High-Resolution Respirometry for Mitochondrial Characterization of Ex Vivo Mouse Tissues

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This article describes methodologies to examine mitochondrial respiration in fresh preparations of mouse tissues, including skeletal muscle, heart, liver, white and brown adipose tissue, and brain. Reference values and tips to maximize experimental efficiencies are also provided. Finally, correction methods and complementary techniques to properly interpret the results are presented and contrasted. © 2015 by John Wiley & Sons, Inc.

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INTRODUCTION

Mitochondrial research has gained momentum due to numerous discoveries revealing the central role of mitochondria in diverse pathophysiological states (Wallace, 2005). Mitochondria serve many functions, not only as the major powerhouses for aerobic performance in the cell, but also in biosynthetic pathways, as well as in apoptotic and stress signaling. Consequently, there is great interest in the study of mitochondrial function and its possible dysfunctions that lead to—or result from—organismal disease.

Mitochondrial function has classically been assessed by measuring the levels and/or activity of isolated mitochondrial marker enzymes, such as citrate synthase, cytochrome *c* oxidase, $NADH_2$ oxidoreductase, and succinate dehydrogenase (Kelley et al., 1999; Simoneau et al., 1999; He et al., 2001; Kelley et al., 2002). Mitochondrial respiratory capacity, however, depends on factors other than just individual enzyme (or complex subunit) content. Rather, there is a qualitative element to mitochondrial respiratory capacity, relying on substrate control, coupling characteristics, post-translational modifications, or super-respiratory complex formation. Therefore, the direct measurement of mitochondrial oxidative phosphorylation might provide a more physiological readout of a tissue's mitochondrial function.

In this article, we describe how to assess oxidative phosphorylation system (OXPHOS) capacity by determining different respiratory states and respiratory control ratios in diverse mouse tissue preparations (e.g., skeletal muscle, heart, liver, white and brown adipose tissue, and brain) through the use of high-resolution respirometry. Basic Protocols 1 to 6 describe how to extract and prepare mouse tissues for respirometry assays. Basic Protocol 7 details a respirometry assay aimed to evaluate coupled and uncoupled respiration

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through Complex I and/or Complex II. The protocols are intended to be exemplifications and not exhaustive, due to space limitations, as it would be unreasonable to cover all mammalian tissues. Similarly, we will exemplify and compare OXPHOS performance of different tissues using a fixed experimental protocol. Needless to say, measurements using a classical Clarke electrode, XF^{e}96 or XF^{e}24 Analyzers (Seahorse Bioscience), or the main instrument used in these protocols (O2k Oxygraph, from Oroboros Instruments) allow multiple variations, so it will be necessary for users to tailor the experiment according to their individual needs. The method described for mouse tissues allows the user to measure, in a single run, uncoupled and coupled respiration through Complex I, the upper limits of respiration through simultaneous activation of Complex I and Complex II, maximal electron transport system capacity, and, finally, maximal Complex II respiratory output. In our experience, this methodology can be successfully applied to rat tissues, and variations of the procedures have also been applied to human samples.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

TISSUE EXTRACTION, STORAGE, AND PREPARATION

In the examples below (Basic Protocols 1 to 6), tissues are obtained from unconscious young (3- to 6-month-old) C57BL/6 J mice, following either injection of ketamine-xylazine or inhalation of isoflurane. Two main buffers are used, one for ex vivo tissue maintenance (BIOPS), and one for respirometry procedures (MIR). The compositions of these buffers are shown in Tables 1 and 2, respectively (see also *http://bioblast.at/images/3/3c/MiPNet03.02_Chemicals-Media.pdf*).

BASIC PROTOCOL 1

Skeletal Muscle Preparation for Respirometry Analysis

After dissection of the specific skeletal muscle to be used for high-resolution respirometry analysis (the most commonly used skeletal muscles are extensor digitorum longus, soleus, and gastrocnemius), the biological sample should be kept in ice-cold BIOPS.

If the muscle sample has to be stored, it is recommended that some preliminary tests be performed to assess the viability of the samples by performing respirometry experiments immediately after dissection of the specimen and at the time at which it is intended to perform the experiments, to be sure that mitochondrial performance is not compromised

Table 1 BIOPS Composition

Compound	Concentration
CaK_2EGTA anhydrous	2.77 mM
K_2EGTA, anhydrous	7.23 mM
Na_2ATP	5.77 mM
$MgCl_2$-$6H_2O$	6.56 mM
Taurine	20 mM
Na_2Phosphocreatine	15 mM
Imidazole	20 mM
Dithiothreitol (DTT)	0.5 mM
MES	50 mM

[a]Further detailed information on stocks preparation can be found online at *http://bioblast.at/images/3/3c/MiPNet03.02_Chemicals-Media.pdf.*

Table 2 MIR Buffer Composition

Compound	Concentration
EGTA	0.5 mM
$MgCl_2 \cdot 6H_2O$	3 mM
Lactobionic acid	60 mM
Taurine	20 mM
KH_2PO_4	10 mM
HEPES	20 mM
d-sucrose	110 mM
BSA, fatty acid free	1 g/liter

[a]Further detailed information on stocks preparation can be found online at *http://bioblast.at/images/3/3c/MiPNet03.02_Chemicals-Media.pdf*.

by storage. Skeletal muscle is a fairly stable tissue, and some reports have shown no difference in mitochondrial function when respirometry studies were performed several hours or days after dissection and preparation of the skeletal muscle from rodents, horses, and humans (kept at 0° to 4°C in BIOPS; Pesta and Gnaiger, 2012).

Skeletal muscle is composed of different fiber types. In mice, four different primary fiber types can be identified based on their myosin heavy chain composition (Type I, Type IIa, Type IIb, and Type IIx). Skeletal muscle fiber types differ in their contractile characteristics, but also and most importantly for the aim of this article, in their metabolic properties and oxidative capacities. Thus, in general, skeletal muscle mitochondrial density per fiber type varies according to the following pattern, from a higher to a lower content: Type I > Type IIa > Type X > Type IIb. Mitochondrial density is an important aspect to take into account to estimate the amount of sample used for high-resolution respirometry.

Materials

C57BL/6 J male mice, 12 weeks old, between 25 and 30 g body weight (Charles River Laboratories)
Isoflurane (inhalant anesthetic) or injectable solution, e.g., Rodent Combo Anesthetic III (ketamine 37.6 mg/ml; xylazine 1.92 mg/ml; acepromazine 0.38 mg/ml)
BIOPS (see recipe in Table 1), ice cold
Saponin (Sigma, cat. no. S7900)
Mitochondrial respiration medium (MIR; see recipe in Table 2)
95% O_2, 5% CO_2 medical gas mixture

Plastic 12-well plate (Corning, cat. no. CLS3512)
Surgical instruments:
- Scalpel handle [Fine Science Tools (FST), cat. no. 91003-12]
- Needle holder (FST, cat. no. 12503-15)
- Small scissors [Marcel Blanc et Cie SA (MB), cat. no. 3250.11]
- Medium scissors (FST, cat. no. 91401-12)
- Big scissors (MB, cat. no. 3010.14)
- Forceps (MB, cat. no. 2000.11)
- Small forceps (FST, cat. no. 91106-12)
- Two thin-edged forceps (MB, cat. no. 2240.05)

Filter paper
Analytical balance (Mettler-Toledo Intl., cat. no. XA 105DU)

O2k Oxygraph high-resolution respirometer (Oroboros Inc.)

1. Anesthetize the mouse, obtain the muscle of interest, and clean it of blood, tendons, and fat content.

2. After cleaning the muscle, place it in a 12-well plate containing 2 ml of ice-cold BIOPS and keep the dish on ice. Use a pair of sharp and thin forceps to skin the muscle fibers by gently separating muscle fiber bundles. Separate fiber bundles by gently pulling the extremes (from the tendon). Continue to separate the fiber bundles until it is not possible to further separate them without overtly breaking the muscle fibers.

   In general, 15 to 20 small muscle bundles can be obtained from a single EDL or soleus muscle. The use of a magnifying lens is highly recommended to allow better visualization of the sample. This skinning procedure facilitates muscle fiber permeabilization by saponin. Muscle bundle separation is a critical step in skeletal muscle sample preparation for high-resolution respirometry. This step of the procedure contributes to a wide range of inter-individual variability due to the skill of the experimenter in skinning the muscle and his/her assessment of when the samples are ready for permeabilization. A way to avoid variability is to use an alternative method to homogenize and permeabilize the muscle sample. The PBI-Shredder SG3 (Pressure BioSciences Inc.; see Alternate Protocol) can be used to homogenize the skeletal muscle sample with the option to add digitonin to the medium to be sure that the sample preparation is fully permeabilized. However, recent studies comparing both methodologies indicate that skeletal muscle fiber preparation by skinning the muscle in muscle bundles and the subsequent saponin-permeabilization is still the most optimal approach (Larsen et al., 2014).

3. Once further muscle bundles cannot be obtained, move them to another well of the 12-well plate containing 2 ml of ice-cold BIOPS and add 50 μg saponin per ml of BIOPS (Kuznetsov et al., 2004). Keep the muscle preparation on ice or at 4°C with gentle agitation for 30 min.

4. After permeabilization, transfer the muscle bundles to a new well of the 12-well plate with ice-cold MIR to wash the sample for 5 to 10 min (this step can be repeated twice).

5. Blot the muscle bundles on filter paper to remove residual external media.

6. Weigh the muscle preparation using a balance with sufficient precision to accurately measure 1 to 3 mg.

   For predominantly oxidative skeletal muscle (rich in Type I and IIa fibers), 1.0 to 1.4 mg of permeabilized skeletal muscle bundles is suggested per 2-ml chamber (O2k chamber volume), but in the case of skeletal muscle composed of fibers that are predominantly glycolytic (rich in Type IIx and IIb fibers), 1.7 to 2.2 mg of permeabilized skeletal muscle bundles per 2-ml chamber is recommended.

7. Transfer the muscle bundles to the O2k chamber filled with MIR (mitochondrial respiration medium).

8. Oxygenate MIR medium (to obtain MIR oxygen concentrations above what is obtained in equilibrium with air) inside the O2k chamber.

   Oxygen enrichment is achieved by generating an air phase on top of the medium in the closed chamber. The air phase is filled with gas (1 to 3 ml of 95% O_2, 5% CO_2 medical gas mixture is used) with a syringe connected to a needle long enough to reach the top of the chamber through the stopper (Fig. 1A). Once the medium reaches an oxygen concentration around 400 nmol O_2/ml, completely close the chamber. Oxygen enrichment takes few seconds and can be followed on the computer running the software supplied by the manufacturer (in this particular example, DatLab, Fig. 1).

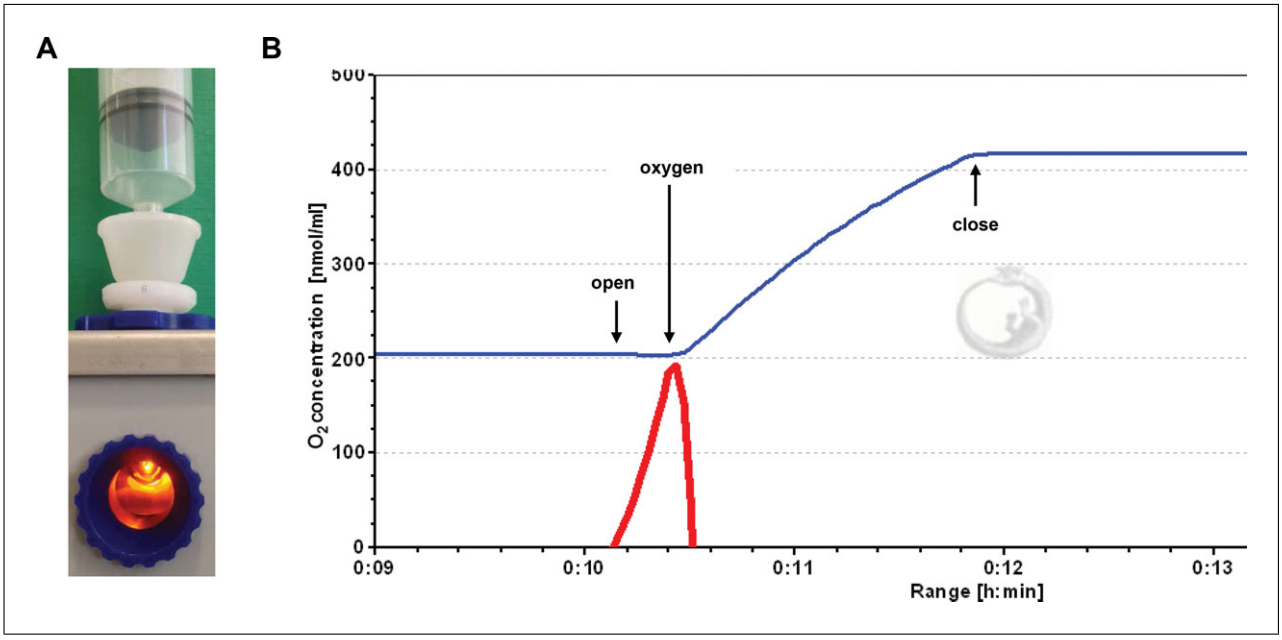

Figure 1 Oxygen enrichment of the mitochondrial respiration medium (MIR) using a medical gas mixture of 95% O_2 plus 5% CO_2. (**A**) Illustrative picture of the syringe filled with the medical gas mixture inserted in the chamber of the O2k respirometer. The tip of the needle is at the top of the chamber in and air phase. (**B**) Representative figure obtained from DatLab (software developed by Oroboros Instruments Corp.). In the figure, the *y* axis represents oxygen concentration, plotted in blue. Time is represented on the *x* axis. In the figure there are three labels: when the chamber was open to generate the air phase ("open"), when the oxygen was injected ("oxygen"), and when the chamber was closed after reaching the desired oxygen concentration ("close").

When working with skinned muscle preparations, O_2 diffusion can be a limiting factor in assessment of mitochondrial respiratory capacity. Therefore, instead of performing experiments starting at air O_2 concentrations, MIR should be enriched in O_2 above these levels. Experiments should be performed between 400 and 220 nmol O_2/ml to avoid limitations in respiration due to a decrease in O_2 diffusion (Gnaiger, 2003). Instruments should be calibrated to determine the background between these O_2 concentrations in order to correct for these values and obtain more precise results. Information on how to perform instrumental background calibrations can be found at the company Web site or in the user's guide of the specific instrument used for mitochondrial respiration determinations. For the methods described in this article, the O2k Oxygraph was used. Oroboros Instruments supplies information about standard operating procedures and protocols. The following link (http://www.oroboros.at/index.php?id=o2k-sop) provides specific information on how to perform background calibration at different O_2 saturation levels in the O2k Oxygraph.

9. Once the chamber is closed, let the system equilibrate for 5 to 10 min. At that point, begin respiratory measurements (Basic Protocol 7).

Preparation of Heart Tissue for Respirometry Studies

BASIC PROTOCOL 2

Heart tissue preparation for high-resolution respirometry studies can be performed using the same materials and following the same procedure used for skeletal muscle (Basic Protocol 1), with minor changes, as described below.

Before beginning this protocol, the experimenter must decide which part of the heart is of interest for the study (for example, left ventricle).

Materials

C57BL/6 J male mice, 12 weeks old, between 25 and 30 g body weight (Charles River Laboratories)

Isoflurane (inhalant anesthetic) or injectable solution, e.g., Rodent Combo Anesthetic III (ketamine 37.6 mg/ml; xylazine 1.92 mg/ml; acepromazine 0.38 mg/ml)
BIOPS (see recipe in Table 1), ice cold
Saponin (Sigma, cat. no. S7900)
Mitochondrial respiration medium (MIR; see recipe in Table 2)

Magnifying lens
Plastic 12-well plate (Corning, cat. no. CLS3512)
Surgical instruments:
- Scalpel handle [Fine Science Tools (FST), cat. no. 91003-12]
- Needle holder (FST, cat. no. 12503-15)
- Small scissors [Marcel Blanc et Cie SA (MB), cat. no. 3250.11]
- Medium scissors (FST, cat. no. 91401-12)
- Big scissors (MB, cat. no. 3010.14)
- Forceps (MB, cat. no. 2000.11)
- Small forceps (FST, cat. no. 91106-12)
- Two thin-edged forceps (MB, cat. no. 2240.05)

Filter paper
Analytical balance (Mettler-Toledo Intl., cat. no. XA 105DU)
95% O_2, 5% CO_2 medical gas mixture
O2k Oxygraph, high-resolution respirometer (Oroboros Inc.)

1. Anesthetize the mouse, dissect out its heart, isolate myocardium (cardiac muscle) from the pericardium (the membrane covering the heart), and cut the specific part of the myocardium for the study into small pieces.
2. Skin the sample into muscle bundles as for skeletal muscle (Basic Protocol 1) under a magnifying lens.

   Cardiac muscle and skeletal muscle both have microfibrils arranged in sarcomeres; however, cardiac muscle bundles are organized in networks rather than unidirectionally, and their fiber length is shorter than in most skeletal muscles.

3. Perform saponin-permeabilization of the cardiac muscle bundles as described for skeletal muscle (Basic Protocol 1, step 3), but only for 15 min.
4. Proceed as in Basic Protocol 1 (steps 4, 5, and 6) by carefully weighing a small amount of cardiac tissue for the respirometry experiments.

   For cardiac muscle 1 to 1.2 mg per 2-ml chamber should be optimal for high-resolution respirometry studies.

5. Quickly place the small piece of permeabilized tissue in the respirometry chamber.
6. Proceed with MIR oxygen enrichment as in Basic Protocol 1, step 8.
7. Close the chamber and proceed with the respirometry study (Basic Protocol 7).

ALTERNATE PROTOCOL

Homogenization of Cardiac Tissue Using the PBI Shredder

Sample preparation is critical for the experiment, and several variables can affect reproducibility. A relevant aspect when working with muscle is the variability in the preparation of separated muscle bundles, which is not always homogeneous between samples and experimenters. Homogenization by the PBI-Shredder instrument provides an alternative for the preparation and permeabilization of different tissues, including cardiac muscle. This instrument is a low-shear mechanical homogenization system that produces a more homogeneous sample than the mechanical muscle bundles preparation described in Basic Protocols 1 and 2 for skeletal or cardiac muscle,

respectively. Contrary to what has been published for skeletal muscle (Larsen et al., 2014), when the method has been optimized for cardiac muscle, mitochondrial damage is minimal, as demonstrated by cytochrome *c* tests (see Troubleshooting, first point). For a more detailed protocol and information about the PBI-Shredder, consult the manufacturer's Web site (*http://www.pressurebiosciences.com/videos/SG3/*). A complete explanation and protocol can also be found at the Oroboros Instruments Web site (*http://oroboros.at/index.php?id=pbi-Shredder-manual*). The PBI-Shredder SG3 instrument is composed of the Shredder-tube with or without a metal insert to disrupt tough cellular structures. Both Shredder-tube types can be used not only for mouse cardiac muscle, but also for liver, adipose tissue, and brain.

The protocol for tissue preparation and homogenization should be selected before starting the study. The main facts to bear in mind are the study aims and researcher skills in preparing skinned muscle bundles. In general, preparation of skinned and saponin-permeabilized muscle bundles requires more time, skills, and training for reproducible results. Sample homogenization using the PBI-Shredder in some tissues produces a lower maximal respiratory capacity when compared with permeabilized skeletal muscle, but allows the researcher to perform studies combining high-resolution respirometry with other analyses, such as ROS production, ATP, or membrane potential measurements. Moreover, the use of the PBI-Shredder is faster, reproducible, reduces variability, and requires fewer skills on the part of the experimenter. All of this should be taken into account before selecting the method that best fits the study and the researcher skills.

Materials

Mitochondrial respiration medium (MIR; see recipe in Table 2)
Tissue sample (e.g., myocardium)

Shredder-tubes (FT500 PULSE tubes by Pressure BioSciences, Inc., composed of: modules, part no. 110530-003; serrated rams, part no. 110532-004PP; and caps, part no.110531-004)
Filter paper
Analytical balance (Mettler-Toledo Intl., cat. no. XA 105DU)
Small scissors [Marcel Blanc et Cie SA (MB) 3250.11]
PBI-Shredder SG3 (Pressure BioSciences)
15- or 50-ml conical centrifuge tubes

1. Close the Shredder-tube cap side with a Shredder-screw cap (*http://www.pressurebiosciences.com/videos/SG3/*).
2. Add 0.5 ml of ice–cold MIR to the ram side of the Shredder-Tube.
3. Blot the tissue on filter paper to remove residual external medium, then weigh the tissue sample (myocardium). Place ∼2 mg (wet weight) myocardium per chamber in the lysis disk at the narrow ram side of the Shredder-Tube and cut into smaller pieces using a small pair of sharp scissors. Distribute the tissue to allow optimal homogenization, always avoiding the edges of the lysis disk.
4. Close the Shredder-Tube by tightly fixing the serrated ram with the tools provided in the kit.
5. Once the tube is closed, place it in the PBI-Shredder SG3 base (a three-position force setting lever), adjust the SG3 driver on the top of the tube, and fix at first position (low force), then proceed with the first homogenization pulse (time = 10 sec).
6. Place the PBI-Shredder SG3 at the second position and homogenize the sample at this increased force (time = 5 sec).

7. Transfer the homogenate to a 15- or 50-ml conical centrifuge tube, depending on the amount of sample homogenate (for one, two, or four chambers), where the final volume of the preparation will be adjusted (see example at the end of the protocol).

8. Rinse the tube thoroughly to ensure all sample is transferred to the conical tube. Adjust the final volume of homogenate preparation with ice-cold MIR to achieve the desired concentration for high-resolution respirometry. Keep in mind that 0.5 ml was already added at the beginning.

   Example: If preparing a heart muscle homogenate for two chambers at a concentration of 1 mg per ml, add 5 mg of heart muscle (that already contains 0.5 ml MIR) to the Shredder tube. To achieve the desired concentration of 1 mg heart muscle homogenate per ml, add 4.5 ml MIR to the conical tube. 4.5 ml MIR in the conical tube plus 0.5 ml MIR transferred from the Shredder tube to the Falcon tube = 5 ml MIR. With this volume, two 2-ml chambers can be safely filled at the desired concentration.

BASIC PROTOCOL 3

Preparation of White Adipose Tissue for Respirometry Studies

This protocol focuses on epididymal white adipose tissue (eWAT). The eWAT is attached to the gonads and can be easily detected as the most prominent adipose depot in the abdominal cavity in mice fed regular chow diets. The eWAT weighs between 1 and 2 g in 6-month old C56BL/6 mice.

Materials

C57BL/6 J male mice, 12 weeks old, between 25 and 30 g body weight (Charles River Laboratories)
Isoflurane (inhalant anesthetic) or injectable solution: e.g., Rodent Combo Anesthetic III (ketamine 37.6 mg/ml; xylazine 1.92 mg/ml; acepromazine 0.38 mg/ml)
BIOPS (see recipe in Table 1) *or* mitochondrial respiration medium (MIR; see recipe in Table 2), ice cold

Dissecting microscope
Surgical instruments (see Basic Protocol 1)
6-well plate
Filter paper
O2k Oxygraph, high-resolution respirometer (Oroboros Inc.)

1. Anesthetize the mouse, isolate the epididymal white adipose tissue, and clean the tissue of capillaries and any non-fat tissue under a microscope.

2. Cut the eWAT into pieces of ~20 mg each.

3. Carefully weigh 50 to 60 mg of tissue and place it into a well of a 6-well plate with 2 ml of either ice-cold MIR buffer, when used for respirometry purposes quickly after extraction, or ice-cold BIOPS for longer periods of time.

   eWAT is a relatively fragile tissue, and declines in respiratory performance are observed within 3 hr of extraction.

   As for skeletal and cardiac muscle, the homogenization step can be carried out using the PBI-Shredder SG3 (see Alternate Protocol). We use the same procedure described for cardiac muscle. However, the amount of eWAT used for high-resolution respirometry is different. We obtain good results with at least 40 mg of tissue per ml of MIR. Therefore, we usually place more than 100 mg of cleaned WAT into the Shredder ram tube to obtain enough sample per two chambers. We then mince the sample with a sharp pair of scissors, homogeneously distribute the tissue for homogenization, and continue with the protocol as described.

4. Remove the excess of BIOPS or MIR buffer from eWAT by quickly blotting the tissue on filter paper. Then, place the pieces of eWAT (generally two to three, ~20 to 30 mg each) in the respirometry chamber. Close the chamber carefully, as eWAT has a tendency to float and can get stuck to the chamber stopper if closed quickly.

5. Proceed with respirometry analyses (Basic Protocol 7).

Preparation of Brown Adipose Tissue for Respirometry Studies

BASIC PROTOCOL 4

Brown adipose tissue (BAT) in mouse is a key tissue for thermogenesis. BAT expresses high levels of a unique protein called UCP1 (for uncoupling protein 1, also known as thermogenin), which is a transmembrane protein that decreases the proton gradient generated in oxidative phosphorylation. UCP1 does this by increasing the permeability of the inner mitochondrial membrane, allowing protons that have been pumped into the intermembrane space to return to the mitochondrial matrix. In this process, the dissipation of the electrochemical gradient produces heat (Cannon and Nedergaard, 2009).

Materials

C57BL/6 J male mice, 12 weeks old, between 25 and 30 g body weight (Charles River Laboratories)
Isoflurane (inhalant anesthetic) or injectable solution: e.g., Rodent Combo Anesthetic III (ketamine 37.6 mg/ml; xylazine 1.92 mg/ml; acepromazine 0.38 mg/ml)
Mitochondrial respiration medium (MIR; see recipe in Table 2)

Surgical instruments (see Basic Protocol 1)
Cordless pestle homogenizer (VWR, cat. no. 47747-370) and micropestle for 1.5- and 2-ml tubes (Eppendorf, cat. no. 022365622)
O2k Oxygraph, high-resolution respirometer (Oroboros Inc.)

1. Anesthetize the mouse, extract the interscapular BAT (Fig. 2), and remove as much surrounding white adipose tissue as possible.

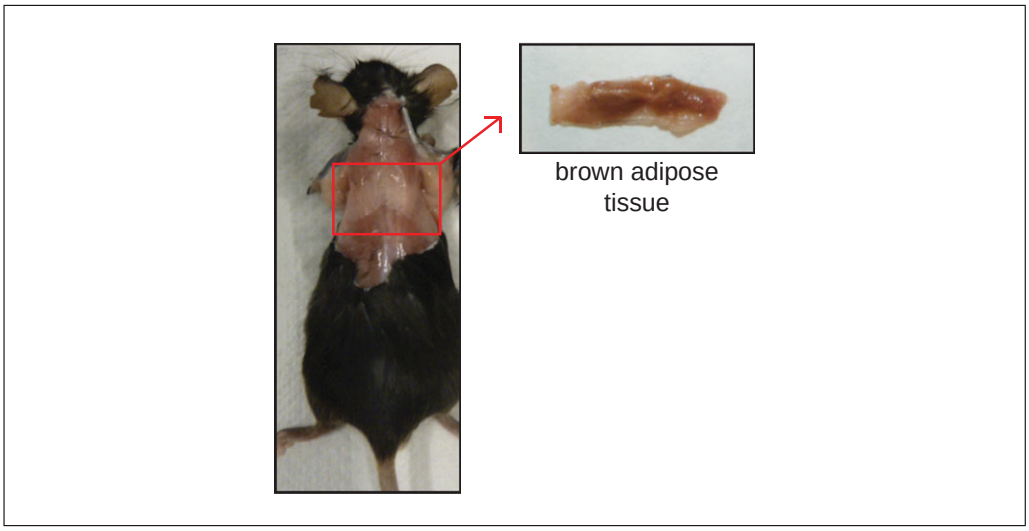

Figure 2 Interscapular brown adipose tissue localization in mice. Interscapular brown adipose tissue in C57BL/6 mice can be identified as an adipose mass on the back of the mouse as highlighted by the red rectangle in the picture. Upon excision of this region, it can be placed upside down, revealing two brown patches (see the image on the right). These patches can be more or less defined, depending on numerous parameters. For example, high-fat feeding promotes fat accumulation in brown fat patches, making it more difficult to define the boundaries between white and brown fat masses. For respiration experiments, the brown fat areas are separated from the surrounding white fat, generally yielding two 20 to 30 mg brown adipose pieces, of which half can be used for Basic Protocol 4.

In most cases, BAT will just consist of two small brown-colored patches weighing around 20 to 30 mg each. Cut each patch in half and weigh one of the two halves (around 10 to 20 mg).

2. Place the piece of tissue in a small microcentrifuge tube with 100 µl of ice-cold MIR. Add MIR buffer to achieve a 1 mg per 10 µl ratio.

   The homogenization can also be done using the PBI-Shredder SG3 (see Alternate Protocol). For this, we use the same procedure described for cardiac muscle. However, the amount of BAT used for high-resolution respirometry is different. We achieve good results with at least 1 mg of tissue per ml of MIR. Therefore, we usually place more than 6 mg of cleaned BAT into the Shredder ram tube to obtain enough sample for two chambers, then cut the sample with a sharp pair of scissors and distribute the tissue homogeneously for homogenization and continue with the protocol as described.

3. Use a pestle homogenizer to gently homogenize the tissue.

   When using a motorized Eppendorf homogenizer, long, intense pulses should be avoided, as this leads to mitochondrial damage.

4. Add 2 mg of tissue (20 µl of homogenate) to the respiratory chamber containing 2 ml MIR.

5. Proceed immediately with respirometry analyses (Basic Protocol 7)

BASIC PROTOCOL 5

Preparation of Liver Tissue for Respirometry Studies

The liver tissue plays a major role in carbohydrate and lipid metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification.

Materials

C57BL/6 J male mice, 12 weeks old, between 25 and 30 g body weight (Charles River Laboratories)
Isoflurane (inhalant anesthetic) or injectable solution: e.g., Rodent Combo Anesthetic III (ketamine 37.6 mg/ml; xylazine 1.92 mg/ml; acepromazine 0.38 mg/ml)
Saline: 0.9% (w/v) NaCl
BIOPS (see recipe in Table 1), ice cold
Mitochondrial respiration medium (MIR; see recipe in Table 2)

Surgical instruments (see Basic Protocol 1)
Analytical balance (Mettler-Toledo Intl Inc., cat. no. XA 105DU)
Filter paper
12-well plate
Cordless pestle homogenizer (VWR, cat. no. 47747-370) and micropestle for 1.5- and 2-ml tubes (Eppendorf, cat. no. 022365622)
O2k Oxygraph, high-resolution respirometer (Oroboros Inc.)

1. Anesthetize the mouse, extract the entire liver, and remove a small piece of no more than 50 mg.

   The rest of the tissue can be used for other experimental purposes or discarded.

   Liver tissue has an amazing ability to shift its metabolic properties according to nutritional outputs (for example, fasting versus feeding), and some of its functions are compartmentalized to specific liver regions. Hence, it is very important that similar liver regions always be used when comparing liver sample respiratory analyses.

2. Remove any excess blood by quickly washing the small (~50-mg) piece of liver in saline (0.9% NaCl) solution and blotting it on filter paper.
3. Weigh the small piece of liver.
4. If the liver will not be immediately used for respirometry purposes, place the piece of liver in a well of a 12-well plate containing 2 ml of ice-cold BIOPS.

   The piece of liver can be kept in BIOPS for at least 6 hr.

5. Place the piece of liver in a small microcentrifuge tube with 100 µl of ice-cold MIR. Add MIR buffer to achieve a 1 mg per 10 µl ratio.
6. Use a pestle homogenizer to gently homogenize the tissue.

   When using a motorized Eppendorf homogenizer, long, intense pulses should be avoided, as this leads to mitochondrial damage.

   The homogenization procedure using the PBI-Shredder SG3 is the same as described for cardiac muscle (see Alternate Protocol). However, the amount of liver used for high-resolution respirometry is different. We achieve good results with at least 1 mg of liver tissue per ml of MIR. Therefore, we usually place more than 6 mg of cleaned liver into the Shredder ram tube to obtain enough sample for two chambers, then cut the sample with a sharp pair of scissors and distribute homogeneously for homogenization and continue with the protocol as described.

7. Add 2 mg of tissue (20 µl of homogenate) to the respiratory chamber containing 2 ml MIR.
8. Proceed immediately with respirometry analyses (Basic Protocol 7).

Preparation of Brain Tissue for Respirometry Studies

BASIC PROTOCOL 6

We have experience using samples from the hypothalamus and cerebellum regions of the brain for high-resolution respirometry and expect that the same procedure could be applied for other brain regions. The protocols described for BAT and liver samples have been proven successful for the preparation of brain samples. However, brain samples are very fragile and sensitive, so samples should be processed as soon as possible after dissection, ideally immediately after dissection.

Materials

C57BL/6 J male mice, 12 weeks old, between 25 and 30 g body weight (Charles River Laboratories)
Isoflurane (inhalant anesthetic) or injectable solution: e.g., Rodent Combo Anesthetic III (ketamine 37.6 mg/ml; xylazine 1.92 mg/ml; acepromazine 0.38 mg/ml)
BIOPS (see recipe in Table 1), ice cold
Mitochondrial respiration medium (MIR; see recipe in Table 2), ice cold

Surgical instruments (see Basic Protocol 1)
Analytical balance (Mettler-Toledo Intl Inc., cat. no. XA 105DU)
12-well plate
Cordless pestle homogenizer (VWR, cat. no. 47747-370) and micropestle for 1.5- and 2-ml tubes (Eppendorf, cat. no. 022365622)
O2k Oxygraph, high-resolution respirometer (Oroboros Inc.)

1. Anesthetize the mouse and extract the brain region of interest (e.g., hypothalamus).
2. Weigh the piece of tissue.

3. If the tissue will not be immediately used for respirometry purposes, place the piece of brain tissue in a well of a 12-well plate containing 2 ml of ice-cold BIOPS.

   Some brain regions (e.g., hypothalamus) should not be stored for more than 1 hr before the respirometry assay. Others, such as the baso-lateral amygdala, can be kept in BIOPS at least for 2 hr.

4. Place the piece of brain in a small microcentrifuge tube with 100 µl of ice-cold MIR. Add MIR buffer to achieve a 1 mg per 10 µl ratio.

5. Use a pestle homogenizer to gently homogenize the tissue.

   When using a motorized pestle homogenizer, long, intense pulses should be avoided, as this leads to mitochondrial damage.

   Another method that we have used successfully for tissue preparation with hypothalamus or cerebellum samples is to follow the homogenization procedure in the microcentrifuge tube (as described) with three gentle strokes of a syringe equipped with a 25-G needle, for full homogenization.

6. Add 2 mg of tissue (20 µl of homogenate) to the respiratory chamber containing 2 ml MIR.

7. Proceed immediately with respirometry analyses (Basic Protocol 7).

BASIC PROTOCOL 7

RESPIROMETRY ASSAY

The procedure is exemplified using a protocol that will allow measurement of the following parameters:

1. Evaluation of proton leak through Complex I.
2. Evaluation of maximal Complex I respiration capacity.
3. Evaluation of maximal respiration by Complex I and Complex II together.
4. Evaluation of maximal electron transport system capacity.
5. Evaluation of maximal electron transport system capacity by Complex II.
6. Evaluation of non-mitochondrial oxygen consumption.

While many instruments can be used for this purpose, the O2k Oxygraph is used as an example in this protocol. Key advantages of the O2k Oxygraph over other respirometry devices are its extremely high resolution [±1 pmol/(sec-ml)], the flexibility to tailor the experiment according to the scientific question, the possibility to titrate substrates, and the ability to inject multiple chemicals during the experiment. The protocols, however, can be adapted to other methodologies, such as the more classical Clark electrode. Respiratory measures of mouse tissues have also been reported using the Seahorse XF24 or XF96 analyzers, but it must be kept in mind that they have a limited number of injection possibilities (up to 4). Guidelines for the preparation and storage of chemical stocks for experiments described below can be found on Table 1. For further information and preparation of other chemical stocks, detailed information can be found online at *http://bioblast.at/images/3/3c/MiPNet03.02_Chemicals-Media.pdf*.

Materials

Tissue sample (from Basic Protocols 1 to 6 or Alternate Protocol)
Chemical stocks as described in Table 3:
- 400 mM L-malic acid
- 2 M pyruvic acid, sodium salt
- 2 M L-glutamic acid
- 500 mM ADP
- 1 M disodium succinate

Table 3 Chemical Stocks for Respirometry Assays

Compound	Stock concentration	Solvent	Storage	Comments
L-Malic acid	400 mM	H_2O	Store at −20°C	Neutralize with 10 N KOH
Pyruvic acid, sodium salt	2 M	H_2O	Freshly prepared	
L-Glutamic acid	2 M	H_2O	Store at −20°C	Neutralize with 5 N KOH
ADP (adenosine 5'diphosphate, potassium salt)	500 mM	H_2O	Store at −80°C	Neutralize with 5 N KOH. To maintain Mg^{2+} constant during the respiration measurement, mix ADP with $MgCl_2$ (0.5 mol/mol ADP).
Succinate disodium salt, hexahydrate	1 M	H_2O	Store at −20°C	Adjust pH to 7 with HCl
FCCP	1 mM	Ethanol	Store at −20°C	
Rotenone	1 mM	Ethanol	Store at −20°C	Difficult to dissolve and light sensitive. Very toxic.
Antimycin A	5 mM	Ethanol	Store at −20°C	Very toxic
Saponin	5 mg/ml	BIOPS	Freshly prepared	
Digitonin	8.1 mM	DMSO	Store at −20°C	Toxic
Cytochrome *c*	4 mM	H_2O	Store at −20°C	

1 mM FCCP
1 mM rotenone
5 mM antimycin A
5 mg/ml saponin
8.1 mM digitonin
4 mM cytochrome *c*

O2k Oxygraph, high-resolution respirometer (Oroboros Inc.)
Hamilton glass microsyringes: (3 × 10 µl, 3× 25 µl, and 2× 50 µl)
Datlab software (*http://www.oroboros.at/index.php?datlabsoftware*)
Statistical analysis software (e.g., Microsoft Office Excel)

1. Place the tissue in the Oxygraph chamber, close the chamber, and allow it to equilibrate with the tissue for 5 to 10 min before adding any substrate.
2. Add 2 mM malate (from 400 mM malic acid stock) using a Hamilton glass microsyringe and let respiration values equilibrate for at least 2 min until O_2 consumption rates are stabilized.

   It has been described for human WAT that a permeabilization step is needed, using digitonin (2 µM) for ∼20 min prior to substrate incubation (Kraunsoe et al., 2010). We have not found this to be a requirement in mouse WAT, as digitonin permeabilization did not significantly affect respiration parameters (data not shown; also see Troubleshooting).

3. Add pyruvate to a final concentration of 10 mM (from 2 M pyruvic acid stock) and let respiration values equilibrate for at least 2 min until O_2 consumption rates are stabilized.
4. Add glutamate to final concentration of 10 mM (from 2 M glutamic acid stock) and let respiration values equilibrate for 3 min until O_2 consumption rates are stabilized.

   The respiration rates at this point illustrate Complex I–driven respiration in the absence of coupled respiration ("leak"). This value is generally relatively low for most tissues,

accounting for a maximum of 10% of maximal respiration rates. A critical exception to this point is brown adipose tissue, where UCP1 can boost this value to almost half of the maximal respiratory capacity. At this point, while traces of ADP from the tissue homogenate might be present in the medium, the concentrations would be insufficient to drive coupled respiration and will be evidenced by a decrease in respiration before reaching a steady respiration rate.

5. Add ADP solution to a final concentration of 5 mM (from 500 mM stock) for most tissue samples. Use 1 mM for BAT samples.

   In most tissues, ADP addition will prompt a large increase in respiration rates (up to 10-fold higher than leak rates). ADP, however, can inhibit uncoupled respiration in brown adipose tissue homogenates. For this reason, ADP is added to only 1 mM in BAT samples. After the increase prompted by ADP addition, respiration rates should be allowed to equilibrate for at least 3 min. The corresponding value (Complex I) will be the sum of Complex I–driven leak plus Complex I–driven coupled respiration. By subtracting the leak to the current value, one can easily quantify maximal Complex I–driven coupled respiration capacity.

6. Add succinate (from 1 M disodium succinate stock) to a final concentration of 10 mM.

   This will stimulate Complex II. In some tissues, such as liver, Complex II will account for a large increase in respiratory rates. In others, such as skeletal muscle, Complex I is largely predominant, and the increases observed upon Complex II stimulation will be moderate.

7. After the increase promoted by Complex II, let respiration rates equilibrate for 3 min.

   The respiration rates at this point will reflect Complex I + Complex II–driven respiration (leak and coupled). Hence, this state is called Complex I + II.

8. In the following step, we will try to evaluate maximal electron transport system (ETS) capacity. This also reflects maximal electron input from Complex I and II on the respiratory chain. To do so, we will uncouple respiration by using an exogenous protonophore compound, in this case carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). One of the major problems when using FCCP is that mitochondrial uncoupling sensitivity is extremely different between different tissues, and can also be affected by dietary interventions or disease states. In addition, excessive FCCP can be toxic and decrease mitochondrial respiration when provided in excess. To solve these two problems, FCCP is titrated by gradually increasing, by 0.5 μM, the concentration of FCCP in the medium. After each addition, respiration rates should be left to achieve equilibrium for at least 1 min. When a follow-up addition of FCCP does not further increase respiration, the value will be generally considered maximal respiration capacity (ETS). The experimenter, however, needs to ensure that oxygen concentrations in the medium are not rate-limiting.

9. Add 0.5 μM rotenone (from 1 mM stock) to inhibit Complex I activity.

   While a quick drop is typically detected, it might take some time for respirometry rates to stabilize. This is especially true in tissues with high Complex II activity, such as liver or white adipose tissue. When the respiration rate is stabilized, it will reflect maximal electron input through Complex II (ETS Complex II).

10. Add 2.5 μM antimycin-A (from 5 mM stock) to inhibit Complex III activity.

    As Complex III is a required acceptor for electrons coming from either Complex I or Complex II, the blockage of Complex III constitutes the complete blockage of mitochondrial electron circulation due to the addition of previous respiratory substrates. Any residual oxygen consumption (ROX), therefore, cannot be attributed to mitochondrial respiration. In general, antimycin-A should quickly promote a dramatic decrease of respiration, close

to absence levels; however, this decrease might be slower in permeabilized muscle fibers or white adipose tissue samples, due to the slow diffusion of the compound.

Some drugs take a long time to diffuse in WAT preparations. This is especially the case for antimycin-A, which can take a very long time (around 15 min) to reach full respiratory inhibition and a steady-state respiration rate.

11. Once respiration values are equilibrated after antimycin-A addition, the corresponding respiratory rate is considered background consumption and should be subtracted from all the previous values for respiratory analyses.

12. Stop the experiment.

Analyze data

Data analysis is tailored by the software used to assess respirometry data. Therefore, it is not possible to supply precise, uniform instructions on how to perform data analysis. Researchers are advised to follow the software manufacturer's instructions. In the case of Oroboros Instruments, the Datlab software, provided by the company, can be used. Further information on this software can be found at the URL *http://www.oroboros.at/index.php?datlabsoftware*. The correction of data from respirometry analyses is key for the final interpretation. This is further discussed in Critical Parameters.

13. Irrespective of the software, the data analysis consists of the following key steps:

    a. Delimit the area to be quantified per respiratory state. This area should be delimited when the specific respiratory state has reached a steady-state rate of oxygen consumption.
    b. Acquire data.
    c. Once the data are obtained, export the data and/or create a figure illustrating the experiment.
    d. Analyze data using software that allows data import and further analysis and statistics (e.g., Microsoft Office Excel).

COMMENTARY

Background Information

Mitochondria are complex organelles that constitute the hotspot of metabolic regulation within the cell. Mitochondrial dysfunction is a hallmark not only of rare inherited mitochondrial disorders but also of most age-related diseases, including those that affect metabolic homeostasis and cognitive health, such as type 2 diabetes and Parkinson's disease (Andreux et al., 2013). Protocols for high-resolution respirometry of mouse tissues offer sensitive diagnostic tests of mitochondrial function, with the powerful advantage of correlating them to dietary, lifestyle, or genetic interventions. Respirometry reflects the function of mitochondria as structurally intact organelles. It provides a dynamic measurement of metabolic fluxes, in contrast to static determination of molecular components, such as metabolite and enzyme levels, redox states, and membrane potential, as well as RNA and DNA levels. Only the combination of these diverse parameters can truly provide a global view of mitochondrial function within a tissue and the reasons behind possible alterations. It must be stressed that, due to the requirement for intact mitochondria, mitochondrial respiratory function cannot be measured on frozen tissue samples, but usually requires minimizing storage times for biological samples, as well as delicate handling procedures. This, together with the throughput limitation of current high-resolution respirometry apparatus, generally makes respirometry assays more complex than the most commonly evaluated parameters (e.g., mitochondrial DNA content evaluation, mitochondrial protein or mRNA levels, or citrate synthase activity). However, respirometry assays provide unique information that cannot be obtained through any other means, such as metabolic fluxes in intact respiratory complexes and supercomplexes, which better mimic native mitochondrial function.

Critical Parameters

One key element when analyzing respiratory rates that is largely unappreciated, especially in high-throughput respirometry approaches, is data correction. Data correction of high-resolution respirometry results has been addressed for human skeletal muscle (Larsen et al., 2012), but the conclusions can be applied to mouse tissues. The three most commonly used methodologies are discussed below, along with advantages and disadvantages of each.

Correction by tissue mass (e.g., pmol/(sec-mg of tissue). This method is extremely easy to apply, as it implies knowing exactly how many mg of tissue are added to the chamber, either as a piece of tissue (WAT, permeabilized muscle fiber) or as homogenates (by knowing the weight of the tissue and homogenization volume). Consequently, this correction method is very fast and does not require any major experimental effort other than properly weighing the tissue before processing. The major disadvantage is that mitochondrial content per milligram of tissue can differ dramatically in different situations. For example, when mice are fed a high-fat diet, their livers can increase in fat content by 5-fold. Consequently, mitochondrial density per mg of tissue will be lower in these tissues, even if the total mitochondrial content in the liver is equal to that in livers from mice fed a non-high-fat diet. The same applies to any other tissue susceptible to fat accumulation (including WAT, BAT, or muscle). Therefore, to draw appropriate conclusions using this correction method, it must be verified whether lipid, protein and mitochondrial content are comparable between samples.

Correction by mg of protein (e.g., pmol/(sec-mg of protein). This method requires that the sample of interest be tested in parallel for respirometry and for protein content. Its advantage over the correction per mg of tissue (see above) is that the possible bias created by differential fat accumulation is avoided. The major disadvantage, however, is that the feasibility of this process is not always evident. While obtaining this information from homogenates (liver, BAT, brain) used for respirometry poses no major problem, the case is different for white adipose samples or skeletal muscle permeabilized samples, as the tissue needs to be extracted after the respirometry analysis for protein measurement, when the integrity of the tissue might have been significantly compromised. However, when assessing protein content from homogenates, the BSA content (1 g/liter; see Table 2) of MIR (homogenization and respiration medium) should be taken into account and subtracted to calculate the protein content of the sample. Finally, one must also keep in mind that total protein content might not be linear with regard to mitochondrial content, especially in tissues that could suffer hypertrophy, such as skeletal muscle. Therefore, mitochondrial content per mg of protein might need to be additionally tested in parallel samples to fully establish conclusions on the respiration rate data. This is a convenient and appropriate method to use with isolated mitochondria.

Correction by mitochondrial content. In this method, respiratory rate values are corrected by an arbitrary factor that accounts for gross mitochondrial content in the sample. This value might be derived from the quantification of mitochondrial DNA content, mitochondrial protein content, or Krebs cycle enzyme activities (e.g., citrate synthase activity). This is a more laborious method for correction, as it implies more laborious techniques, but it also provides the most accurate method to determine whether respiratory rate changes derive from a qualitative and/or quantitative change in mitochondrial function.

Troubleshooting

This section provides the experimenter with a few practical tips to ensure the reproducibility and quality of the assay.

• When working with tissue homogenates or isolated mitochondria, it is necessary to control for the quality of the preparation, as there may be preparations where mitochondria are partially damaged. Upon damage of the outer mitochondrial membrane, cytochrome *c* is released. This depletion reduces the respiratory flux capacity, and can be restored after addition of 10 μM cytochrome *c* (Gnaiger and Kuznetsov, 2002). It is therefore strongly suggested that all homogenates and mitochondrial preparations be controlled for mitochondrial integrity. Cytochrome *c* addition should ideally be made on the coupled state (e.g., Complex I or Complex I + II), as, for some reason, cytochrome *c* can trigger respiration in the leak state, irrespective of mitochondrial integrity. Therefore, experimental runs showing a preparation with induced cytochrome *c* release (i.e., a significant increase in respiration after cytochrome *c* addition) should be excluded from the final data set. In perfectly prepared muscle fibers, cytochrome *c* should have no stimulatory effect on respiratory activity. In liver biopsies, a small effect (around

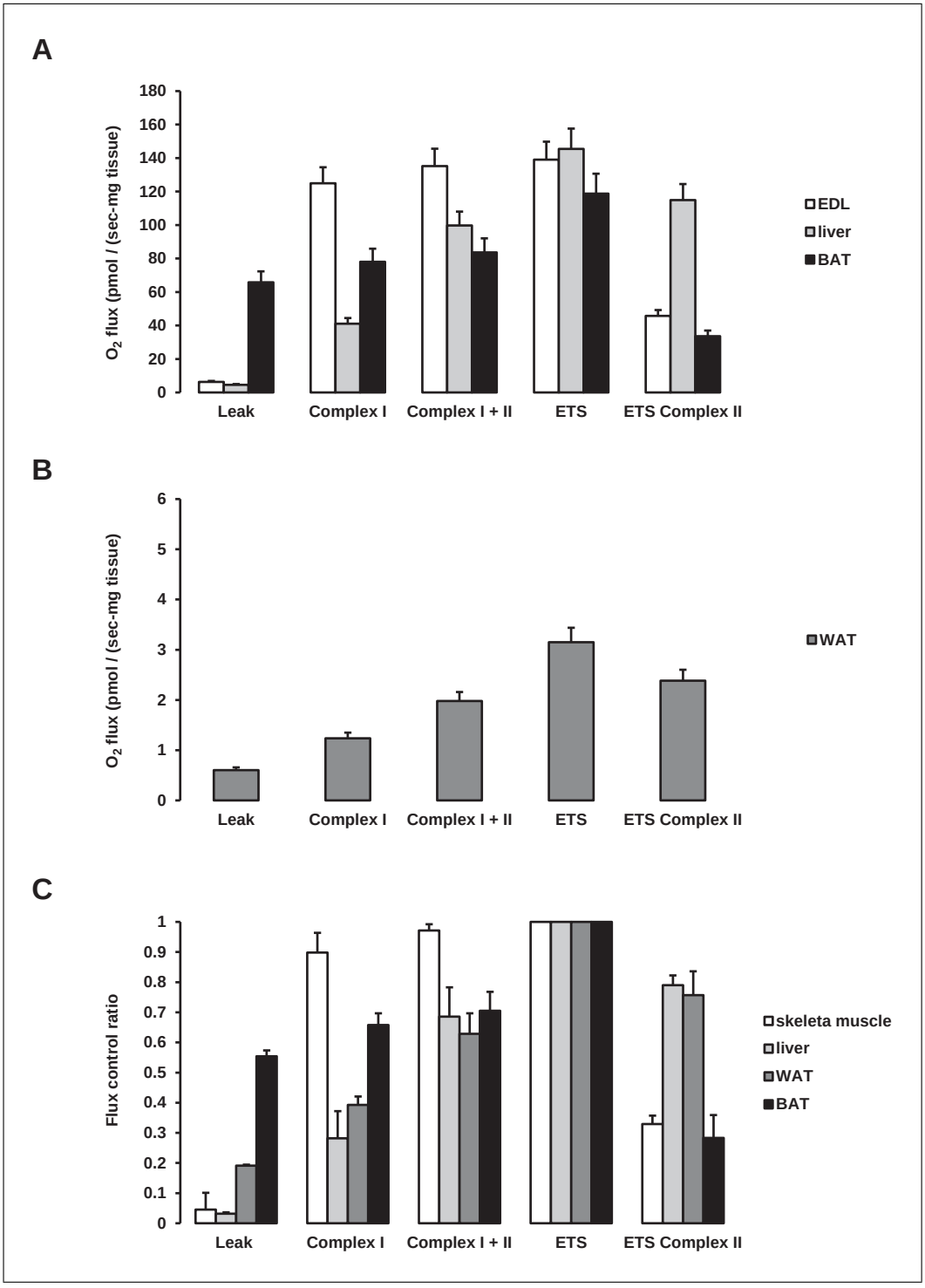

Figure 3 Respiratory characteristics of different mouse tissues. 24 week-old male C57BL/6 N mice were anesthetized using isoflurane and the tissues indicated were collected. All tissues were maintained and treated before respirometry as described in Basic Protocols 1 to 6. Then, (**A**) 2 mg of liver or brown adipose tissue, between 1.8 and 2.2 mg of permeabilized EDL muscle, and (**B**) between 56 and 68 mg of epididymal white adipose tissue were used for the respiratory measurements, as described in Basic Protocol 7. In all of the presented data, ROX values have been subtracted and corrections per mg of tissue have been applied. In (**C**), we illustrate the different relative contribution of each respiratory state to maximal electron transport system (ETS) capacity All values are presented as mean $\pm$ SEM of $n = 8$ mice.

5%) is observed, even in carefully prepared samples.

• Oxygen levels can easily become rate-limiting in permeabilized muscle fibers and white adipose tissue samples. It is very important to make sure that oxygen is not rate-limiting in all preparations. This can be done by reoxygenating the chamber and remeasuring respiration rates.

• While we have not observed any differential effect in mouse WAT, it has been reported that addition of digitonin to human WAT preparations is required to maximize respiration rates (Kraunsoe et al., 2010). To verify that the sample was fully permeabilized, digitonin can be added after succinate. A respiration increase after digitonin addition indicates that the sample was not fully permeabilized. If this is the case, digitonin can be added to the medium immediately after the addition of the samples. It is important to let digitonin permeabilize the sample for 10 to 15 min before starting the protocol. The optimized digitonin concentration should be determined in preliminary studies.

• Respiratory Flux Ratios (RFR). Respiratory flux ratios are the contribution of each respiratory state to maximal respiratory capacity. This is calculated by dividing the oxygen consumption rate of each respiratory state by the ETS value (maximal respiratory capacity value). It is an extremely valuable calculation as it evaluates qualitative changes in mitochondrial respiration, especially with regard to the contribution of individual complexes to maximal respiration activity. Figure 3C clearly illustrates how skeletal muscle has a remarkable coupling capacity, as coupled respiration can account almost completely for maximal electron transport capacity. In BAT, it is clearly observed how the initial leak is much higher than in any other tissue, as is expected with the specialization of this tissue on uncoupled respiration. Finally, liver and WAT hold stronger positions for Complex II–driven electron transport, in line with their massive capacity for fat metabolism.

Anticipated Results

All tissues display different respiratory profiles, reflecting qualitative differences between mitochondria in different tissues. Figure 3 shows how the profiles of EDL muscle, brown adipose tissue, liver, and brain strongly differ. Further comparisons between tissues can be found in published references (e.g., Holmström et al., 2012). The extreme fat accumulation of WAT makes respiratory rates relatively low. Maximal respiratory rate values generally range between 3 and 5 pmol/(sec-mg tissue), rarely surpassing 10 pmol/(sec-mg tissue) in regular mice.

Time Considerations

O2k Oxygraphs produce high-quality data, but with low throughput capacity. An O2k Oxygraph has two chambers. Hence, only two samples can be measured simultaneously. Most laboratories therefore use several O2k Oxygraphs in parallel to increase the number of samples that can be processed per day.

Oxygenation of the medium takes at least 1 hr. It is therefore recommended that the chambers oxygenate while the experimenter performs mouse dissection tasks. The preparation of skeletal muscle or heart samples (Basic Protocols 1 and 2) will take ~2 hr, as samples need to be carefully dissected, and muscle bundles isolated and permeabilized. In contrast, all other tissues can be prepared much more quickly. The respiration measurements (Basic Protocol 7) generally require 1.5 to 2 hr to perform. Our laboratories have efficiently performed two rounds of measurements per day on each O2k Oxygraph, for example by measuring liver and muscle samples from the same mouse. For this purpose, all tissues are kept in BIOPS buffer upon extraction and sequentially processed thereafter. For the experienced researcher, it should not be difficult to perform respirometry assays on liver during the morning and on muscle during the afternoon.

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Conflict of Interest

C.C. is an employee of the Nestlé Institute of Health Sciences S.A.

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