Innovative Methodology—American Journal of Physiology-Regulatory, Comparative, and Integrative Physiology

Measuring mitochondrial respiration in intact single muscle fibers.

Rosemary A. Schuh¹,², Kathryn C. Jackson³, Ramzi J. Khairallah⁴, Christopher W. Ward⁴, Espen E. Spangenburg³

¹Research Service, Maryland VA Health Care System, Baltimore, MD 21201

²University of Maryland, School of Medicine, Department of Neurology, Baltimore, MD 21201

³University of Maryland, School of Public Health, Department of Kinesiology, College Park, MD 21045

⁴University of Maryland, BioMet and School of Nursing, Baltimore, MD 21201

Running title: Skeletal muscle mitochondrial function

Corresponding Author:

Espen E. Spangenburg, Ph. D.
University of Maryland
School of Public Health
Dept of Kinesiology
College Park, MD 20742
301-405-2483 (office)
301-405-5578 (fax)
espen@umd.edu
Abstract

Measurement of mitochondrial function in skeletal muscle is a vital tool for understanding regulation of cellular bioenergetics. Currently, a number of different experimental approaches are employed to quantify mitochondrial function, with each involving either mechanical or chemical-induced disruption of cellular membranes. Here, we describe a novel approach that allows for the quantification of substrate-induced mitochondria-driven oxygen consumption in intact single skeletal muscle fibers isolated from adult mice. Specifically, we isolated intact muscle fibers from the flexor digitorum brevis muscle and placed the fibers in culture conditions overnight. We then quantified oxygen consumption rates using a highly sensitive microplate format. Peak oxygen consumption rates were significantly increased by 3.4-fold and 2.9-fold by simultaneous stimulation with the uncoupling agent, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), and/or pyruvate or palmitate exposure, respectively. However, when calculating the total oxygen consumed over the entire treatment palmitate exposure resulted in significantly more oxygen consumption compared to pyruvate. Further, as proof of principle for the procedure we isolated fibers from the mdx mouse model, which has known mitochondrial deficits. We found significant reductions in initial and peak oxygen consumption of 51% and 61% compared to fibers isolated from the wild type (WT) animals, respectively. In addition, we determined that fibers isolated from mdx mice exhibited less total oxygen consumption in response to the FCCP + pyruvate stimulation compared to the WT mice. This novel approach allows the user to make mitochondrial-specific measures in a non-disrupted muscle fiber that has been isolated from a whole muscle.
Introduction

The understanding of mitochondrial function in skeletal muscle is a critical component for delineating mechanisms behind muscular adaptation and their contribution to various chronic health conditions. It is now well established that mitochondrial function contains some plasticity and deficiencies in function are critical to the pathogenic progression of numerous muscle diseases.

Several approaches have been used to assay mitochondrial function in muscle and each has limitations and benefits. For example, it has become common to use measurements of single mitochondrial enzymes as a surrogate to measuring mitochondrial function. Unfortunately, due to the complexity of mitochondrial activity these measures do not provide a complete physiological readout of mitochondrial function, but instead only provide information about the activity of that specific enzyme. It is assumed that the changes in single enzyme activity frequently correlate with changes in overall mitochondrial function. However, there are often circumstances when not all mitochondrial enzyme activity is altered to the same magnitude under a chosen condition making interpretation of the data difficult.

Measurements of mitochondrial respiration provide a more appropriate indication of the function of mitochondria. Historically these measures have been made using amperometric oxygen electrodes (i.e., Clark electrode) to determine changes in oxygen tension within a buffer. With this approach, mitochondrial respiration studies in skeletal muscle have been typically conducted in isolated mitochondria or in permeabilized muscle fiber bundle preparations. The isolated mitochondrial approach requires fresh muscle samples that are mechanically homogenized and subsequently exposed to a differential centrifugation approach resulting in a reasonably pure mitochondrial fraction (12). The isolation yields a high mitochondrial purity, but evidence suggests that the isolation procedure can result in the loss of specific complexes within the electron transport chain (27). Furthermore, recent evidence has documented that this approach can disrupt or even over estimate mitochondrial function (26).

Due to limitations with isolated mitochondria preparations, permeabilized fiber bundles are often used to measure oxygen consumption of mitochondria. Fiber bundles are mechanically dissected from the whole muscle and chemically treated to permeabilize the sarcolemma without damaging other organelle membranes (20, 30). This approach allows investigators to measure mitochondrial function in the context of the whole muscle fiber with minimal mechanical disruption. While maintaining control over the intercellular milieu, a disadvantage is that disruption of the muscle fiber membrane necessitates measures be made immediately upon tissue removal. In addition, in this technique since the cytoplasm and external media become equilibrated any substances within the equilibration media could affect mitochondrial function. Thus, great care must be taken to ensure proper media formulation. Both of these considerations place time and experimental constraints on the experiment which affects the throughput of this approach. However, some groups have shown that prior to permeabilization it is possible to cyropreserve isolated fiber bundles for subsequent measures with minimal effect on mitochondrial function (19, 34). This approach would allow for increased sample
collection without the need for immediate processing, but it is unclear why this procedure has not resulted in more utilization.

Mitochondrial function has also been assessed in intact non-disrupted tissue/cellular preparations and important information has been gained. The main benefit of this approach is that it allows mitochondrial function to be assessed in its most native state. The main drawback of whole muscle experiments with classic amperometric oxygen probes is the diffusion limitation of oxygen in the tissue and bath. More recently, miniaturized self-referencing or amperometric electrodes have proved particularly useful in overcoming diffusion limitations by allowing the assay of oxygen metabolism in single cultured cell preparations (23), however the techniques are highly demanding and thus result in lower throughput. For example, Elzinga et al eloquently demonstrated methodology using oxygen electrodes and isolated single fibers from frog muscle where they demonstrated increasing oxygen consumption as a function of contraction frequency (8). It was determined that the maximum rate of oxygen consumption by the fibers was related to activity levels of the mitochondrial protein succinate dehydrogenase (35).

Recently, systems have been developed to make measurements of mitochondrial respiration in the same small volumes within a multi-well microplate format coupled with substantially enhanced sensitivity compared to standard amperometric oxygen probes (25). With this approach, measurement sensitivity in both isolated mitochondria and primary cell cultures have been greatly enhanced allowing for increased throughput enabling treatments and replicates to be conducted rapidly. Here we describe the use of this approach to assay single intact skeletal muscle cells.

We have adapted the use of intact individual muscle fibers isolated from a whole muscle to measure mitochondrial respiration. This approach allows for similar measures to be made as previously documented, however since the fiber membranes are not disrupted it allows the user the ability to place the fibers in culture removing the need to make immediate measures after the isolation procedure. In addition, this approach assesses the ability of the mitochondria within the fibers to consume oxygen at a physiological temperature. Finally, after the respiration experiments the user can utilize the cells for immunohistochemical techniques or isolate the cells for procedures such as westerns, q-PCR, or activity assays.
**Materials and Methods:**

**Animals:** For the first series of studies, 10-16 week old male C57/BL6 mice were used. In our second studies, the murine model of Duchenne’s muscular dystrophy (C57Bl/10 ScSn-mdx) (mdx) and their control counterparts (C57Bl/10 ScSn), now referred to as wildtype (WT) mice were used at 5 months of age. All experiments were approved by the University of Maryland Animal Care and Use Committee.

**Chemicals:** All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

**Single fiber isolation:** After euthanasia (CO₂ inhalation), FDB muscles were harvested bilaterally from C57/BL6, mdx or WT mice. Single skeletal muscle fibers were enzymatically isolated from the flexor digitorum brevis (FDB) muscle. An example microscope brightfield image of the isolated intact single fibers and a high magnification image of an individual fiber stained with mitotracker (green)/dapi (blue) are shown in Figure 1. The isolation procedure was modified from work previously published with this method in muscle from rats and mice (2-5, 22, 28). In brief, surgically excised FDB muscles were incubated in dissociation media (DM) containing DMEM (Invitrogen), gentamycin (50 µg/ml), FBS (2%, ATCC, #30-2020 Rockville, MD), and collagenase A (4 mg/ml, Roche). Two FDB muscles were placed in a 35mm disposable culture dish with 4 ml of DM and then in an incubator (37°C, 5% CO₂) for 1.5-2 hours. Following the dissociation, muscles were placed in a new 35mm plate with warmed media containing gentamycin and FBS but without collagenase. FDB muscles were triturated with a small bore (~1mm) fire polished glass transfer pipette to yield single FDB myofibers. 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 min and the process was repeated. Following trituration, large debris (nerve, un-digested FDB muscle) was removed with forceps. Seahorse XF24 cell culture V7 microplates (Seahorse Bioscience, Billerica, MA) were coated with 5 µl of extracellular matrix (ECM, Sigma-Aldrich, E1270, St. Louis, MO) previously diluted 1:1 in DMEM. Following application of ECM in the wells, the lid was placed on the V7 microplate and the plate vigorously tapped horizontally against an open hand to spread the ECM throughout the wells. The plate was allowed to briefly air dry for 10-30 min. Following a thorough dispersion of single fibers in the 35 mm dish, 75µl aliquots of the media were taken randomly and deposited into each well with the goal being to allow the fibers to cover ~60% confluency of the well bottom. The confluency was determined through light microscope visualization. Single myofibers were verified as being fully adherent within 5 min. The cells were placed in a 95% air/5% CO₂ humidified incubator at 37°C overnight in DM media without the collagenase.

**XF24 Microplate-Based Respirometry:** Bioenergetic analyses of intact single cultured muscle fibers were performed using an XF24-3 Extracellular Flux Analyzer (Seahorse Bioscience). As previously described, the Seahorse Bioscience technology provides an innovative and more sensitive way to assess mitochondrial function compared to traditional amperometric oxygen probes, which results in real time sensitive measures with high throughput due to the microplate format (13, 25).

**Intact Fibers:** The assay measurement buffer (MB) at ~37°C contained 120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 1 mM MgCl₂, 5 mM HEPES (pH 7.4) supplemented with 2.5 mM D-glucose (Sigma G7528) and 0.5 mM L-carnitine (Sigma CO158). Prior to measurements, pre-warmed MB was
gently added to the fibers and the fibers were placed in an unbuffered, humidified incubator at 37 °C for 2 hours to allow temperature and pH equilibration. Since, the Flux Analyzer performs direct injections of substrates to the media in the wells, it is absolutely critical that the investigator know the initial volume of media in each well prior to the addition of the MB. Thus, 1000 µl of MB was removed from each well and 600 µl of MB was added per well prior to placing the plate in the analyzer resulting in a final volume of 675 µl. Each plate contains specific temperature control wells, which have no fibers in the wells. These wells had 675 µl of MB added to each well. Following this incubation period, the calibration plate was loaded onto the instrument and calibration initiated. Following calibration, the plate containing the fibers replaced the calibration plate in the instrument to begin the experimental run. After an equilibration step, basal oxygen consumption rates (OCR, pmoles/min) and extracellular acidification rates (ECAR, mpH/min) were recorded using 3-min mix, 2-min wait and 3-min measure (looped 3 times) cycles prior to injection of substrate plus carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma C2920) to induce maximal oxygen consumption. Three more measurement loops were recorded following FCCP injection and prior to injection of antimycin A (inhibitor of mitochondrial respiration, Sigma A8674). Two measurement loops were recorded after the injection of antimycin A. The drugs prepared in MB (75 µl volumes) were preloaded then sequentially injected as indicated through ports in the XF24 calibration cartridge to final concentrations of 400 nM FCCP, substrates supplied were either sodium pyruvate (10 mM, Sigma P8574) or albumin (Roche, Indianapolis, IN, 03117405001) conjugated to sodium palmitate (Sigma P9767, 200 µM), and antimycin A (1 µM). Upon completion of the runs, all cells were scraped into Mueller buffer (50 mM Hepes (pH 7.4), 0.1% Triton X-100, 4mM EGTA, 10mM EDTA, 15 mM Na₄P₂O₇•H₂O, 100 mM β-glycerophosphate, 25 mM NaF, 50µg/ml leupeptin, 50 µg/ml pepstatin, 40 µg/ml aprotinin, 5mM Na₃VO₄, and 1 mM PMSF) to lyse the cells to normalize the data to total protein (32). Unfortunately, the amount of total protein detected in each well was below the minimal detection limits of the assay (BCA Assay, Pierce) and thus we were unable to use this as a normalization approach.

Permabilized Fibers: For these experiments, we followed the previously developed protocol with a few minor changes (20). We isolated the fibers as previously described above and the fibers were allowed to adhere overnight to ECM coated plates. The following morning the fibers were placed in buffer A (10 mM Ca-EGTA buffer (2.77 mM of CaK₂EGTA + 7.23 mM of K₂EGTA) free concentration of calcium 0.1 µM, imidazole 20 mM, taurine 20 mM, K-MES 49 mM, K₂HPO₄ 3 mM, MgCl₂ 9.5 mM, ATP 5.7 mM, phosphocreatine 15 mM, leupeptin 1 µM, pH 7.1, adjusted with 5 N KOH) at room temperature for 5 mins. Buffer A was replaced with Buffer A containing saponin (50µg/ml) and allowed to incubate for another 30mins. The buffer was then changed to Buffer B (EGTA 0.5 mM (0.19 g liter⁻¹), MgCl₂.6H₂O 3 mM (0.61 g liter⁻¹), taurine 20 mM (2.502 g liter⁻¹), KH₂PO₄ 10 mM (1.361 g liter⁻¹), HEPES 20 mM (4.77 g liter⁻¹), BSA 1 g liter⁻¹, potassium-lactobionate 60 mM (120 ml of 0.5 M K-lactobionate stock solution liter⁻¹), mannitol 110mM (20.04 g liter⁻¹), dithiothreitol 0.3 mM (0.046 g liter⁻¹), pH 7.1, adjusted with 5 N KOH) and placed in the Extracellular Flux Analyzer for specific measures.

Statistics: All experiments were conducted three-five times with at least five replicates per experiment. All data are expressed as means ± SE. Statistical significance was determined using a t-test for comparing
FCCP stimulated OCR rates compared to basal OCR rates, AUC OCR measures, and for comparison across time points of OCR between the WT and mdx fibers. A two-way ANOVA was utilized for spare respiratory capacity measures utilized between the WT and mdx fibers (see fig 3D). With the ANOVA, if an interaction was found the test was followed by a Holm-Sidak post-hoc test. A \( P \) value of <0.05 was considered significant.

**Results:**

*Pyruvate and palmitate-induced respiration.* In the first set of experiments, we isolated intact single fibers from 10-16 week old male C57/BL6 mice. After the fibers were dissociated, we plated the fibers on ECM coated XF24 V7 microplates overnight. The next day the intact single fibers were removed from the incubator, the media was removed and replaced with MB containing low glucose concentrations. The fibers were returned to a 37°C non-CO2 buffered incubator for 2 hrs. The fibers were then placed in the XF24-3 analyzer for analysis. In these experiments, we sought to determine if respiration could be stimulated in intact muscle fibers utilizing commonly used substrates, sodium pyruvate and albumin conjugated-palmitate. Since both substrates are transported across the sarcolemma, we supplied the substrates in excess simultaneously with the mitochondrial uncoupler FCCP, to the MB as indicated in Figure 2. Under basal conditions, the energetic demand of the fibers is low thus to enhance substrate utilization by the mitochondria we provided the cells with FCCP. We found that both substrates resulted in significant increases (\( P < 0.001 \)) of approximately 3-fold in oxygen consumption by the single fibers (Figure 2A). To confirm that the increase in oxygen consumption was specific to the mitochondria, we next added antimycin A, an inhibitor of Complex III of the electron transport chain. As can be seen at the point indicated in Figure 2A, respiration was returned to baseline after antimycin treatment confirming that the increase in oxygen consumption mediated by the substrate exposure was due to increased oxygen consumption by the mitochondria. We found no difference in the extracellular acidification rate (ECAR) when the intact single fibers were treated with either pyruvate or palmitate (Figure 2B). ECAR is an indirect measure of glycolytic activation, measured as milli-pH per minute, with the assumption that in the cultured media any increase in acidification of the media driven by accretion of protons is derived by lactate (25). Thus, any increases in ECAR indicate an increased acidification of the medium potentially due to enhanced lactate production. There was no significant difference in the peak oxygen consumption induced by either substrate, however when considering the total amount of oxygen consumed as calculated by the area under the curve (AUC, Figure 2C), more total oxygen was consumed when the fibers were treated with palmitate.

To determine if the isolated single fibers could be used in the extracellular flux analyzer in a similar approach as previously described for the permeabilized fiber bundles, we conducted experiments in which we saponin treated the isolated fibers to permeabilize the sarcolemma. Saponin is a cholesterol specific detergent that in muscle cells will selectively permeabilize the sarcolemma without damaging the mitochondria (20, 30). Unfortunately, we found the permeabilized single fiber approach
is not compatible with the Extracellular Flux Analyzer due to a temperature sensitivity issue. Specifically, unlike our results in the intact fibers we were unable to reliably measure substrate or FCCP driven increases in OCR when the fibers were permeabilized (data not shown). When we placed the permeabilized fibers in the Extracellular Flux Analyzer and exposed the fibers to 10mM Glutamate and 5mM malate we found no changes in OCR even if we injected a saturating dose of 2 mM ADP which should induce a maximal State 3 respiration (20). We also were unable to induce an FCCP response in these permeabilized fibers, while intact fibers that were run in parallel showed normal FCCP responses (data not shown). Visual examination of the permeabilized fibers found that they were not tolerating the higher, but physiological temperature of 37°C compared to the intact single muscle fibers. However, we did find that at temperatures equivalent to room temperature and lower, the permeabilized fibers remained viable which is what is expected based on previous publications (20, 30). Unfortunately, the Extracellular Flux Analyzer is designed to collect the experimental data at 37°C making the permeabilized fiber approach challenging in the single fiber model. However, using the muscle fiber bundle model as previously described is more conducive to experiments at physiological temperatures (1, 20, 26). To appreciate this one must consider the methodological differences between the muscle fiber bundle approach and the single muscle fiber model. Muscle fiber bundles are isolated from a section of muscle with the muscle fibers being mechanically separated to maximize surface area while still retaining some form of attachment to each other in a bundle formation (1). Single muscle fibers are fibers that are completely dissociated from each other after isolation of the whole muscle, thus there is no contact with previous neighboring fibers. The advantage to the single muscle fiber approach is the ability to culture the fibers in a multi-well format for multiple days, which is not conducive to the muscle fiber bundle approach since the bundle is non-adherent. Thus, when fiber bundles are isolated and permabilized it is done so in ice-cold solutions until right before the mitochondrial measures are made which allows the bundles to tolerate physiological temperatures long enough to complete the experiments. However, since single muscle fibers are maintained under culture conditions and in a multi-well format, mimicking the ice cold buffer approach prior to the measures is not possible which limits the amount of time we can maintain the permabilized single fibers at physiological temperatures. Unfortunately, after permeabilizing the fibers we were unable to run the experiments fast enough to achieve reliable data. However, it should be clear that the single muscle fibers will tolerate physiological temperatures when the sarcolemma is intact and the fibers are not permabilized. It is well documented in the literature that when single fibers are permabilized or ‘skinned’ they rapidly deteriorate at physiological temperatures, however they remain viable at temperatures that are room temperature and below (6, 33, 36). In fact, to overcome this limitation many investigations have examined the role of temperature on permabilized (or ‘skinned’) single fibers by using a temperature jump method to rapidly raise the temperature of the fiber, or by conducting their experiments in a much short time span than our approach (less than 30mins) (29). Finally, others have shown that permeabilized single fibers housed at higher temperatures release significant amounts of reactive oxygen species, which is associated with reduced force production and likely contributes to the deterioration (7).

Mitochondrial function deficiency in mdx fibers. To show application of our approach for measuring mitochondrial oxygen consumption, we examined mitochondrial respiration responses in intact single fibers isolated from mdx vs WT animals. It has been previously demonstrated that maximal
mitochondrial respiration in skeletal muscle of mdx mice is approximately 2-fold lower compared to WT mice, which appears to be mediated by a loss in specific mitochondrial protein content (21). In the present study, we isolated intact fibers from age-matched WT and mdx mice to determine if we could detect a mitochondrial deficiency using our approach. The fibers from mdx mice exhibited significantly lower basal (P<0.001) and maximal (P<0.001) mitochondrial respiration rates compared to fibers from the WT mice (Figures 3A and 3D). In addition, we detected significantly lower (P<0.01) ECAR rates in the mdx fibers compared to the WT mice suggesting potential defects in glycolysis in the mdx fibers (Figure 3B). However, when we calculated the OCR/ECAR ratio no differences were found (data not shown). When total oxygen consumption was calculated there was a trend (P = 0.08) for a lower response to the FCCP + pyruvate in the mdx mice (Figure 3C). We calculated the average initial oxygen consumption rates and the peak oxygen consumption rates in the WT versus the mdx mice (Figure 3D). The intact muscle fibers from mdx mice demonstrated reduced initial and peak oxygen consumption rates (Figure 3D). Further, we found that the fold-increase in oxygen consumption in response to the FCCP + pyruvate stimulation was 3.9 and 2.9 in the WT and mdx, respectively. In addition, we calculated the mitochondrial spare respiratory capacity as previously described (15, 38), in the fibers and found a 62% reduction in mdx fibers compared to the WT fibers. Spare respiratory capacity is defined as the difference between the basal and the maximal (uncoupled) OCR and was suggested to approximate the ability of mitochondria to upregulate OCR in response to an increased demand for ATP (15, 38). Thus, using the intact single fiber model, we present results that are consistent with previous findings that the mdx murine model does exhibit mitochondrial dysfunction.

**Discussion:**

Determining mitochondrial function in skeletal muscle has become a commonly employed methodology. In these experiments, we sought to determine if mitochondrial respiration measurements could be determined in cultured intact single muscle fibers in a novel microplate format. The use of this approach enables the investigator to make measurements with minimal disruption of the myofibers. Additionally, by using appropriate culture conditions, the intact single fibers can be maintained overnight in a standard CO₂-incubator for subsequent measures (4). In these experiments, intact adult muscle fibers were isolated and cultured overnight. The plates were removed from the incubator the next morning and mitochondrial respiration was determined in a microplate format, in which 20 different wells were assayed simultaneously. Mitochondrial oxygen consumption was induced by supplying the fibers excess concentrations of substrate (i.e. PYR or PA) and simultaneously uncoupling the mitochondria through addition of FCCP. As expected, we found that PYR and PA were equally effective at inducing oxygen consumption, but the PA ultimately consuming more total oxygen over the entire measurement time. To ensure the increase in OCR was representative of mitochondrial
oxygen consumption, antimycin was delivered to the fibers resulting in complete loss of the enhanced OCR. Thus, our method provides an approach to measure mitochondrial-specific respiration in enzymatically isolated single muscle fibers from an adult animal without major disruption of the membrane.

Current approaches to quantify mitochondrial respiration either rely on mechanical disruption of the whole muscle followed by differential centrifugation or chemical permeabilization of the sarcolemma. Recent findings have suggested the isolation of mitochondria-enriched fractions from skeletal muscle can result in alterations in mitochondrial function. In an elegant study, Picard et al found that there are alterations in the stoichiometry of proteins within the electron transport chain in response to the isolation process (27). In addition, the isolation process further results in enhanced reactive oxygen species production in response to substrate delivery (27). The data confirm the importance of studying mitochondrial function in preparations which result in minimal disruption to the cell. Further, Picard et al confirmed this concept in skeletal muscle taken from aged animals (26). Specifically, they found that using standard mitochondrial isolation conditions resulted in an over-exaggeration of any mitochondrial deficiency in the aged animals when compared to the same measures made in permeabilized fibers which demonstrated no outward deficiency in mitochondrial function (26). Thus, the data of Picard et al. would suggest that using a permeabilized fiber bundle approach is an advantageous approach to consider when studying mitochondrial function in skeletal muscle (26). Here, we provide an alternative to the muscle fiber bundle approach by using intact single muscle fibers (Figure 1). These cells are isolated from a whole muscle with all membranes intact and can be maintained in standard cell cultures for a short duration while still retaining their phenotype (~48 hrs). The cells can be cultured longer with adjustments to the culture conditions (4). By using substrates that can cross the sarcolemma, we demonstrate that successful mitochondrial measures can be made in this model without disruption of the cell membrane. A limitation of the approach is that we cannot add substrates unless they are permeable to the cell membrane, thus it is difficult to achieve certain measures. Comparisons of state 3 capacity is one of the most commonly reported parameters for assessing mitochondrial function (20), however in the intact fiber approach it is not possible to determine a true state 3 respiratory capacity compared to using isolated mitochondria or permeabilized fiber bundles due to an inability to regulate ADP concentrations. We use FCCP-stimulated respiration as a means to induce a maximal respiratory response, which allows us to determine the spare respiratory capacity, defined as the difference between maximal OCR and the basal OCR (25). Thus, our approach assesses the ability of the cell to function as a whole, whereas other approaches are directly working with the electron transport machinery. As with any approach, there are inherent advantages and disadvantages to each approach.

In these experiments, we also employed a novel microplate approach. To our knowledge, this is the first demonstration using this technology to measure mitochondrial respiration in muscle fibers taken from an adult animal. Albeit, others have examined single fiber respiration measures in individual fibers in an isolated bath system (8, 35). An advantage of this system is the ability on an individual plate to supply fibers isolated from the same muscle with different substrates (i.e. PYR or PA) to drive respiration. Simultaneous measurements of mitochondrial respiration and the metabolic flexibility of
the mitochondria in the muscle fibers results in enhanced throughput (see Figure 2A). Metabolic
flexibility is defined as the ability of a cell to adjust fuel oxidation to fuel availability (10). The term was
originally defined by Kelley and Mandarino as “the capacity to switch from lipid oxidation and high rates
of FA uptake to suppressed lipid oxidation and increased glucose uptake and oxidation under insulin-
stimulated conditions (17).” A critical aspect of this definition is the ability of the mitochondria within
the tissue to oxidize substrates and clearly in our approach we are measuring only a small aspect of the
definition. Thus, by placing the cells in the same environment but only changing the substrate it is
feasible to quantify the oxygen consumption rates response to the different substrates (see Figure 2C).
This response can then be compared across conditions to determine if the mitochondria within the
fibers lack an inherent ability to use different substrates. Due to the complexity of mitochondria, there
are numerous points within specific metabolic pathways that could contain a defect. For example, it is
feasible to imagine that if there was a limitation in β-oxidation and the experimental protocol provided
substrate to the muscle fibers that did not use β-oxidation (i.e. pyruvate) then the limitation would be
overlooked. In fact, Koves et al have suggested that incomplete oxidation of fatty acids can contribute
to the development of insulin resistance in skeletal muscle (18). Additionally, it has been suggested that
decreases in carnitine concentrations could result in poor lipid utilization due to poor lipid entry into the
mitochondria (24). By examining more than one type of substrate an investigator gets a more complete
understanding of the function of their mitochondria within their experimental paradigm. Further, by
developing an approach using intact muscle fibers, an investigator can use this as a first pass screen to
determine if substrate flux limitations into the cells contribute to their phenotype. Specifically, if an OCR
response is reduced in the intact cells, but their response is normal when the cells are treated with a
membrane permeable substrate (i.e. transport independent), it would suggest that the limitation may
be occurring at the sarcolemma. For example, analyzing substrate flux across the cell membranes could
be achieved using octanoic acid (C8), a medium chain fatty acid which can bypass transport-mediated
movement into the cell (16). CD36/FAT is a well described lipid transporter in the muscle (14). Thus, if
the muscle is experiencing an inability to traffic lipid into the muscle a potential defect could be
determined using octanoic acid in parallel experiments with palmitic acid. If no defect was detected
using octanoic acid, this would suggest a transport mediated effect. However if the defect remained,
then it would suggest that beta-oxidation may be the limiting factor. The technique is also amenable to
quickly screen single muscle fibers with various pharmacological agents that may affect metabolic
function. This allows examination of the agents’ effect on adult cells as opposed to using cells that may
exist in a more embryonic state. Overall, we believe we have identified a novel approach for measuring
mitochondrial function in intact cultured adult muscle fibers.

As a proof of principle assay, we chose to analyze mitochondrial respiration measures from mdx
mice, the murine model of Duchene muscular dystrophy. While the loss of dystrophin is a primary
deficit responsible for the initiation of the skeletal muscle dysfunction present in these mice, the
pathogenic progression of the disease involves documented mitochondrial dysfunction. Experiments
that used either saponin-permeabilized muscle fiber bundles or isolated mitochondria from the
quadriceps muscle demonstrated impaired rates of maximal respiration compared to age-matched
control mice (21). Using our approach, we confirmed this finding in that initial or basal rates for oxygen
consumption were substantially reduced in the mdx mice compared to WT mice. In addition, there was
a substantially lower maximal OCR in response to the PYR + FCCP stimulation in the mdx mice compared to the WT mice. It is likely that the differences we detected are due to the described mitochondrial dysfunction and not plating differences. We were extremely careful to visually monitor the pre- and post-plating of the cells. We saw no indications of over or under plating the cells in either condition (data not shown). However, we also feel that we would have been able to detect a plating effect in our experiments. Specifically, we induced peak respiration by providing the cells with a maximal dose of FCCP + PYR, which would provide a maximal response from the mitochondria present in that well. If our differences were due to a plating density response, we should have seen a similar percent increase from initial (baseline) to peak (FCCP) (Figure 3D). However, when calculating the fold change from initial to peak the mdx mice increase by 2.9-fold in response to stimulation while the WT increase by 3.9-fold suggesting we are able to detect a mitochondrial myopathy in the mdx using this approach. Further, when we calculate the spare respiratory capacity we detected a 62% deficiency in the fibers isolated from the mdx mice compared to the WT mice. The spare respiratory capacity is the difference between the basal and the maximal (uncoupled) OCR and can be used as a surrogate readout for the ability of the mitochondria to increase oxygen consumption in response to an increased demand for ATP. Thus if the difference we detected was entirely due to plating density, one would predict that when normalizing to the different starting points (i.e. initial OCR) all the significant differences would be lost. Since previously published data have shown that a number of the respiratory chain components are significantly reduced in the mitochondria from the mdx compared to WT mice (9, 11, 21), we feel that our technique has successfully confirmed that mdx mice do exhibit poor functioning mitochondria compared to WT mice.

Interestingly, we also found a substantially lower ECAR response in the fibers from the mdx mice compared to the WT mice. ECAR is a measure of the rate of extracellular acidification and it is expected that cells that have a high ECAR are exhibiting a glycolytic phenotype. Our data demonstrate that the initial ECAR between the fibers from the mdx and WT animals is equivalent; however the peak stimulated ECAR response is significantly lower in the mdx compared to WT groups. This suggests that glycolysis in the mdx fibers may be compromised compared to the WT fibers. Indeed, Wehling-Henricks et al. (37) previously demonstrated that phosphofructokinase (PFK) activity was significantly lower in the mdx mice compared to WT mice, an effect that was mediated by loss of neuronal nitric oxide synthase (nNOS). Thus, our data provide a confirmatory and functional readout that suggests glycolysis may be impaired in fibers isolated from the mdx mice compared to the WT mice. To the best of our knowledge there is very little evidence concerning glycolytic flux measures in mdx and WT mice and may be an area that needs to be further considered in the mdx phenotype.

A key consideration when using this approach includes the muscle fiber type. It is well documented that metabolic variation is large across and within different muscle fiber types (31). If utilizing a muscle or model that is likely to have large variations in fiber type, it would be critical to consider performing immunohistochemistry or gel-based fiber typing.

**Limitation of this approach:** To make accurate conclusions over experiments that demonstrate differences in OCR it is critical to normalize the data to determine if there is a potential internal mitochondrial deficiency or if the difference is due to a reduction in the muscle fiber mitochondrial
density. A number of normalization procedures are possible including total protein, estimates of mitochondrial density through PCR based techniques, or a cellular imaging based technique. Albeit, in our intact fiber experiments we have found that we were unable to extract enough protein from each well to accurately quantify total protein using a standard colormetric protein assay (data not shown). Thus, it would be prudent to consider a more sensitive approach such as PCR. Thus, if differences in mitochondrial DNA were detected between groups it would indicate a reduction in mitochondrial volume per fiber, however if no differences were found in the mitochondrial DNA then it would suggest an internal defect may exist within the mitochondria requiring further experiments. The approach we are describing provides a powerful means to rapidly determine if there are differences in mitochondrial function in intact skeletal muscle fibers, however the approach cannot accurately predict what the existing deficiency is without further follow up experiments. Further, we feel strongly that our results are not due to differences in the amount of material plated per well from each group. The XF24-3 Extracellular Flux Analyzer utilizes retractable probes that contain O₂ sensors which enter each well simultaneously sealing off a small volume of medium to assess O₂ consumption. Thus, since the probes measure a small area of the well and not the entire well if our results were due to differences in material we would have had to consistently under load one small area across an enormous number of measured wells. In our experiments to ensure reliability of measures we visually assessed each well to ensure the well is not under or overloaded for fiber number. We also performed a high number of replicates per condition from each muscle. For example, in the WT and mdx experiments, the OCR and ECAR measures are an average of 10 replicates per animal (n=5 animals/group), which should ensure that any differences we detect are actual physiological or biochemical differences and not due to material amount on the plates.

In conclusion, we believe we have developed an innovative way to assess mitochondrial respiration in skeletal muscle using a microplate format. This method enables maintenance of the intact single muscle fibers in standard cell culture conditions with minimal disruption. In addition, since the measures are made on live intact cells, the tissue can then be used in follow up experiments such as westerns, Q-PCR, or imaging studies.
Acknowledgments:

Grant funding is acknowledged from Rehabilitation R&D REAP and Biomedical R&D CDA-02 from the VA Research Service (RAS), and the National Institutes of Health (RC2 NR011968 to C.W.W.) (R21 AR059913 to E. E. S.) and KCJ was supported by NIH AG000268. Also, the authors wish to thank Dr. Matt Hulver for insightful advice.

Figure Legends

Figure 1 (A-B) Visual representation of intact single muscle fibers isolated from the FDB of adult male mice. A. Brightfield image of cultured single fibers after dissociation (10x magnification). Black line = 500µm. B. Dual labeling of a single fiber with mitotracker (green) and DAPI (blue) (40x magnification).

Figure 2 (A-C) Basal and stimulated oxygen consumption rates (OCR) of cultured adult intact single muscle fibers. A. Initial OCR was measured in the single fibers, followed by peak OCR which was induced (first arrow) by direct exposure to FCCP (400nM) and either pyruvate (PYR (10mM); ●) or albumin-conjugated palmitate (PA (200µM); ●). Subsequently, to ensure the measured peak OCR was due to increased mitochondrial respiration the fibers were treated with antimycin A (second arrow). *, # indicates significantly different than initial OCR of PYR or PA at all points measured, respectively (P<0.001). B. Extracellular acidification rates (ECAR) of muscle fibers after direct exposure to FCCP and either pyruvate (PYR; ●) or albumin-conjugated palmitate (PA; ●). C. Total oxygen consumption was significantly higher in the fibers treated with PA compared to PYR. Oxygen consumption was determined by calculating the total area under the curve (AUC). † indicates significantly different than PYR AUC (P<0.05).

Figure 3 (A-D) Basal and stimulated OCR was significantly lower in the fibers from mdx mice compared to the WT mice. A. Initial and FCCP + PYR-stimulated peak OCR was significantly lower in the mdx fibers (●) compared to the WT fibers (●). *Indicates significantly different than initial or baseline WT-OCR, # Indicates significantly different than initial or baseline mdx-OCR, † significantly different compared to WT all points under horizontal line (P<0.001). B. ECAR was significantly reduced in mdx muscle fibers compared to WT after exposure to FCCP and PYR. *Indicates significantly different than initial OCR (P<0.001) C. Total oxygen consumption tended to be lower in the mdx fibers compared to the WT fibers (P=0.08). Oxygen consumption was determined by calculating the total area under the curve (AUC). D. Initial and peak OCR was significantly lower in the mdx fibers compared to the WT fibers. *Indicates significantly different than initial WT, † significantly different WT peak OCR, # significantly different than initial mdx OCR. (P<0.001 for all).
References


Figure 1 (A-B).
Figure 2 (A-C).

A

B

C

*  

#  

†  

OCR (pMoles/min)

ECAR (pMph/m/M)

TIME (min)

TIME (min)

AUC OCR (pMoles x 10^3)

PYR

PA
Figure 3 (A-D)

A

OCR (pMoles/min)

WT

MDX

†

* # #

TIME (min)

B

ECAR (mP/min)

WT

MDX

†

* # #

TIME (min)

C

AUC OCR (pMoles x 10^3)

WT

MDX

D

OCR (pMoles/min)

Initial

Peak

WT

MDX

†, #