Video Article

Using Isolated Mitochondria from Minimal Quantities of Mouse Skeletal Muscle for High throughput Microplate Respiratory Measurements

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Abstract

Skeletal muscle mitochondria play a specific role in many disease pathologies. As such, the measurement of oxygen consumption as an indicator of mitochondrial function in this tissue has become more prevalent. Although many technologies and assays exist that measure mitochondrial respiratory pathways in a variety of cells, tissue and species, there is currently a void in the literature in regards to the compilation of these assays using isolated mitochondria from mouse skeletal muscle for use in microplate based technologies. Importantly, the use of microplate based respirometric assays is growing among mitochondrial biologists as it allows for high throughput measurements using minimal quantities of isolated mitochondria. Therefore, a collection of microplate based respirometric assays were developed that are able to assess mechanistic changes/adaptations in oxygen consumption in a commonly used animal model. The methods presented herein provide step-by-step instructions to perform these assays with an optimal amount of mitochondrial protein and reagents, and high precision as evidenced by the minimal variance across the dynamic range of each assay.

Video Link

The video component of this article can be found at https://www.jove.com/video/53216/

Introduction

The main physiological role of skeletal muscle mitochondria is to produce ATP from oxidative phosphorylation[1]. Importantly, skeletal muscle mitochondria play a specific role in exercise capacity[2], aging[3], degenerative disease[4] and Type II diabetes[5]. As an aging society, and Type II Diabetes being the 7th leading cause of death in the United States[6], the need for methods that assess mitochondrial function has become increasingly more prevalent in biomedical research[7,8]. Specifically, the measurement of oxygen consumption has exceptional utility in the assessment of mitochondrial function since it represents the coordinated function between mitochondrial and nuclear genomes to express functional components of oxidative phosphorylation[9].

Several technologies exist that enable the measurement of oxygen consumption in intact cells and isolated mitochondria[7-10]. In addition, assays have been developed for multiple cell types and tissues, and in a variety of species that allow for the measurement of mitochondrial pathways and respiratory control[9,11,12]. However, there is currently a void in the literature in regards to the compilation of all of these assays using isolated mitochondria from mouse skeletal muscle for use in microplate based oxygen consumption technologies. Importantly, use of microplate based respirometric assays is growing among mitochondrial biologists and allows for high throughput measurements using minimal quantities of isolated mitochondria[9]. Therefore, a collection of microplate based respirometric assays were developed that allow pinpointing of where abnormalities and/or adaptations may be occurring in the electron transport chain (ETC). In addition, two additional microplate based respirometric assays were developed that enable the assessment of the coordination between the tricarboxylic acid (TCA) cycle and the ETC, and between ß-oxidation and the ETC. Importantly, the presented methods provide a clear and concise way to measure mechanistic changes in mitochondrial function in a commonly used animal model.

Protocol

NOTE: This protocol begins after one has isolated mitochondria from red skeletal muscle. Mitochondria were isolated as previously described from ~ 75 - 100 mg of red skeletal muscle[13]. Between 5 - 10 µg/µl of mitochondrial protein will be attained from this amount of tissue by resuspending the final mitochondrial pellet in ≤50 µl of isolation buffer for mitochondria (IBM) 2 (See Frisard *et al*[13]).

1. Setup

1. Prepare working stock solutions (**Table 1**) and mitochondrial assay solution (MAS) mixes (**Table 2**) for subsequent assays.
   Note: Most of the preparation for the assays can be done in advance as most stocks solutions and MAS can be stored.
2. Hydrate a respirometric assay cartridge in 1 ml calibration solution the night before the experiment in a non-CO_2 incubator at 37 °C.
3. The day of the experiment, take out frozen stocks (substrates and mitochondrial modulators, MAS mix, MAS w/o BSA, **Table 1**) and thaw in 37 °C bath or incubator.
4. Prepare all substrate solutions (**Table 3**) and injections (**Table 4-5**) and adjust pH as desired (pH 7.4). After the pH is adjusted, store on ice.
5. Program the multi-well oxygen consumption measurement machine to proper mix, wait, and measure parameters (See **Table 6**) and label background and group/condition wells by first entering the analysis software, followed by the "Standard" mode.
   1. Enter into the "Guest" forum and click on the "Assay Wizard". Once in the "Assay Wizard", click on "Protocol" to change mix, wait, and measure parameters. Label the background wells under the "Back. Correction" tab and be sure to highlight "Do background correction".
   2. Label conditions by first clicking on the "Groups & Labels" tab, followed by the "Group Info" tab. After these parameters are entered, click "End", followed by "Save Template".

2. The Assay Run

1. Load the injection solutions into an assay run cartridge and start the calibration by first entering the analysis software, followed by entering the "Standard" mode. Enter into the "Guest" forum. Open the template saved in Step 1.5 under the "XF" drive, under the "Templates" tab. Once open, click "Start" to begin calibration.
   Note: The assay run cartridge must be entered into the machine with the notched corner to the bottom left. This takes about 30 min. Take care to inject solutions into the proper ports. Injections in **Table 4-5** are listed in order of loading.
2. Gently, but thoroughly mix the mitochondria stock by stirring the solution with a 200 µl pipette tip, followed by gently triturating the stock with a 200 µl pipette tip that has had its orifice widened by cutting ~ 3 mm off the end of the tip with scissors.
3. Perform a bicinchoninic acid (BCA) assay to determine the protein concentration of the mitochondrial stock.
4. Using the stock concentration acquired from step 2.3, resuspend 10 µg of mitochondrial protein/200 µl of the succinate/ rotenone substrate mix (**Table 3**). Resuspend 14 µg of mitochondrial protein/ 200 µl of the remaining substrate mixes (This must be done for *each* substrate mix) (**Table 3**). Place all mitochondrial protein/substrate mixes on ice.
   Note: The optimal amount of mitochondrial protein per well for each assay was determined as previously described[9]. It was determined that the pyruvate /malate, palmitoyl carnitine/malate, glutamate/malate and electron flow assays utilizes 3.5 µg of mitochondrial protein, while the succinate/rotenone assay utilizes 2.5 µg of mitochondrial protein per well. Therefore, the researcher needs to resuspend enough mitochondrial protein for 4 replicates in this step since either 2.5 µg or 3.5 µg per 50 µl is loaded per well (See step 2.6).
5. Mix the mitochondria/ substrate solutions gently, but thoroughly by stirring the solution with a 200 µl pipette tip, followed by gently triturating the stock with a 200 µl pipette tip that has had ~ 3 mm of its end cut off.
6. Place a cell culture plate on ice and in triplicate, load 50 µl/well of each of the mitochondria/substrate mixes. The developers of the microplate based respirometric assay recommend leaving a minimum of two blank (no mitochondria) wells, preferably on either side on the cell culture plate.
7. Spin the cell culture plate at 2,000 g for 20 min at 4 °C. While the plate is spinning, warm up the substrate solutions in a 37 °C water bath.
8. After the spin is complete, carefully load 450 µl of each substrate solution on top of its respective wells (*i.e.*, load the pyruvate/malate substrate to the wells that have mitochondria initially resuspended in this substrate solution). Be sure to load the plate at RT. Note: Blank wells should be loaded with 500 µl of substrate. Blank wells designated for the electron flow assay should be loaded with the electron flow substrate (Pyruvate/Malate + FCCP), while the coupled assay blank wells can be loaded with any coupling assay substrate.
   Note: If performing the coupled and electron flow assays on the same plate, the researcher must reassign background wells to the respective assays after the assay run is complete using the "XF Reader" platform via the "Instrument" tab. Once within the "Instrument" setting, click on the "Administration" tab, followed by "Background Correction". Hit "End Instrument Setup Mode" when complete. This is because the combination of ascorbate/TMPD can consume O_2, thus a separate background correction is needed for each assay type.
9. After the 450 µl of substrate is added to each well, view the layer of mitochondria in the well to ensure the mitochondria are dispersed evenly in a single monolayer (20X magnification [See Rogers[9], **Figure 4**]). Wells that do not appear to have an evenly distributed monolayer can be removed *post hoc*.
10. After checking for mitochondrial adherence, insert the microplate into the respirometric assay instrument and start the assay run by clicking "OK".
    Note: The calibration from step 2.1 must be complete before the run will begin. Once the researcher clicks "OK", the machine will eject the utility plate that was used for calibration.
    1. Remove the utility plate and place the cell culture plate that contains the mitochondria on the tray. The blue notch on the cell culture plate should be placed in the bottom left corner of the tray. Click on, "continue" to start the assay run.
11. After the run is complete, eject the cell culture plate and cartridge by pressing "Eject" on the screen.
    1. Once the plate is ejected, remove and discard the cell culture plate and cartridge and click "Continue" on the screen. The run will be automatically saved as an .xls file in the "Data" file.
12. Open this file and change the display from "O2" to "OCR" by hitting the downward arrow under the "Y1:" marker.
    1. Next change "Middle Point" to "Point-to-Point Rates" under the "Rate Data Displayed As:" option. Click "OK" to apply these changes.
13. Click the "Well Group Mode" tab on bottom left of the screen to group wells of similar conditions together. Finally, click the "Sample Mean/ Standard Error" tab toward the middle of the screen, to the left. Alternatively, these commands can be setup before the assay begins in the "Assay Wizard" setting.
    Note: Each condition will have a mean ± standard deviation displayed for each rate and each condition. There are rate measurements taken per condition (State 2[Coupling]/State3u [Electron Flow] respiration followed by four injections). Use the mean of the State 2 rate of the

second measurement, determine State 4o at the minimum point after oligomycin injection, use the maximum point for State 3 and State 3u after ADP and FCCP injection, respectively, and use the minimum point for Antimycin A induced respiration for the Coupling Assays. Use the maximum point for pyruvate/malate induced State 3u respiration, use the minimum point for rotenone induced respiration, use the maximum point for succinate induced State 3u respiration, and use the minimum point for Antimycin A induced respiration for the Electron Flow assay. All experiments were performed 3 times and the data shown are the results of a representative tracing.

Representative Results

Figure 1 represents oxygen consumption rates (OCR) for the pyruvate/malate, succinate/rotenone, palmitoyl carnitine/malate, and glutamate/malate assays (Coupling Assays). These assay tracings are displayed as Oxygen Consumption Rate, or OCR, vs. time, are background correceted, and are displayed as point-to-point rates. Each panel represents oxygen consumption in different mitochondrial states as described by Chance and Williams[14]. The first panel represents basal oxygen consumption, or State 2. The second panel, after injection of ADP, represents maximal coupled respiration, or State 3. The third panel, after injection of oligomycin A (an inhibitor of Complex V), represents respiration due to proton leak, or State 4o. The fourth panel, after injection of FCCP, represents maximal uncoupled respiration, or State 3u. Finally, the fifth panel, after injection of Antimycin A, represents the inhibition of oxidative respiration. Notably, all mitochondrial states have minimal standard deviation. This is due to thorough mixing of the mitochondrial stock and the mitochondria/substrate mixes, and the attainment of a single monolayer of mitochondria after the adherence spin (Step 2.6). On the other hand, loading unequal mitochondria in each well and not attaining a single monolayer of mitochondria in the well of the microplate leads to increased standard deviation in each state as displayed in **Figure 2**.

The tracings in **Figure 1** display plateaus for each mitochondrial state and for each substrate. The plateau attained after two measurement cycles suggests good mitochondrial quality and that the mitochondria remain adhered to the well throughout the duration of the assay. In addition, the attainment of plateaued maximal rates may be more desirable since this allows the researcher to take the average OCR at these mitochondrial states, thus reducing bias that may occur by arbitrarily selecting a point.

The amount of mitochondria per well was determined by optimization trials. The optimal amount of mitochondria per well should result in State 2 rates between 100-200 pmol/min/well and state 3 rates < 1,500 pmol/min/well, since these values are within the dynamic oxygen sensing range of the multi-well oxygen consumption measurement machine. Loading too much mitochondrial protein per well may result in OCRs beyond the dynamic range of the instrument (**Figure 3A**). **Figure 3** depicts loading 3.5 µg of mitochondrial protein per well (blue tracing) compared to loading 2.5 µg of mitochondrial protein per well (red tracing) for the succinate/rotenone assay. Loading too much mitochondrial protein per well can also lead to the exhaustion of oxygen within the microchamber of the well, thus preventing accurate measurement of OCR for each successive measurement[9] (**Figure 3B**). Point-to-point OCR is the instantaneous rate of change of the OCR. If flat, the OCR is steady/stable, but if decreasing, then there may be a biologic or technical issue. The steep decline in OCR in State 3 and State 3u respiration (**Figure 3A**) is caused by the mitochondria exhausting the oxygen supply before the end of the measurement (**Figure 3B**).

Figure 4 represents OCR vs. time for the electron flow assay. The tracing is corrected and displayed as described for **Figure 1**. The first panel in this assay represents State 3u respiration on pyruvate/malate via Complex I. The second panel, after injection of rotenone, represents inhibition of Complex I mediated respiration. The third panel, after injection of succinate, represents substrate stimulated State 3u with electrons entering the ETC at Complex II (Complex II mediated respiration). The fourth panel, after injection of Antimycin A, represents inhibition of Complex III and thus total respiration. Finally, the fifth panel, after injection of ascorbate/TMPD, represents Complex IV mediated respiration. Similar to the tracings in **Figure 1**, all mitochondrial states have minimal standard deviation, and each rate has or nearly has attained a plateau.

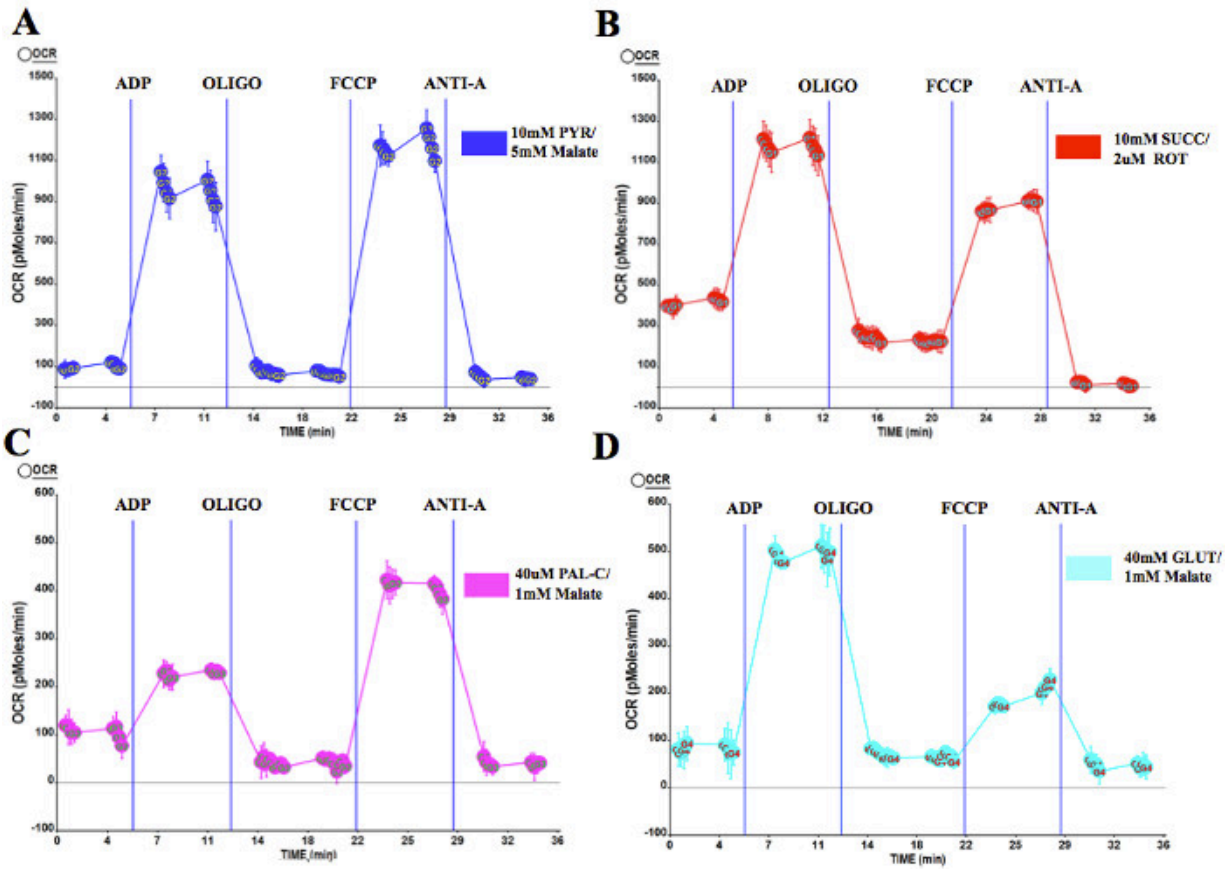

Figure 1. Coupling Assays. **(A)** 10 mM Pyruvate/ 5 mM malate, **(B)** 10 mM succinate/ 2 µM rotenone, **(C)** 40 µM palmitoyl carnitine/ 1 mM malate, and **(D)** 10 mM glutamate/ 10 mM malate coupled mitochondrial respiration assay tracings as determined by multi-well measurement of oxygen consumption. Values are expressed as mean ± SD. Mitochondrial protein loaded per well was 3.5 µg for all assays except succinate/ rotenone, which utilizes 2.5 µg of mitochondrial protein per well. Data represents n=3 paired biological replicates. OCR = Oxygen Consumption Rate; ADP = Adenosine diphosphate; Oligo = Oligomycin A; FCCP = Carbonyl cyanide-*4*- (trifluoromethoxy) phenylhydrazone; Anti-A = Antimycin A; PYR = Pyruvate; SUCC = Succinate; ROT = Rotenone; PAL-C = palmitoyl carnitine; GLUT = glutamate. Please click here to view a larger version of this figure.

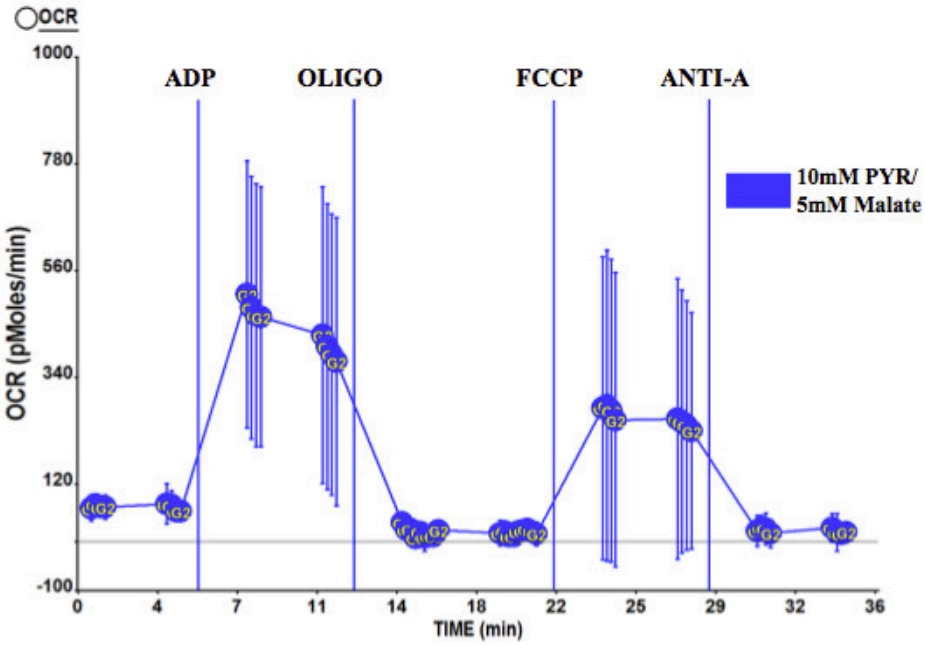

Figure 2. Highly Variable Pyruvate/Malate Assay. Highly variable 10 mM pyruvate/ 5 mM malate assay caused by incompletely mixing mitochondria from the mitochondrial stock in the substrate/MAS mix, thus leading to variable mitochondrial protein loading in each well. Mitochondrial protein loaded per well was 3.5 µg. Data represents n=3 paired biological replicates. OCR=Oxygen Consumption Rate; ADP= Adenosine diphosphate; Oligo= Oligomycin A; FCCP= Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone; Anti-A= Antimycin A; PYR=Pyruvate. Please click here to view a larger version of this figure.

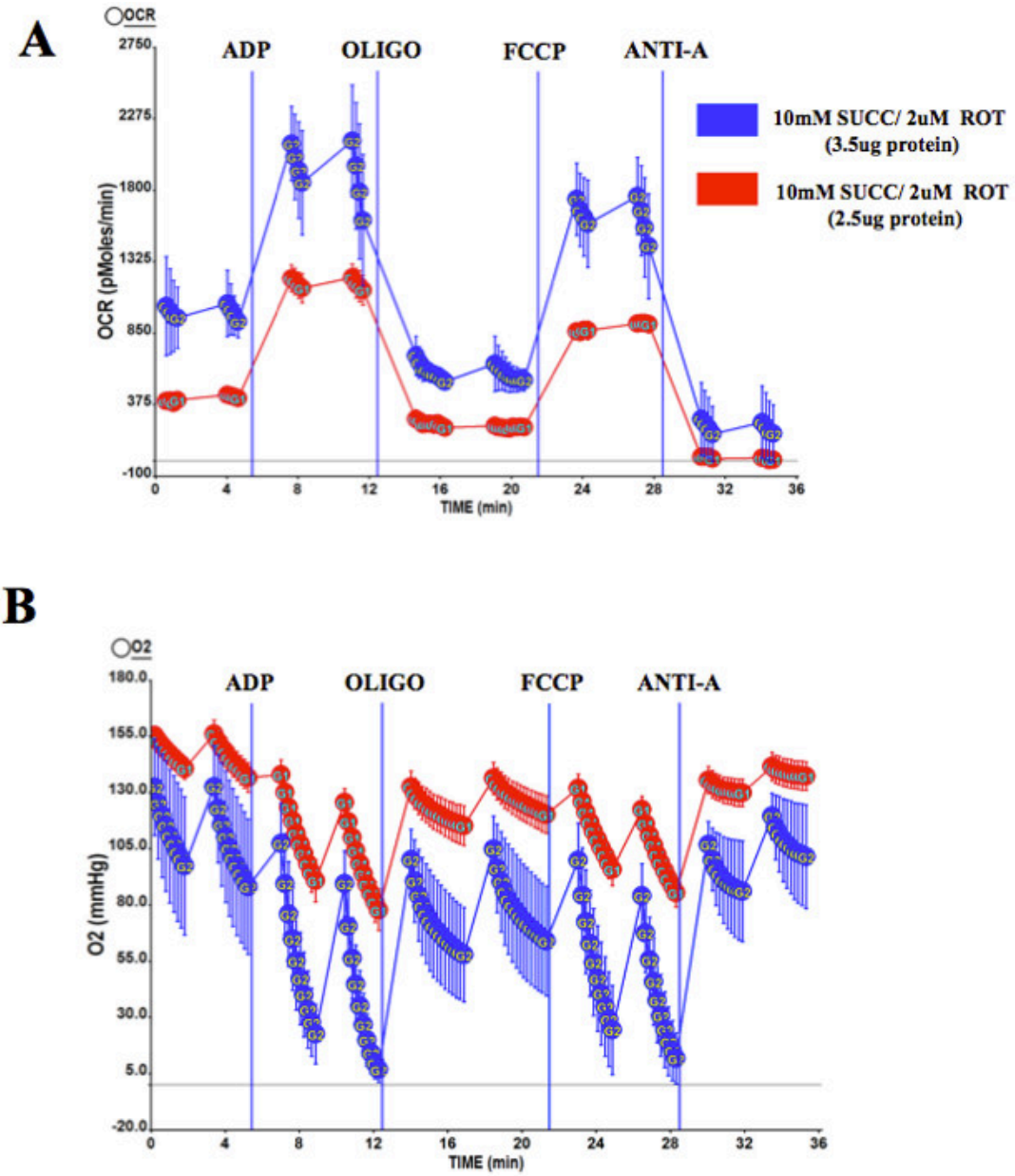

Figure 3. Overloading Mitochondrial Protein for the Succinate/Rotenone Assay. (A) Oxygen consumption rates outside the dynamic range of the multi-well oxygen consumption measurement machine caused by loading 3.5 µg of mitochondrial protein per well (blue tracing) compared to loading 2.5 µg of mitochondrial protein per well (red tracing). **(B)** Oxygen tension approaching zero following ADP and FCCP injections caused by loading excessive mitochondrial protein (3.5 µg) per well (blue tracing) compared to loading an optimal amount (2.5 µg) of mitochondrial protein per well (red tracing). Data represents n = 3 paired biological replicates per mitochondrial protein amount. OCR = Oxygen Consumption Rate; ADP = Adenosine diphosphate; Oligo = Oligomycin A; FCCP = Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone; Anti-A = Antimycin A; SUCC = Succinate; ROT = Rotenone; O_2 = Oxygen; mm Hg = millimeters of mercury. Please click here to view a larger version of this figure.

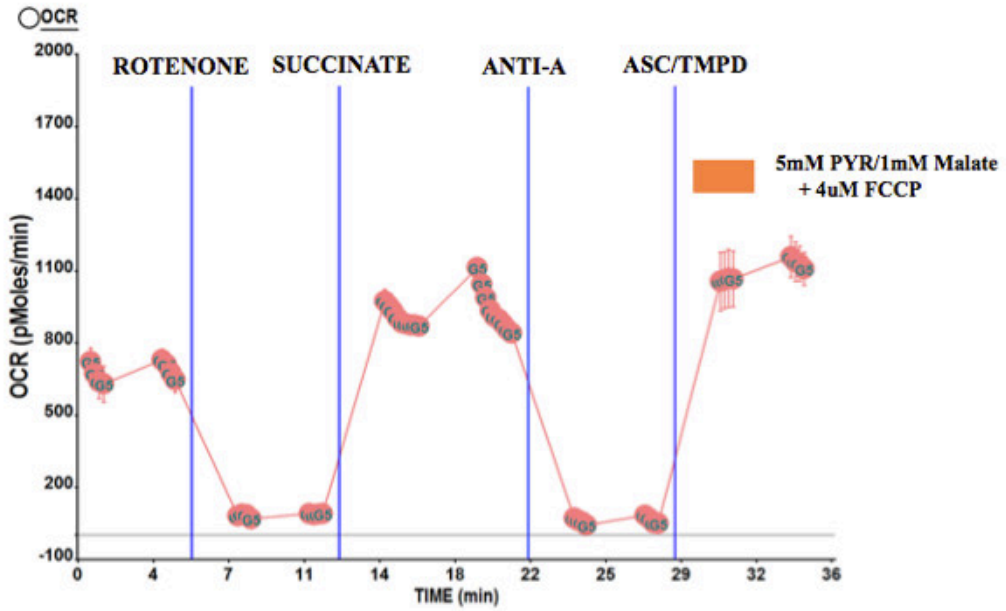

Figure 4. Electron Flow Assay. 5 mM pyruvate/ 1 mM malate+ 4 µM FCCP, electron flow mitochondrial respiration assay tracing as determined by multi-well measurement of oxygen consumption. Values are expressed as mean ± SD. Mitochondrial protein loaded per well was 3.5 µg. Data represents n=3 paired biological replicates. OCR = Oxygen Consumption Rate; Anti-A = Antimycin A; Asc = Ascorbate; TMPD = *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. Please click here to view a larger version of this figure.

Reagent	Stock Concentration	MW	Final Volume	Mass Added	Comments/ Description
	(M)	(g/mol)	(ml)	(g or ml)	
EGTA, pH 7.2	0.1	380.35	100 ml of 1M Tris Base	3.801 g	Store at 4 °C
HEPES	1	238.3	250 ml of DiH_2O	59. 57 g	Store at 4 °C
$MgCl_2$, hexahydrate	1	203.31	250 ml of DiH_2O	50.82 g	Store at 4 °C
Pyruvate pH 7.4	0.1	88.06 (Comes as 14.11 M solution)	40 ml of DiH_2O	0.283 ml of pyruvic acid	Make 1 ml aliquots and store at -20 °C, make fresh every two weeks
Succinate pH 7.4	0.5	118.09	100 ml of DiH_2O	9.4 g of succinic acid	Make 1 ml aliquots and store at -20 °C
Malate, pH 7.4	0.5	134.09	100 ml of 95% Ethanol	6.7 g of malic acid	Make 200 µl aliquots and store at -20 °C
TMPD	0.01	164.25	10 ml	0.0164 g	Make 300 µl aliquots and store at -20 °C; Mix with an equimolar amount of ascorbate to keep TMPD reduced
Palmitoyl L-carnitine chloride	0.01	436.07	1.14 ml of 95% Ethanol	0.005 g	Make 40 µl aliquots and store at -20 °C
Oligomycin A	0.006	791.06	0.987 ml of 95% Ethanol	0.005 g	Make 20 µl aliquots and store at -20 °C
FCCP	0.01	254.17	3.9 ml of 95% Ethanol	0.01 g	Make 40 µl aliquots and store at -20 °C
Rotenone	0.001	394.4	10 ml of 95% Ethanol	0.0039 g	Store at -20 °C
Antimycin A	0.005	548.63	9.12 ml of 95% Ethanol	0.025 g	Store at -20 °C

K^+ ADP	0.5	501.32	3.9 ml of DiH_2O	1.0 g	Store at -20 °C
Malic Acid, pH 7.4	0.5	134.09	40 ml	2.68 g	Make 200 µl aliquots and store at -20 °C

Table 1. Stock Solutions

Reagent	Stock Concentration (M)	Mass Added (g or ml)	Final Molarity/Percent
Sucrose	--	11.98 g	70 mM
Mannitol	--	20.04 g	220 mM
Potassium phosphate monobasic	--	0.34 g	5 mM
$MgCl_2$, hexahydrate	1	2.5 ml	5 mM
HEPES	1	1.0 ml	2 mM
EGTA	0.1	5.0 ml	1 mM
Essentially Fatty Acid Free- BSA	--	1.0 g	0.20%

Table 2. MAS Mix. pH 7.4, 500 ml: Aliquot 25 ml and store at -20 ºC *Note: Exclude BSA for MAS mix used for assay injections.

Substrate Medium	Final Concentration	Amount of stock (µl)	Amount of MAS* (ml)
Pyruvate/Malate	10 mM/5 mM	Pyruvate: 1,000	9
		Malate: 100	
Succicinate/Rotenone	10 mM/2 µM	Succinate: 200	10
		Rotenone 20	
*Pyruvate/Malate + FCCP	5 mM/1 mM/4 µM	Pyruvate: 500	10
		FCCP: 4	
		Malate: 20	
Palmitoyl L-carnitine/Malate	40 µM/1 mM	Palmitoylcarnitine: 40 Malate: 20	10
Glutamate/Malate	10 mM/10 mM	Glutamate: 400	10
		Malate: 200	

Table 3. Substrate Solutions pH 7.4: Make fresh the day of the experiment. *Electron flow assay solution.

Injection Medium	Concentration	Amount of stock (µl)	Amount of MAS (ml)	Amount injected into Cartridge	Final Concentration
					(After injected in plate)
ADP	50 mM	300 µl	3	50 µl	5.0 mM
Oligomycin A	20 µM	10 µl	3	55 µl	2.0 µM
FCCP	40 µM	12 µl	3	60 µl	4.0 µM
Antimycin A	40 µM	24 µl	3	65 µl	4.0 µM

Table 4. Injections for Coupled Assays. pH 7.4: Make fresh the day of the experiment. *Coupling assays include (but are not limited to) pyruvate/malate, succinate/rotenone, palmitoyl carnitine/malate, and glutamate/malate.

Injection Medium	Concentration	Amount of stock (g or ul)	Amount of MAS (ml)	Amount injected into Cartridge	Final Concentration (After injected in plate)
Rotenone	20 µM	60 µl	3	50 µl	2.0 µM
Succinate	50 mM	300 µl	3	55 µl	5.0 mM
Antimycin A	40 µM	24 µl	3	60 µl	4.0 µM
TMPD/Ascorabte	1 mM, 100 mM	TMPD : 300 µl	3	65 µl	100 µM, 10mM
		Ascorbate: 0.059 g			

Table 5. Injections for Electron Flow Assay. pH 7.4: Make fresh the day of the experiment

Command	Time (min)	# of cycles

Calibrate		
Wait	10 min (to allow plate to warm from adherence step)	
Mix	1 min	2
Measure	2 min	
Inject A		
Mix	1 min	2
Measure	2 min	
Inject B		
Mix	1 min	2
Measure	2 min	
Inject C		
Mix	1 min	2
Measure	2 min	
Inject D		
Mix	1 min	2
Measure	2 min	

Table 6. Instrument Run Protocol.

Discussion

The methods presented in this article provide step-by-step instructions for the performance of a collection of microplate based respirometric assays using mitochondria isolated from 75 - 100 mg of mouse skeletal muscle. These assays can be performed with high precision as evidenced by the tight standard deviation between triplicate wells. Importantly, these respirometric assays allow pinpointing of where abnormalities and/or adaptations may be occurring in the ETC, TCA cycle, β-oxidation pathway, substrate transporters, etc. in a commonly used animal model.

It is important to highlight the rationale for using various fuels and inhibitors used in this protocol. The pyruvate/malate and glutamate/malate respirometric assays allow for the assessment of Complex I mediated respiration, as well as the assessment of their respective transporters, and in the case of glutamate, the deaminase[15]. Alternatively, the combination of succinate/rotenone allows the assessment of mitochondrial respiratory flux through Complex II of the ETC since rotenone inhibits complex I and succinate provides electrons to Complex II via the reduction of flavin adenine dinucleotide ($FADH_2$)[15]. These assays provide substrate specific information as to coupling efficiency and maximal respiration. The electron flow assay is unique in that the combination of substrates and inhibitors allows for the assessment of multiple complexes during mitochondrial respiratory flux[9]. The initial substrate mix of pyruvate/malate + FCCP allows for the evaluation of maximal respiration driven by Complex I, while the injection of rotenone followed by succinate allows for the assessment maximal respiration driven by Complex II. The injection of Antimycin A, an inhibitor of Complex III, followed by the injection of ascorbate/TMPD allow the for the evaluation of respiration driven by Complex IV since the Ascorbate/TMPD is an electron donor to Cytochrome C/Complex IV. While no information on coupling efficiency is obtained, the method is ideal for very small sample sizes that preclude running multiple substrates independently. Finally, the use of palmitoyl carnitine/malate allows for the assessment of the coordination between β-oxidation and the ETC since the reducing equivalent produced from the oxidation of palmitic acid (β-oxidation) feed into the ETC through the electron transferring flavoprotein[15]. It should be noted that the Coupling and Electron Flow assays could also be used in tandem to identify changes in mitochondrial function due to some intervention (drug treatment, genetic manipulation).

The high precision attained for these assays is primarily due to thorough mixing of the mitochondria, whether it is prior to protein determination, or with the substrate solutions. Along these lines, once the mitochondria is resuspendend in the substrate solutions, it is critical to mix this solution thoroughly prior to loading the cell culture plate as described in Step 2.2 with a widened orifice pipette tip. Failure to mix the mitochondria thoroughly will lead to large variation within the assay. In addition, using a narrow orifice pipette tip will create shearing forces while mixing the mitochondria and increases the potential to damage the mitochondrial membranes and release of Cytochrome C. The adherence step (2.7) is also a critical step in this protocol. Failure to spin the loaded cell culture plate long/fast enough will result in incomplete adherence of the mitochondria to the well, thus leading increased variability between wells and measurements.

The described protocol has been optimized to include: loading an optimal amount of mitochondrial protein per well, using the correct concentrations/preparation methods to make stock and substrate solutions, altering the assay run to ensure mitochondrial state plateaus, and appropriate mixing of the mitochondrial stock and mitochondrial/substrate mixes. Prior to these optimization efforts, the authors encountered pitfalls in the assay run. The following discusses troubleshooting methods/modifications that were helpful in optimizing this protocol. With respect to optimal loading, loading too little mitochondria will not elicit a robust response, while loading too much mitochondria will exhaust the oxygen within the microchamber and lead to inaccurate measurements. Rogers *et al*[9] provides examples of determining optimal loading amounts of mitochondria per well for microplate based respirometric assays. More often, too much mitochondria is loaded per well as evidenced by state 2 rates over 100-200 pmol/min/well and state 3 rates >1500 pmol/min/well. If over loading occurs, perform an experiment with varying concentration of mitochondrial protein (*e.g.*, between 1 - 10 µg) to elicit OCRs within the dynamic range of the oxygen consumption measurement machine. Preparing and using the correct concentrations of substrates and stocks is of utmost importance. Always use the acid

form of substrates/injections and adjust the pH with potassium hydroxide or HCl; sodium buffers/solutions are not recommended. In addition, resuspending substrates/stocks in DMSO or 100% ethanol will result in measurement failure or error. Be sure to use 95% ethanol where noted. It is common for palmitoyl carnitine to precipitate out of the 95% ethanol after thawing the frozen stock, thus causing large variability. Be sure to warm up the palmitoyl carnitine stock and vortex well prior to use. In addition, a highly variable pyruvate/malate assay result may be due to the pyruvate stock being >2 weeks old. Be sure to remake frozen aliquots of pyruvate every 2 weeks. The assay run was modified to 2-min measurements to ensure mitochondrial state plateaus. If observation of exhaustion of ADP is desired, the researcher may extend the measurement time under the "Protocol" tab under the "Assay Wizard" forum. Finally, large variability occurs when the mitochondrial stock and mitochondria plus substrate solutions aren't homogenized fully prior to loading. If variability between wells is high after the assay run, be sure to fully mix the substrate solution prior to the next experiment. Never vortex the mitochondria/substrate solutions, rather stir, mash, and gently triturate with a widened orifice pipette tip.

There are some limitations of this technique that are worth noting. First, the number of wells on the cell culture plate used for these assays is relatively low (*i.e.*, 24 wells and at least 2 designated for blank wells). If it is desired to perform all 5 of these assays on one plate, the researcher is only able to examine the responses from one mouse at a time. However, it should be noted that 96 well instruments are available for higher throughput. Secondly, there are inherent strengths and weaknesses in assessing mitochondrial dysfunction in isolated mitochondria compared to in intact cells[1]. Some weaknesses include having less physiological relevance compared to intact cells and inducing artifacts from the isolation process. Finally, the success of this method is contingent on the quality of the mitochondrial isolation process.

Although some of these assays have been either developed in different systems or have been validated in other animal models, the methods presented herein are the first to synthesize all of the aforementioned assays for the optimal use in a multi-well oxygen consumption measurement machine using mouse skeletal muscle. Importantly, all 5 of these assays can be performed with the amount of mitochondria isolated from ~75 - 100 mg of mouse skeletal muscle, thus providing high throughput with minimal material. Of great significance, the ability of multi-well oxygen consumption technologies to perform assays with minimal quantities of mitochondria, combined with an optimized isolation method, allows the researcher to perform a multitude of other experiments with the remainder of skeletal muscle tissue (*e.g.*, western blots, RT-PCR, enzymatic assays, etc.), which is often a struggle with this animal model.

In conclusion, the methods presented herein provide step-by-step instructions for the performance of a collection of microplate based respirometric assays using minimal quantities of mouse skeletal muscle. Importantly, the presented methods require minimal quantities of tissue and mitochondria. Once mastered, the techniques described herein will allow researchers to determine a potential mechanism of a compound or gene product on mitochondrial oxygen consumption in a commonly used animal model.

Disclosures

George W. Rogers is an employee of Seahorse Bioscience that manufactures the instrument for which these assays were developed. Open Access fees were provided by, Seahorse Bioscience, a part of Agilent Technologies.

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