochondrial state III.
    4. Subtract nonmitochondrial O_2 consumption from the respiration values post injection of the complex V inhibitor oligomycin (injection B) to obtain mitochondrial state IVo.
    5. Calculate mitochondrial state IIIu by deducting nonmitochondrial O_2 consumption from respiration post FCCP injection (injection C).
    NOTE: FCCP is a potent mitochondrial oxidative phosphorylation uncoupler. ATP synthesis is bypassed in its presence, and the ETC attains maximal activity.
    6. Subtract basal respiration values from the mitochondrial state IIIu values to obtain mitochondrial spare capacity, which is the capacity to generate extra ATP in case of increased energy demand.
    7. Divide mitochondrial state IIIu by state IVo values to obtain the Respiratory Control Ratio (RCR).
    NOTE: Negative or null RCR values indicate that mitochondrial coupling is affected.
2. In reference to EFA, perform the following calculations:
    1. Obtain residual activity by calculating the mean value post rotenone and Antimycin A injection (injections A and C).
    2. Calculate complex I to IV (CI-CIV) activity by subtracting residual activity from the basal values (measurement points 1 and 2).
    3. Subtract residual activity from the values post injection of the CII substrate succinate (injection B) to obtain CII-CIII-CIV activity.

4. Calculate CIV activity by deducting the residual activity from the values obtained after injecting the cytochrome c-reducing agents, ascorbic acid and TMPD (injection D).

3. Represent all results using bar plots, as in **Figure 4**, to extract appropriate conclusions.

Representative Results

The protocol presented here allows the *in vivo* analysis of mitochondrial respiration through the isolation of mitochondria from mouse skeletal muscle. An outline of the method is shown in **Figure 1**. After dissecting skeletal muscles from the hindlimbs (**Figure 2**), tissues are homogenized and mitochondria purified, under isotonic conditions, through serial centrifugations. The purity of the different fractions obtained during the isolation process can be analyzed by western blot using antibodies against different organelle markers. **Figure 3A** shows the general protein profile from the different fractions showing distinct protein populations in the fractions isolated.

Figure 3B shows that all mitochondrial content is in the mitochondrial fraction, as evidenced by the strong signals for markers of the outer (VDAC and TOMM20) and inner (TIMM23) mitochondrial membranes, and even for nucleoid-associated proteins (mtTFA). All nuclei and cytoskeleton (β-actin) are present in Fraction N, which represents nuclei and undisrupted material (**Figure 3C**). Moreover, most of the endoplasmic reticulum (ER) (Calnexin), plasma membrane (Na^+/K^+-ATPaseα1), or cytoplasm markers (LDHA, HSP70, or AKT) remain in the SN1 or SN2 fractions, highlighting the high purity obtained during isolation (**Figure 3C**). However, there are a few traces of ER contamination in the mitochondrial fraction, possibly due to the proximity of these organelles. Given the results obtained in subsequent respiratory assays, the isolation procedure described here yields highly pure, viable mitochondrial preparations.

Both CA and BOX assays permit the calculation of different mitochondrial states in the presence of several substrates that stimulate specific metabolic pathways. Specifically, these assays are performed in the presence of pyruvic acid or palmitoyl-L-carnitine chloride. Pyruvic acid is a substrate of the Krebs cycle; malic acid is a Krebs cycle intermediary and an NADH inductor, while palmitoyl-L-carnitine is a substrate of the fatty acid β-oxidation pathway. (**Figure 4A,B**). In both assays, the first state that can be measured is the basal respiration rate, which represents mitochondrial respiration in the presence of the substrates initially added to the assay medium. Then, mitochondrial State III is achieved, which represents ATP production from ADP and inorganic phosphate, showing the maximal respiration in a coupled state. Thus, there is an increase in oxygen consumption after ADP addition, as observed in **Figure 4A,B**.

Further, State IVo indicates the proton leak associated with the inhibition of ATP synthase by oligomycin, which leads to a decrease in respiration, as observed in the CA and BOX graphs (**Figure 4A**,**B**). The addition of FCCP leads to State IIIu, which shows the maximal respiratory capacity that mitochondria can display in an uncoupled state when oxygen consumption reaches its highest level in these assays. To calculate all these states, it is fundamental first to determine nonmitochondrial respiration, which is obtained at the end of the assay when Antimycin A and rotenone are injected to inhibit oxidative respiration. As shown in **Figure 4A**,**B**, these values need to be always below basal respiration and very close to zero in the case of assays with isolated mitochondria like these assays described here. Finally, the RCR parameter

must be calculated to assess mitochondrial integrity. It reflects whether mitochondria can react to ADP by producing ATP at a high rate with a low proton leak. The mean RCR values found in the CA and BOX assays were 5.78 ± 1.03 and 3.47 ± 0.42 (**Figure 4A,B**), respectively, which are in agreement with optimal RCRs reported previously[21,22,23,24].

Additionally, the EFA examines the activity of the ETC complexes individually or in combination through the injection of specific substrates and inhibitors (**Figure 4C**). CI-CIV activity represents mitochondrial respiration based on pyruvate and malate substrates when no complexes are inhibited; in this case, most of the electrons go through CI. As rotenone is a specific inhibitor of CI, CI is blocked after its addition, resulting in decreased oxygen consumption (**Figure 4C**). CII-CIII-CIV activity shows respiration when only CI is blocked, and CII is activated: succinate, injected through port B, is a specific substrate of complex II (succinate dehydrogenase). Thus, CII is reduced, initiating the electron flow from CII to CIV, while CI is still inhibited by the previous rotenone injection, producing an increase in oxygen consumption (**Figure 4C**). Antimycin A addition inhibits CIII, blocking the electron flow through the ETC and reducing oxygen consumption. Further, CIV activity is determined when it is stimulated by cytochrome C oxidation after being reduced by the addition of ascorbic acid/TMPD; **Figure 4C** shows that CIV activation produces an increase in oxygen consumption. Finally, residual activity refers to oxygen consumption when the oxidative phosphorylation chain is inactivated after rotenone and Antimycin A addition. This value establishes the background for the calculation of the activity of the complexes as indicated in protocol steps 2.2-2.4 in section 4.

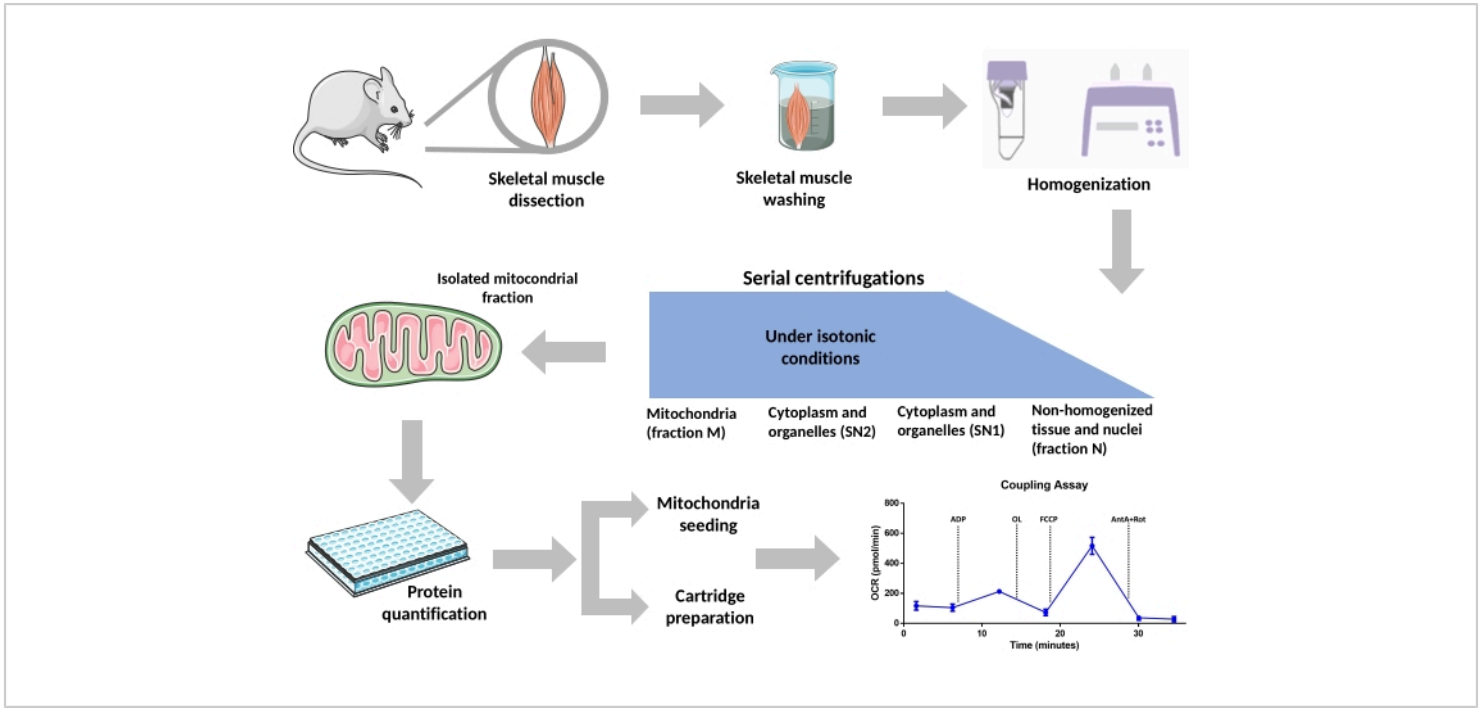

Figure 1: Schematic visualization of the method. Abbreviations: OCR = oxygen consumption rate; ADP = adenosine diphosphate; OL = oligomycin; FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; AntA = Antimycin A; Rot = rotenone. Please click here to view a larger version of this figure.

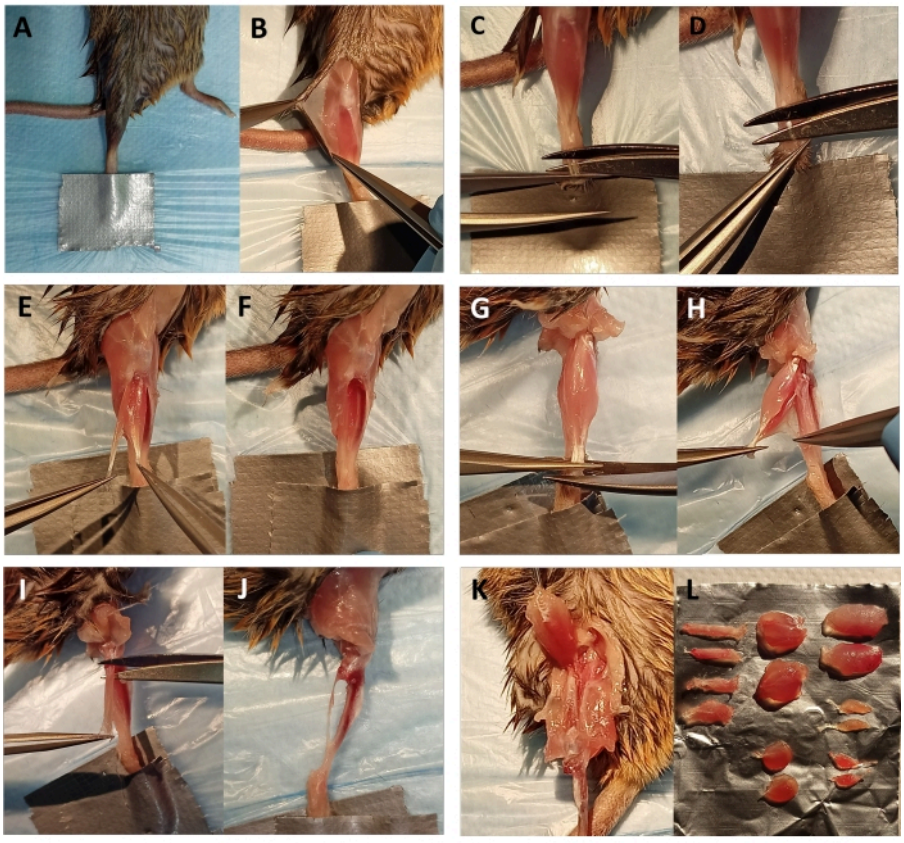

Figure 2: Dissection and isolation of murine skeletal muscles from hindlimbs for mitochondrial respiration analyses. (**A**) The right hindlimb was stretched and immobilized with tape. (**B**) An incision was made through the skin from next to the ankle, stopping proximal to the knee. Connective tissue overlying the TA was carefully removed. EDL (**C**) and TA (**D**) tendons were sectioned close to their insertions. The TA tendon was pulled up to ease the muscle away from the underlying musculature and bone. Then, the TA was cut proximally, close to the ventral crest to the tibia. Similarly, the EDL muscle was eased away, and the proximal tendon was meticulously cut at the side of the knee. (**F**) Right hindlimb after TA and EDL dissection. (**G**) In the posterior hindlimb, the Achilles tendon was sectioned to dissect GA and soleus muscles. (**H**) GA and soleus were carefully liberated from the tibial bone. (**I**) The remaining muscles were collected from ankle to knee until only the fibula and tibial bones remained. (**J**) Hindlimb after dissection. (**K**) Quadriceps was dissected. (**L**) All skeletal muscles collected for posterior mitochondrial isolation. The process was performed for both hindlimbs. Abbreviations: TA = tibialis anterior; EDL = extensor digitorum longus; GA = gastrocnemius. Please click here to view a larger version of this figure.

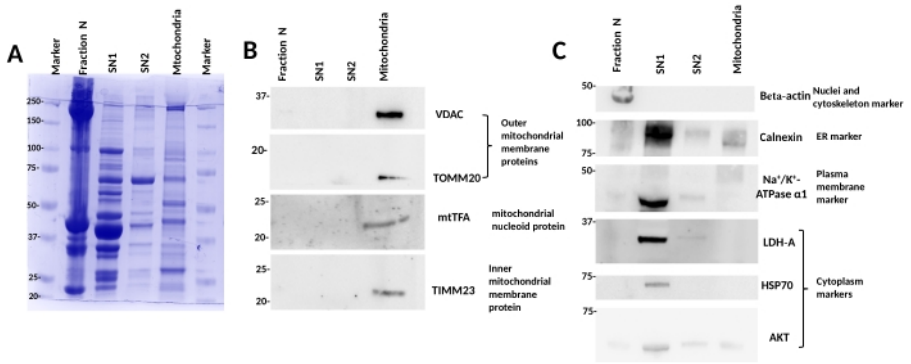

Figure 3: Purity of the fragmentation process. (**A**) General protein profile stained with Coomassie blue. (**B**) Mitochondrial protein purity profile. (**C**) Nonmitochondrial markers. The different fractions obtained during mitochondria isolation were loaded and incubated with antibodies against different proteins in specific subcellular fractions. Abbreviations: fraction N = non-homogenized tissue and nuclei; SN1 = supernatant number 1: cytoplasm + organelles; SN2 = supernatant number 2: cytoplasm + organelles; VDAC = voltage-dependent anion channel; TOMM20 = translocase of outer mitochondrial membrane 20; mtTFA = mitochondrial transcription factor A; TIMM23 = translocase of inner mitochondrial membrane 23; LDHA = lactate dehydrogenase A; HSP70 = heat shock protein 70; ER = endoplasmic reticulum. Please click here to view a larger version of this figure.

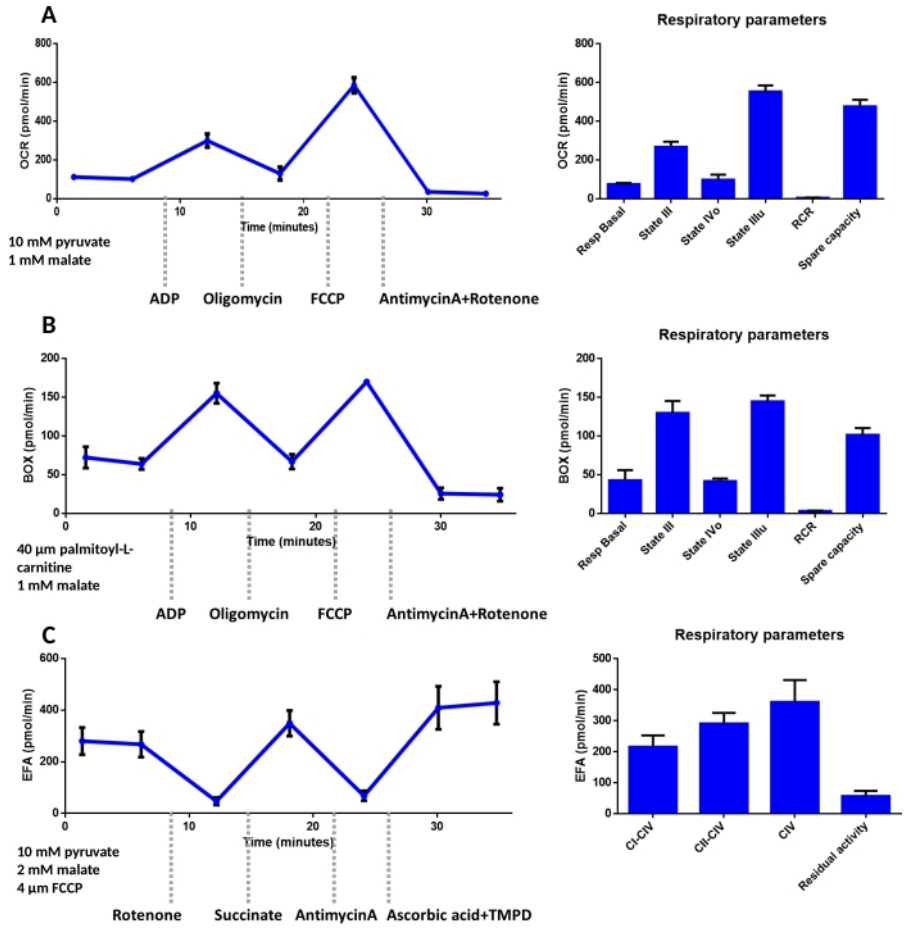

Figure 4: Representative results. Bioenergetic profiling of mitochondria isolated from hindlimb muscles of a 13-month-old male wild type C57BL/6N mouse. The injection points of the different substrates and inhibitors are indicated in each assay. The performance of the ETC and OXPHOS machinery can be analyzed using pyruvate and malate, or palmitoyl-L-carnitine and malate as substrates in the Coupling Assay (**A**) or β-oxidation of FA assays (**B**), respectively. (**C**) The electron flow assay allows the study of individual mitochondrial complexes in the presence of pyruvate, malate, and FCCP; 10 µg of mitochondria were plated per well. Results are displayed following the middle-point representation option to visualize results in an aggregated form. This is in contrast to the point-to-point option, which would enable the visualization of the 9 consecutive measurements usually performed during each measurement step. The type of visualization can be changed after completing the assay under the **Kinetic line chart** options in the analysis software. Abbreviations: FA = fatty acids; OCR = oxygen consumption rate; ADP = adenosine diphosphate; FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; RCR = respiratory control ratio; EFA = Electron Flow Assay; BOX = β-oxidation of FA.

Please click here to view a larger version of this figure.

Coupling Assay						
Port	Inhibitor	[Stock]	[Injection Port]	[Final]	Recipe	
					Inhib	CAM-BSA
A	ADP	100 mM	50 mM	5 mM	650 µL	650 µL
B	Oligomycin	4 mM	31.6 µM	3.16 µM	11.3 µL	1418.7 µL
C	FCCP	20 mM	60 µM	6 µM	4.68 µL	1555.32 µL
D	Antimycin A // Rotenone	5 mM // 4 mM	40 µM // 50 µM	4 µM // 5 µM	13.52 µL // 21.12 µL	1655.36 µL
Electron Flow Assay						
Port	Inhibitor	[Stock]	[Injection Port]	[Final]	Recipe	
					Inhib	EFAM-BSA
A	Rotenone	4 mM	50 µM	5 µM	16.25 µL	1283.7 µL
B	Succinate	500 mM	100 mM	10 mM	286 µL	1144 µL
C	Antimycin A	5mM	4 µM	4 µM	12.48 µL	1547.5 µL
D	Ascorbate // TMPD	1 M // 50 mM	100 mM // 1 mM	10 mM // 100 µM	169 µL // 33.8 µL	1487.2 µL
β-Oxidation Assay						
Port	Inhibitor	[Stock]	[Injection Port]	[Final]	Recipe	
					Inhib	BOX-BSA
A	ADP	100 mM	40 mM	4 mM	520 µL	780 µL
B	Oligomycin	4 mM	31.6 µM	3.16 µM	11.3 µL	1418.7 µL
C	FCCP	20 mM	60 µM	6 µM	4.68 µL	1555.32 µL
D	Antimycin A // Rotenone	5 mM // 4 mM	40 µM // 20 µM	4 µM // 2 µM	13.52 µL // 8.45 µL	1668 µL

Table 1: Preparation of inhibitor solutions for the different assays. The **[Stock]** column shows the concentrations of the stock solutions of the compound indicated in the **Inhibitor** column. **[Injection Port]** refers to the concentration of the inhibitor

solutions to be loaded in the different ports of the cartridge, while the **[Final]** column indicates the final concentration of the inhibitors in the wells. Finally, the **Recipe** columns specify the volumes of stock inhibitor solutions and BSA-free media that must be mixed to prepare the solutions to be loaded in the injection ports. Abbreviations: ADP = adenosine diphosphate; FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; TMPD = *N,N,N′,N′*-tetramethyl-para-phenylene-diamine; Inhib = inhibitor; BSA = bovine serum albumin; CAM-BSA = BSA-free coupling assay medium; EFAM-BSA = BSA-free electron flow assay medium; BOX-BSA = BSA-free β-oxidation of fatty acid assay medium.

Port	Volume injected	Stock prepared (26 wells)
A	50 µL	1300 µL
B	55 µL	1430 µL
C	60 µL	1560 µL
D	65 µL	1690 µL

Table 2: Calculation of injection volumes.

Action	Time (minutes)	Repetition	Port
Calibrate	15-20		
Wait	10		
Mix + Wait	1 + 3	× 2	
Mix	1		
Measure + Mix	3 + 1	× 2	
Inject			A
Mix	1		
Measure	3		
Mix	1		
Inject			B
Mix	1		
Measure	3		
Mix	1		
Inject			C
Mix	1		
Measure	3		
Mix	1		
Inject			D
Mix + Measure	1 + 3	× 2	

Table 3: Protocol settings for the different assays.

Discussion

All methods used to study mitochondrial respiration have their limitations; hence, it is crucial to select the method that best suits a specific experimental question. This work provides an updated and detailed protocol to isolate mitochondria from mouse skeletal muscle to perform different respiratory assays to investigate mitochondrial function. Indeed, the study of mitochondrial bioenergetics in isolated mitochondria using microplate-based technologies is valuable to study tissue-specific respiration in terms of reproducibility, reliability, and complexity. Further, the use of isolated mitochondria confers control over substrate availability, facilitating the understanding of the underlying biological processes. Another positive aspect of using isolated mitochondria is that

it is possible to study State III because the ATP generated after ADP injection is not converted again into new ADP as most of the transmembrane ATPases are lost. Moreover, in the assays described here, oligomycin is added to block potential nonspecific ATP synthesis[20].

Several important considerations need to be highlighted for this protocol. First, isolated mitochondria are extremely sensitive and should be handled with care. The high content of sucrose and mannitol in the buffers used ensures the optimal osmotic conditions to protect the isolated mitochondria from damage. Nevertheless, the assay duration should be kept at a minimum to preserve mitochondrial viability and prevent detachment from the microplate once seeded. Thus, given that protocol steps, such as the preparation of assay media and substrate and inhibitor solutions, or the loading of the inhibitors in the respirometric assay cartridge, are time-consuming, every step should be perfectly coordinated. Further, unlike other methods based on enzymatic digestion[21,24] and mortar and pestle homogenization[17,19,21,23,24], the method described here relies on the use of an automated homogenizer, enabling fast and efficient sample homogenization. Importantly, mitochondrial suspensions are less sensitive if highly concentrated. Thus, only prepare dilutions immediately before using them. Finally, it is essential to work quickly during the respirometric assay proper. Usually, when analyzing cells in culture in these assays, three consecutive measurement steps are performed after the injection of substrates or inhibitors, which results in protocols that extend between 40 and 50 min., To ensure the viability of the isolated mitochondria throughout the whole procedure, the number of measurement steps is often kept to a minimum due to their vulnerability[19,23,25], reducing the time it takes to complete the respirometric analysis to ~20 min. The protocols described in this work (**Table 3**) follow this trend, even though other previously published methods report efficient OCR profiles with longer and more standard measurement steps[26].

For example, if two consecutive assays are going to be run with the same set of samples on the same day, prepare all reagents for the second assay during the first one except for the mitochondrial dilutions: only dilute, seed, and spin mitochondria immediately before starting the second assay, while the instrument is being calibrated. Once the experiment is completed, the RCR parameter (section 4, step 1.7) must be calculated to assess the quality of the mitochondrial preparations used. Usually, RCR values between 3.5 and 5 denote that mitochondrial integrity is optimal and show functional coupled oxidative respiration[25]. Assays with lower RCR values must be discarded because mitochondrial integrity was compromised during isolation. In these cases, consider washing the mitochondrial pellet twice (section 2, steps 19-21)[22], as previously reported.

Second, maintaining the correct pH throughout the procedure is critical to obtaining successful results. Ensure that the pH of all solutions is set as indicated, that is, pH 7.2, and that the pH is always adjusted with KOH unless otherwise stated. Notably, the pH of BSA-containing buffers should not be directly measured with the pH meter as BSA can damage the probe. Thus, adjust the pH in the corresponding buffers before adding BSA. To avoid changes in the pH due to BSA addition, always use FFA BSA. Further, some ultrapure H_2O batches could be acidic, so the use of commercially purified, pH 7 H_2O is recommended. Furthermore, it is necessary to ensure that the pH meter is correctly calibrated and that it is the appropriate model to measure the pH of the chemicals to be prepared.

Third, it is crucial to correctly perform the sample collection step for protein measurement of the final mitochondrial suspension (section 2, step 21) to prevent mitochondrial destruction. Resuspend the pellet quickly but thoroughly, collect an aliquot, and add FFA BSA to the remaining suspension to conserve the osmotic balance and protect the mitochondria from damage. If FFA BSA were already present in the resuspension buffer, and thus, during protein quantification, the high protein content would confound the quantification results. This is why FFA BSA needs to be added after setting an aliquot aside for quantification.

Fourth, this method is highly versatile and can be adjusted to the experimenter's needs. For example, if the animal model under study is characterized by reduced muscle mass (e.g., sarcopenic mice), additional muscles can increase mitochondrial yield. Although an adult wild-type male C57BL/6N mouse was used in this work, animals of any gender, age, or genetic background, as well as specific muscle types, can be employed instead.

Fifth, respiratory values can differ between experiments due to slight variations in the composition of buffers and reagents, which need to be prepared fresh every time. Age, sex, genetic background, or environmental conditions can also have a profound impact on the results. However, a successful experiment should present increases in oxygen consumption after the injection of complex substrates such as ADP and succinate, decreases after the injection of inhibitors such as oligomycin or rotenone, and marked increases when the potent uncoupler FCCP is added. Moreover, to prevent high technical variability, ensure thorough resuspension of mitochondrial pellets to obtain homogeneous suspensions before seeding them in the microplate.

Sixth, if basal respiratory values are low, consider performing a titration experiment to adjust the amount of protein for seeding. Finally, because this work provides a method to obtain crude mitochondrial extracts, a certain grade of impurity associated with other organelles, mainly ER, is expected. A more aggressive fragmentation process to remove these impurities would be detrimental to mitochondrial viability, hindering the performance of respiratory assays. If purity is a concern, especially if it is not consistent between samples, assay results can be normalized relative to mitochondrial purity after running western blots against different organelle markers (**Figure 3**). To our knowledge, this is the first method that highlights concerns about mitochondrial purity in crude extracts. Usually, in other protocols for respirometric assays with isolated mitochondria, the same amount of protein extract is seeded in the wells to compare samples, assuming that the same amount of mitochondria is used in all cases. This is a reasonable assumption considering that isolation is performed in parallel in all samples. However, the genetic or environmental background of the samples under study may lead to considerable differences in the purity of the mitochondrial extracts. For example, if a given genetic mutation causes increased development of cytoplasmic ER, crude mitochondrial extracts from these animals could contain greater than expected ER content, and consequently, lower than expected mitochondrial protein. Thus, even if the same amount of total protein from control and mutant samples are seeded in the microplate, oxygen consumption of the mutants would be underestimated. Hence, it is recommended that the purity of the extracts of all the conditions under study must be determined. If the values are comparable, no normalization is needed. However, if purity values among samples differ considerably beyond the statistical error the

experimenter is willing to accept, they should be used to normalize oxygen consumption results.

The present protocol has some limitations. Of note, this method has been optimized for skeletal muscle tissue isolated from mice. Adjustments may need to be made if using different tissues or skeletal muscle from different species. Additionally, when analyses are performed on isolated mitochondria, the normal cellular microenvironment is lost due to the absence of cellular context and the other organelles that could interact with mitochondria. This could lead to results that slightly deviate from physiological conditions. Finally, as the mitochondria are centrifuged, they adhere to the bottom of the wells. While completely necessary, the potential effects of centrifugation on their normal state are unknown.

It is important to take into account that, after performing the respirometric assay analyses, a reduction in oxygen consumption associated with ATP-linked or FCCP-stimulated respiration in isolated mitochondria could indicate potential mitochondrial alterations that could be explained by[20]: 1) restricted transport of substrates across the inner mitochondrial membrane, 2) decreased activity of the enzymes involved in the rate-limiting reactions of specific metabolic pathways such as the ETC, Krebs cycle, or β-oxidation, or 3) impaired ETC function, among other possible explanations.

In summary, the updated method described here represents an adequate and reliable way to assess ETC and OXPHOS function from skeletal muscle tissue. It has multiple applications to evaluate how genetic modifications or the environment could affect mitochondrial respiration contributing to a specific phenotype. Remarkably, the present protocol could be adapted to other model organisms or be used with human samples to evaluate potential mitochondrial dysfunctions associated with human disease.

Disclosures

The authors declare that they have no conflicts of interest to disclose.

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