

# Measuring mitochondrial respiration in intact skeletal muscle fibers

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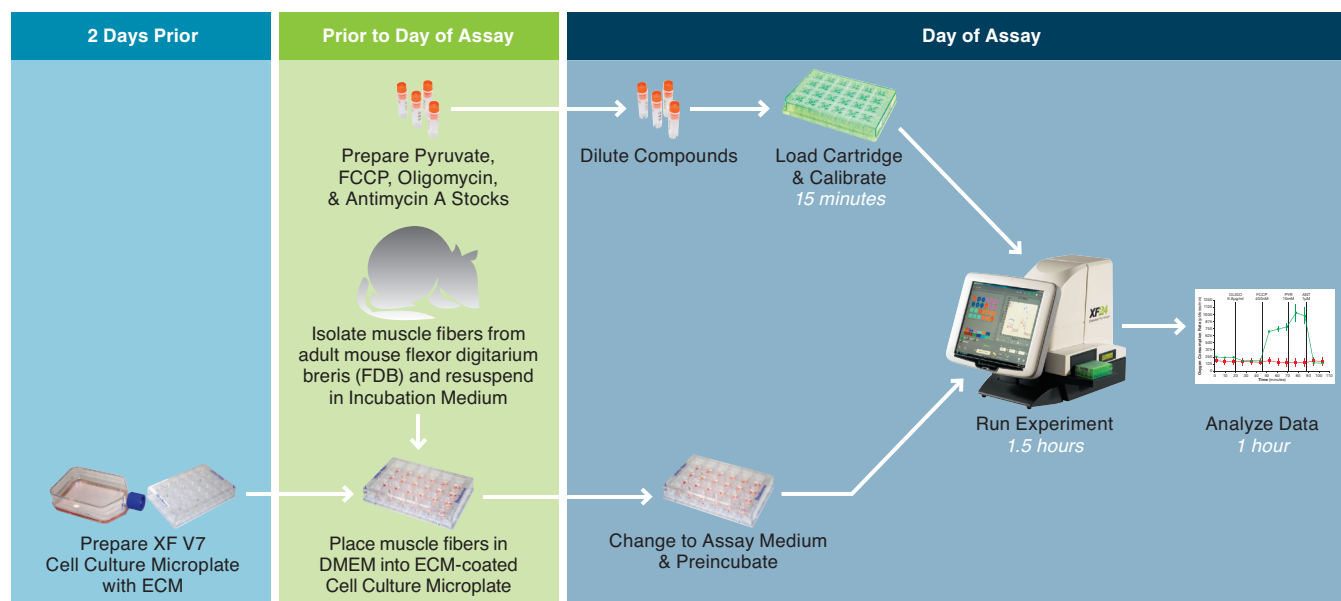
Assessment of mitochondrial function in skeletal muscle is a powerful tool for modeling neuromuscular conditions including muscular dystrophy, aging and cachexia. Similarly, skeletal muscle plays an important role in normal and pathological metabolism, such as obesity and diabetes. The majority of investigations use measurements of single mitochondrial enzymes as a surrogate to measuring mitochondrial function which does not provide a complete physiological readout of the mitochondria.

To overcome this, permeabilized fiber bundles<sup>1</sup> or isolated mitochondria<sup>2</sup> are being used as a means to measure oxygen consumption in mitochondria. However, these systems are disadvantaged by from a number of limitations. Isolated

mitochondria lack the modulating effects of the cellular environment and involve the risk of analyzing a subset of the mitochondrial population. Permeabilized fibers lack most of the cytoplasm and suffer from the possibility of partial permeabilization and diminished biological response, or excessive permeabilization and loss of cytochrome C.

In this technical brief, we present a novel approach to assess mitochondrial respiration using intact, single cultured muscle fibers. This approach allows the user to utilize dissected, intact muscle fibers from an isolated whole muscle, with minimal disruption to the integrity of the cell. In addition, the fibers can remain in culture for up to a week prior to analysis, enabling the investigator to make chronic measurements.

**Figure 1 | Flow Chart of XF Assay**



## Materials and Methods

### Reagents:

1. Dissociation medium (DM): Dulbecco's Modified Eagle Medium (D-MEM) high glucose, no sodium pyruvate or phenol red (Invitrogen, #21063-029), gentamycin [50µg/ml] (Sigma, #G1397), FBS [2%], and collagenase A [4 mg/ml] (Roche #11088785103) pH 7.2.
2. Incubation medium (IM): Dissociation medium (above) without collagenase A.
3. Assay medium (aCSF): 120 mM NaCl, 3.5 mM KCl, 1.3 mM  $\text{CaCl}_2$ , 0.4 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{MgCl}_2$ , 5 mM HEPES and 15 mM D-glucose (Sigma, # G7528) Adjust to pH 7.4.
4. Injection reagents [10X] (Sigma):
  - Oligomycin [8 ug/ml]
  - FCCP [4uM]
  - Pyruvate [100mM]
  - Antimycin A [10uM]
 Make 100X stocks at pH 7.4 and freeze at -20 °C.
5. Growth Factor Reduced BD Matrigel™ Matrix (BD Biosciences, #354230).

### Supplies:

1. Seahorse XF24 FluxPaks (Seahorse Bioscience, #100850-001), including Seahorse XF24 Assay Cartridges, Seahorse XF24 Cell Culture Microplates, and XF calibration plates
2. 35mm disposable culture dishes

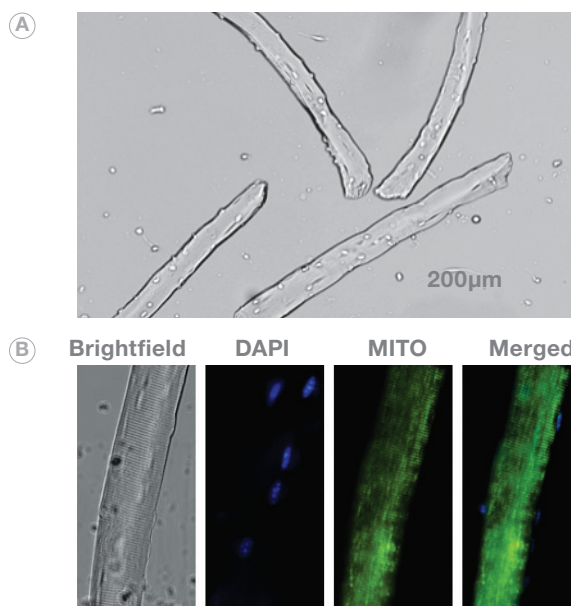
### Isolation and Dissociation of Diaphragm Muscle–Flexor Digitorum Brevis (FDB) muscle:

Single skeletal muscle fibers were enzymatically isolated from FDB of 8 week-old C57BL/6 mice. The procedure is a modification of work previously published with muscle from rats and mice<sup>3-8</sup>. In brief, surgically excised FDB muscles were incubated in dissociation media (DM). It was important to carefully dissect the whole muscle to ensure no induction of mechanical damage to the muscle before isolating the fibers. The muscle was gently rinsed with PBS. Four muscles were placed in a 35mm disposable culture dish with 4ml of DM, then placed in an incubator (37°C, 5%  $\text{CO}_2$ ) for 1.5-2 hours. Following the dissociation, the muscles were placed in a new 35mm plate with warmed incubation media containing gentamycin and FBS without collagenase. FDB muscles were triturated with a small bore (~1mm) fire polished glass transfer pipette to yield single FDB myofibers. A wide bore p1000 pipette may be used in place of

the transfer pipette. If trituration did not yield a significant number of disassociated single fibers after 5-10 passes, the muscles were returned to DM for 15-30 minutes and the process was repeated. Following trituration, large debris (nerve, un-digested FDB muscle) was removed with forceps.

### Seeding of Dissociated Muscle Fibers:

Seahorse XF24 Cell Culture Microplates were coated with 3µl of Growth Factor Reduced Matrigel™ Matrix diluted 1:1 in DMEM. Following application of a 3µl drop of Matrigel in the wells, the lid was placed on the microplate and the plate vigorously tapped horizontally against an open hand to spread the Matrigel across the bottom of the wells. The plate was then air-dried for 10-30 minutes. Following a thorough dispersion of single fibers in the 30mm dish, 90µl aliquots were taken randomly and deposited into each well, where they attach by sedimentation, with the goal of allowing the fibers to cover ~50-60% of the well bottom (Figure 2). The confluency was determined through light microscope visualization. Fibers were then placed in an incubator until use. Single myofibers were verified as being fully adhered within 5 minutes.



**Figure 2 | Intact Isolated Muscle Fibers**

A. Brightfield image of isolated flexor digitorum brevis muscle fibers immediately after plating on Growth Factor Reduced Matrigel-coated XF24 Cell Culture Microplates.

B. A single muscle fiber subsequently stained with DAPI (blue-nuclei) and MitoTracker (green-mitochondria) showing integrity of the muscle fiber.

## XF Bioenergetic Analysis

Bioenergetic analyses of cultured intact single muscle fibers were performed in the Seahorse XF24 Extracellular Flux Analyzer 24 hours after isolation (Figure 1). In this experimental design, the importance of optimizing substrate availability to obtain maximal respiratory capacity, is highlighted by the addition of subsequent pyruvate after the uncoupling agent FCCP. Results of the addition of pyruvate are discussed in the section on Interpretation of Results.

### Load the Seahorse XF Assay Cartridge:

Load the drug injections ports of the Seahorse XF Assay Cartridge with the assay reagents at 10X. Ports A-D add 75 $\mu$ l oligomycin, 83 $\mu$ l FCCP, 92 $\mu$ l pyruvate and 102 $\mu$ l antimycin A, yielding final concentrations of 0.8 $\mu$ g/ml, 400nM, 10mM, 1 $\mu$ M respectively.

### Media Exchange & Calibration:

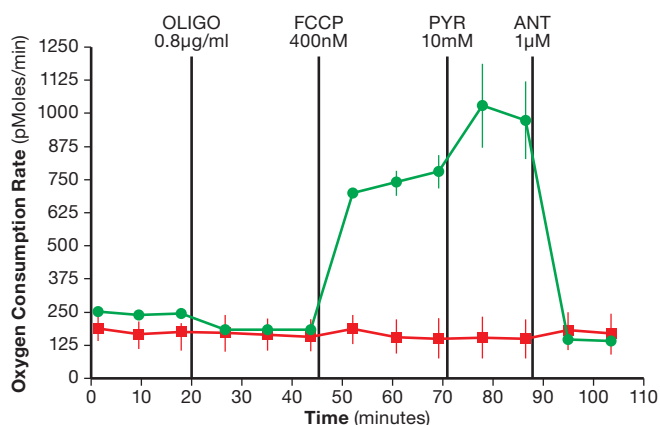
Exchange incubation medium with assay medium prior to measurements. Aspirate 40 $\mu$ l of the incubation media/well, leaving 50 $\mu$ l, and add 950 $\mu$ l of pre-warmed assay medium (aCSF). Aspirate 950 $\mu$ l of the media per well and add 625 $\mu$ l/well of aCSF to a final volume of 675 $\mu$ l. Add 675 $\mu$ l of aCSF to the temperature control wells (without fibers) and equilibrate the microplate in a CO<sub>2</sub> free incubator at 37°C for 1 hour. During this equilibration period, calibrate the Seahorse XF Analyzer with a calibration plate using the standard calibration protocol.

### Muscle Fiber Assay:

Following calibration, replace the calibration plate with the plate containing the fibers and begin the experimental run. Follow the Instrument Run Protocol as described in Table 1.

### Data Analysis:

At the end of the XF experiment, the software generates two files (“.xfd” and “.xls”). The xfd file is analyzed using the XF reader software and the xls file analyzed using excel. Both file types requires the installation of the XF software on the PC being used for analysis. Analyzing using excel provides the most options.



**Figure 3 | Oxygen Consumption Rates (OCR) of Isolated Muscle Fibers in Response to Bioenergetic Modulators**

Muscle fibers treated with Oligomycin [A], FCCP [B], sodium pyruvate [C], and antimycin [D] (green line). FCCP stimulates respiration in mitochondria by uncoupling ATP synthesis from electron transport, while oligomycin and antimycin both inhibit respiration by inhibiting ATP synthase and oxidation of ubiquinol in the electron transport chain, respectively. When the isolated fibers are provided a mitochondrial substrate such as pyruvate, oxygen consumption is stimulated, illustrating the importance of optimizing substrate when determining maximal respiratory capacity. N = 4 separate wells; experiment performed 24 hours after isolation. Similar results have been achieved 48hrs after isolation (data not shown). Control cells were treated with medium only [red Line]

## Interpretation of Results

The assay described is a variation of the Seahorse XF Cell Mito Stress Test in which several questions about mitochondrial function/dysfunction are queried and assessed (Figure 3). The Seahorse XF Mito Stress Test first measures basal respiration to obtain the resting state of the fibers. ATPase activity is subsequently blocked using oligomycin, and the drop in Oxygen Consumption Rate (OCR) reflects the respiration needed to sustain ATP consumption in the fibers. The remaining respiration reflects the proton leak of the mitochondria (i.e. the flow of protons across the inner mitochondrial membrane (IMM) which generates heat and not ATP). This rate can be influenced by the composition of the IMM (lipids, proteins). It is also highly influenced by the oxidative stress level of the system.

Next, the uncoupler, FCCP is added. FCCP is a compound that carries protons across the IMM and dissipates the electrochemical gradient (membrane potential) that drives ATP synthesis. In order to maintain the membrane potential, the mitochondria needs to increase the flow of electrons and thus oxygen consumption. By optimizing the concentration of the uncoupler FCCP, maximal respiration for the sample is obtained. This measure reflects how a system reacts to an increased ATP demand. If the maximal respiration is diminished it may lead to an energetic crisis for the fiber.

Finally, antimycin A is added, inhibiting complex III and stopping all mitochondrial respiration. This is used to subtract the non-mitochondrial sources of oxygen consumption, and calculate the mitochondrial-specific component of respiration.

In this experiment, pyruvate is added after FCCP. This provides the fibers with additional substrate. In some systems, running glucose alone is far from optimal as pyruvate or amino acids are needed for replenishment of Krebs cycle components (anaplerosis).

To achieve maximal respiration subsequent to uncoupler addition, it is important to ensure the sample is not substrate limited. Optimizing substrates is critical for proper interpretation of stress test results. This is particularly important if the questions asked could be related to how well substrate pathways operate under a given experimental or disease state. To answer this, the model system needs to be optimized for all substrates.

In summary, the Seahorse XF Cell Mito Stress Test can be employed to address a number of questions related to mitochondrial competence. Using the system described in this brief, muscle fibers from many disease models can be analyzed.

Table 1 | Instrument Run Protocol

Start Protocol			
Command	Time (min.)	Port	Repeat
Calibrate			
Mix	3:00		
Wait	2:00		
Mix	3:00		
Wait	2:00		
Mix	3:00		
Wait	2:00		3-5
Measure	3:00		
Inject		A	
Mix	3:00		
Wait	2:00		2-4
Measure	3:00		
Inject		B	
Mix	3:00		
Wait	2:00		2-4
Measure	3:00		
Inject		C	
Mix	3:00		
Wait	2:00		2-4
Measure	3:00		
Inject		D	
Mix	3:00		
Wait	2:00		2-∞
Measure	3:00		
End Protocol			

Assay Optimization Hints

1. The first run should be a titration of the numbers of muscle fibers to be used. Numbers will vary between species and tissue used. It is advisable to run volumes and amount around the numbers reported here. Basal rates between 200-400 pmoles/min is a good starting point.
2. For optimization of the concentration of reagents added, follow the guidelines in the Seahorse XF Cell Mito Stress Test Kit (Seahorse Bioscience #101706-100).
3. It is important that the fibers adhere well. Monitor seeding to make sure the fibers adhere.
4. In general, 3-5 pre-injection measurements should be made to get a stable baseline. For the first runs, performing 3-4 measurements between injections is recommended to determine the amount of time needed to generate a complete response. The number of rate measurements can then be adjusted accordingly.
5. In the protocol referenced here, glucose is being used as a substrate with later addition of pyruvate. The substrate makeup of the assay buffer can be adjusted to reflect the type of analysis being performed. Glucose, pyruvate, glutamine and other amino acids as well as fatty acids can be present in the assay media from the beginning.

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## Acknowledgements

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