TITLE:

Robust Mitochondrial Isolation from Rodent Cardiac Tissue ~~for Use in High-Resolution Respirometry~~

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SUMMARY:

Bioenergetic and metabolomic studies on mitochondria have revealed their multifaceted role in many diseases, but isolation methods of these organelles is variable. The method detailed is capable of purifying high-quality mitochondria from multiple tissue sources. Quality is determined from respiratory control ratios and other metric determined with high-resolution respirometry.

ABSTRACT:

Mitochondrial isolation has been practiced for decades following procedures set by pioneers in the fields of molecular biology and biochemistry to study metabolic impairments and disease. Consistent mitochondrial quality is necessary to properly interrogate mitochondrial physiology and bioenergetics; however, there are many different published isolation methods available for researchers. Although different experimental strategies require different isolation methods, the basic principles and procedures are similar. Here is detailed a protocol capable of extracting well-coupled mitochondria from a variety of tissue sources from small animals and cells. The steps outlined herein describe organ dissection, mitochondrial purification, protein quantification, and various quality control checks. The primary quality control metric used to identify mitochondria of high quality is the respiratory control ratios (RCR). The RCR is the ratio of the respiratory rate during oxidative phosphorylation and the rate in the absence of ADP. Alternative metrics are discussed. While high RCR values relative to their tissue source are obtained using this protocol, there are several steps that can be optimized to suit the individual needs of researchers. This procedure is robust and has consistently resulted in isolated mitochondria having above average RCR values across animal models and tissue sources.

INTRODUCTION:

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Isolation of Mitochondria from Rodent Cardiac Tissue for High-Resolution Respirometry

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Mitochondria are subcellular organelles which establish cytoplasmic energetic conditions that are optimized for specific cell functions. While cellular, tissue, and organism level studies can be informative concerning mitochondrial function, isolating the organelles establishes a level of experimental control not possible otherwise. Mitochondrial isolations have been performed since the 1940s, allowing mechanistic studies of metabolism and respiration across a variety of cells and tissues[1,2]. The historical relevance of mitochondria is additionally well-documented[3]. As the main producers of ATP, mitochondria play many key roles which are vital for optimal cellular and organ function[4] ~~(PMID: 31900386)~~. Within the mitochondrial matrix, substrates are oxidized by the TCA cycle which produce reducing equivalents and mobile electron carriers such as NADH and UQH_2 ~~(PMID: 34621061; 15134745)~~[5,6]. Cytochrome c is the third main mobile electron carrier in the mitochondrial biochemical reaction network ~~(PMID: 32023432)~~[7]. These molecules are then oxidized by the transmembrane complexes of the electron transport system (ETS) embedded in the inner mitochondrial membrane ~~(PMID: 29464561)~~[8]. Redox reactions of the ETS are coupled to proton translocation from the matrix to the intermembrane space. These processes establish an electrochemical proton gradient that is used phosphorylate ADP with P_i by F_1F_O ATP synthase and produce ATP[9,10]. The individual processes that occur at each complex can be explored with high-resolution respirometry using Clark-type electrodes or microplate oxygen consumption assays[11,12]. Additionally, disease models and treatments with isolated mitochondria can also be used to determine the impact or importance of mitochondrial function in progression of certain pathologies. This has proven fruitful in the field of cardiology where alterations in fuel and substrate delivery have been used to elucidate how mitochondrial dysfunction influences heart failure[13-16]. Mitochondria are also known to impact the development of other disease states such as diabetes, cancer, obesity, neurological disorders, and myopathies[17,18]. Therefore, the use of isolated or purified mitochondria enables mechanistic investigations of oxidative metabolism and ATP production in the source tissue.

There is no shortage of mitochondrial isolation protocols due to their importance in bioenergetic research. Additionally, highly specific methods can be found that are tailored to subpopulations of mitochondria within tissues and cells[19,20]. The basic procedural steps are similar between isolation methods, but variations can be made to buffer composition, homogenization steps, centrifugation spins to improve the amount and quality of mitochondria. Changes to these aspects are based off of metabolic demand of the tissue, overall organ function, mitochondrial density, and other factors. In tissues such as liver and skeletal muscle, handheld Teflon homogenizers are used to preserve mitochondrial integrity and limit damage to the mitochondrial membranes[21]. However, when isolating from kidneys, some protocols suggest the use of manually driven homogenization or the use of commercial kits to yield better results[22]. Although both methods yield functional mitochondria, the quality of the organelles can become compromised by the additional time it takes to complete isolations using these protocols. Centrifugation is also vital to the extraction of mitochondrial protein as it separates cellular components such as nuclei and other organelles from mitochondria[23]. During the isolation process, it is debated whether differential or density-based centrifugation should be implemented to obtain purer isolates[24]. While density centrifugation can separate mitochondria from organelles of similar specific gravity such as peroxisomes, it is not well-established if

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mitochondria from these methods better represent *in situ* organelle function relative to mitochondria isolated using differential centrifugation. In the field of mitochondrial physiology, density-based centrifugation is preferred and can be easily altered to increase isolate purity. Whether changes to g forces, centrifugation duration, and number of centrifugation spins are incorporated should be thought of before experimentation due to their influence on mitochondrial purification. Furthermore, mitochondrial resuspension, arguably the most crucial step during isolation, differs greatly between studies with the use of scraping, vortex-based mixing, or homogenization[23,25,26]. Mechanical resuspension of these types can be too abrasive and impair membrane integrity of mitochondria. For this reason, gentle washing should be performed to correct of this. Despite the plethora of modulations and suggested methodology, there are fewer comprehensive protocols with high-reproducibility and adaptability for rodent models.

The methods herein describe a detailed, robust, and highly reproducible protocol that will yield purified, well-coupled mitochondria from small animal cardiac tissue. As demonstrated, this method is easily adapted to accommodate the specific needs of each experiment and/or laboratory environment to isolate mitochondria from kidneys, liver, and cultured cells. Further alterations can be made to isolate mitochondria from tissues and other animals not listed here. Provided are buffer recipes that are used for all isolations listed throughout and can be modified if needed. Much like other published protocols, the use of motorized homogenization and differential centrifugation is implemented but adjustments are made to both the sheering time and force at which the samples are centrifuged to consistently deliver high-quality mitochondrial isolates depending on the isolation source. Of note, this protocol differs from others as it uses gentle washing via pipetting to resuspend pelleted mitochondria. This allows for mitochondrial membrane integrity to be preserved and maintains overall functionally of the organelles. Mitochondrial protein is quantified either by total protein determination and/or measuring citrate synthase activity. The utility and broad applicability of this isolation method is further supported by the values of respiratory control ratios (RCR) which are achieved across organisms and tissue sources.

PROTOCOL:

The use and treatment of all vertebrate animals was performed in accordance with approved protocols reviewed and accepted by the Institutional Animal Care & Use Committee (IACUC) at Michigan State University. This protocol was designed using both male and female Hartley albino guinea pigs and Sprague Dawley (SD) rats. For isolation of cardiac mitochondria from guinea pigs, animals were sacrificed between 4 – 6 weeks of age (300 – 450 g). Cardiac mitochondria from SD rats of both sexes were obtained between the ages of 10 – 13 weeks (250 – 400 g).

Recipes for buffers are described in Table 1 and are to be prepared in advance.

1. Experimental preparation

1.1 Before starting the isolation, label two 50 mL centrifugation tubes as “Spins 1, 2” and “Spin 3”.

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1.2 Place tubes, freshly thawed isolation buffer (IB), sharp mincing scissors, assembled homogenization probe, and a 5 mL beaker or equivalent sized container on ice.

1.3 Place freshly thawed respiration buffer (RB) in incubator to warm for subsequent respiratory assays.

1.4 Pre-chill a refrigerated centrifuge to 4° C.

1.5 Ensure that all equipment is arranged and 20 µL of 7-15 U/mg protease from *Bacillus licheniformis* has been added to the tube labeled "Spins 1 and 2".

1.6 Set up a gravity-dependent pressure system for perfusion via cannulation using CB, glass cannula, and plastic tubing with stopcock valve attached.

1.7 Arrange surgical tools and include proper scissors and forceps for dissection and cannulation of the heart.

NOTE: All water should be of pure quality (18.2 MΩ·cm)

2~~1~~. Tissue Dissection

2.1 Inject animal with sterile heparin sulfate intraperitoneally at a dose of 500 U/kg.

2.2 Allow animal to sit in induction chamber for 15 minutes after administration of heparin. During this time, supply 2 L/min of pure O_2 gas to fully oxygenate the animal, calm them, and minimize any stress that may have adverse consequences on tissue of interest.

2.3 Start anesthesia induction by a continuous flow of isoflurane at 0.5%. After one minute, increase to 1%. Continue to increase by 1% every minute until 5% is reached. Once at 5%, wait for one minute and monitor the animal's breathing pattern.

2.4 Once breathing has slowed and becomes labored (approximately at the 6.5-minute mark) turn off isoflurane and oxygen flow.

2.5 Quickly remove the animal from induction chamber and check for appropriate anesthetic depth by squeezing a paw and checking for the corneal reflex. If the animal responds to either stimulus, then place back in induction chamber, readminister anesthesia, and repeat anesthetic depth check.

2.6 Once the proper depth of anesthesia is reached, quickly decapitate with a guillotine to severe the cervical spine and place prone body on ice.

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2.7 Make two parallel vertical incisions from the clavicles proceeding along the lateral rib cage down the length of the thorax. Ensure that the incisions are deep enough to cut through the ribs on the lateral thorax but take care to avoid damaging intrathoracic structures such as the heart or great vessels.

NOTE: Vertical incision sizes depend on the animal being used. If using guinea pigs and rats, cut to the diaphragm (approximately 6.35 cm for rats and 11 cm for guinea pigs). If using mice, perform a standard thoracotomy[27].

2.8 Expose the heart using a hemostat to displace the anterior thorax and pack the exposed thoracic cavity with ice. This step minimizes warm ischemia time and enhances viability of the isolated organelles.

2.9 Use tweezers to blunt dissect the thymus and pericardium and fully expose the heart.

2.10 Using forceps, apply gentle inferior traction on the heart to expose and identify the aorta. The aorta is the thickest great vessel branching out from the base of the heart. Other large vessels, such as the pulmonary vein, are noticeably more translucent than the aorta.

2.11 Cut the aorta approximately 4-6 mm above the aortic root but below the carotid branching points.

2.12 Cannulate the aorta and perfuse the heart[28,29] ~~(PMID: 29091971; 26910432)~~ with ice-cold cardioplegia (CB) solution using a gravity-dependent pressure head until the coronary arteries are cleared of blood and the organ appears blanched.

NOTE: For large rodents, a cannula diameter of 1.8 – 2.2 mm works well, while for smaller rodents, a diameter range of 1.4 – 1.8 mm is recommended. Retro-perfusion should take no more than 15-30 seconds for the coronary vessels to clear of blood.

2.13 Isolate the ventricular myocardium by dissecting away the atria, cartilaginous valvular tissue, and fatty tissues.

2.14 Place ventricles in a pre-chilled 10 mL beaker containing 0.1 – 0.2 mL ice-cold IB.

2.15 Mince tissue with sharp surgical scissors until pieces are approximately 1 mm^3.

3~~2~~. ~~-~~Mitochondrial Purification and Protein Quantification

3.1 Transfer minced tissue into the pre-chilled centrifuge tube labeled “Spins 1 and 2” containing the protease solution.

3.2 Add ice-cold IB to a final volume of 25 mL.

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3.3 Using a motorized handheld rotor-stator homogenizer, disperse the tissue at 18,000 rpm on ice for 20 – 25 seconds.

3.4 Centrifuge homogenized tissue in tube labeled “Spins 1 and 2” at 8,000 x g for 10 minutes at 4 °C.

3.5 Discard the supernatant (contains protease) by pouring into waste carboy and gently rinse the pellet with 5 mL of IB to remove residual protease.

3.6 After discarding the rinse, resuspend the pellet with fresh ice-cold IB to a final volume of 25 mL by gentle vortex.

3.7 Centrifuge at 800 x g for 10 minutes at 4 °C.

3.8 Remove the supernatant (contains mitochondria) by gently pouring into a pre-chilled 50 mL tube labeled “Spin 3”. While pouring, take care to avoid dislodging the loose upper portion of the pellet. As an alternative, use a transfer pipette or stripette to collect the supernatant.

3.9 Centrifuge the supernatant at 8000 x g for 10 minutes at 4 °C.

3.10 Discard the resulting supernatant and retain the mitochondria-containing pellet.

3.11 Use a lint-free wipe to absorb excess supernatant from the inside wall of the tube, taking care to avoid disturbing the pellet. Keep the pellet at 4 °C on ice as much as possible.

3.12 To resuspend the mitochondria, add 80 µL of ice-cold IB to the bottom of tube. Gently resuspend the pellet by repeatedly washing IB over the pellet.

3.13 To avoid creating bubbles, set a micropipette to aspirate and deliver between 40 – 60 µL of volume.

3.14 As the mitochondrial pellet disperses, increase the micropipette volume to efficiently resuspend the pelleted mitochondria. Avoid touching the pellet with the pipette tip and avoid making bubbles.

3.15 Once resuspended, transfer the mitochondria to a pre-chilled microcentrifuge tube and label it as stock mitochondria. Make note of the total resuspension volume.

3.16 To determine the mitochondrial protein concentration in the sample, conduct a total protein assay using the well-known BCA or Bradford protein assays as defined by the specifications of the manufacturer.

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NOTE: An alternative, or complementary, strategy to assess yield is to determine the citrate synthase activity. For reference, mitochondrial content can be quantified by following the protocol described in Vinnakota et al.[30].

43. Quality Control Checks

4.1 In a pre-chilled microcentrifuge tube, dilute the mitochondrial stock to desired working concentration with IB.

NOTE: Mitochondrial stocks are diluted to 40 mg/mL to work at 0.1 mg/mL final concentration for respirometry assays when using isolates from cardiac tissue.

4.2 Rinse oxygraph chambers, stoppers, and microliter syringes ten times with distilled water to clean before use in respirometry assays.

4.3 To test viability and quality of mitochondrial isolates, load 2.3 mL of respiration buffer (RB) into oxygraph chambers and allow the oxygen consumption signal to equilibrate at 37 °C for about 10 minutes or when the rate is near 0.

4.4 Once signal is equilibrated, push down the stoppers and aspirate the excess buffer that emerges from the capillary of the stopper.

4.5 Add 1 mM EGTA using a microliter syringe to chelate any residual calcium in the buffers or mitochondrial sample.

4.6 To fuel respiration, add 5 mM sodium pyruvate and 1 mM L-malate.

4.7 Following addition of substrates, add a bolus of diluted mitochondria to achieve working concentration and allow respiration to occur for 5 minutes. This period is termed LEAK or State 2 respiration.

4.8 At the 5-minute mark, add a bolus of 500 µM ADP to initiate State 3 respiration, also termed OXHPOS, and allow the mitochondria to respire until anoxia.

4.9 Calculate the respiratory control ratio (RCR) by dividing the maximal rate of oxygen consumption during State 3 by the rate of oxygen consumption just before the addition of ADP in State 2 (see Figure 1).

NOTE: An alternative RCR expression of 1 – 1/RCR can also be used as a metric of quality which is bounded between 0 and 1; however, it makes it difficult to differentiate quality using this metric when the RCR > 10 (see Figure 2).

4.10 Rinse chambers and stoppers 10 times with pure water to clean the oxygraph for subsequent assays. If respirometry is complete, fill chambers with 70% ethanol and place stoppers in chambers until next use.

REPRESENTATIVE RESULTS:

Upon completion of mitochondrial isolation, the quality and functionality of the isolates should be tested via quantifying rates of oxygen consumption (J_{O2}) using high-resolution respirometry. To do so, mitochondrial stocks were diluted to 40 mg/mL to allow for working concentrations of 0.1 mg/mL in 2 mL of RB for all respirometry assays using isolated cardiac mitochondria. Respiration was fueled by 5 mM sodium pyruvate and 1 mM L-malate in the presence of 1 mM EGTA, a calcium chelator, and was allowed to equilibrate for 5 minutes to establish State 2 respiration. During this state, rates of oxygen consumption should average 45 – 55 pmol/mL/sec or 27 – 33 nmol/mg/min. Be aware of the electrode-dependent oxygen consumption rate and perform the appropriate background corrections when necessary. Oxidative phosphorylation (State 3) is initiated at the 5-minute mark by a bolus of 500 µM ADP. Substrate additions and a representative tracing are detailed in Figure 1. Functional mitochondria will have an immediate increase in J_{O2} after the addition of ADP that ranges according to the tissue source as shown in Figure 2. Without the use of EGTA, residual calcium has variable effects on maximal rates of J_{O2} during OXPHOS which depends on mitochondrial concentration, substrate availability, and other environmental factors. Buffer composition is important for preservation of mitochondrial membrane integrity and functionality. All buffer recipes mentioned throughout the protocol are further detailed in Table 1 and can utilized for all mitochondrial preparations described herein.

Successful isolation of mitochondria is denoted by obtaining RCR values that lie within the given range for each species and tissue source as shown in Table 2. Based on the data collected using this protocol, if isolating cardiac mitochondria from guinea pigs, rats, or mice, RCRs should be ≥ 16, 8, and 5, respectively. If isolating from rat liver or kidney, RCRs should be ≥ 6, while RCRs from cells are considered acceptable if above 3.8. If RCR values fall below these ranges or if there are qualitative differences in the respirometry tracings, it is recommend performing an additional assay with new RB and substrates to rule out issues from contamination. Although the 1-1/RCR transform bounds values between 0 and 1, this metric is not advised when comparing mitochondrial quality across sexes or species when the RCR value is greater than 10. For this reason, standard RCR values (State 3/State 2 or OXPHOS/LEAK) were quantified during all experimentation. Information regarding modulations that can be made to this protocol to better isolate mitochondria from mouse hearts, liver, kidneys, and cells are detailed in Table 3.

FIGURE AND TABLE LEGENDS:

Figure 1. ~~Ex~~Regions of interest for~~perimental setup~~ ~~of~~ quality control checks using high-resolution respirometry. Respiratory chambers were loaded with 2 mL of RB and allowed to equilibrate at 37 °C until the rate of oxygen consumption was near 0. Once equilibrated, 1 mM EGTA, a calcium chelator, was added along with 5 mM sodium pyruvate and 1 mM L-malate. Following the addition of these substrates, mitochondria were added at the desired working concentration (0.1 mg/mL) and allowed to respire for 5 minutes to achieve State 2 respiration

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(yellow). At the 5-minute mark, 500 µM ADP was added to initiate State 3 respiration (red). RCRs were determined by time averaging the rates of oxygen consumption denoted by the red and yellow boxes to compare State 3 to State 2. State 4 is denoted by the green box and represents the period of extramitochondrial ATPase hydrolyzation of ATP and can be used to assess outer membrane integrity in cytochrome c assays. Data displayed was collected using SD rat cardiac mitochondria.

Figure 2. Representative J_{O2} tracings and respiratory control ratios across animal and tissue sources. Quality of isolated mitochondria was tested by quantifying rates of oxygen consumption using high-resolution respirometry. Respiration was fueled by 5 mM sodium pyruvate and 1 mM L-malate in the presence of 1 mM EGTA. Mitochondria and substrates were allowed to respire for 5 minutes for State 2 respiration to stabilize. Maximal rates of oxygen consumption after the addition of ADP were compared to State 2 rates of oxygen consumption before ADP to calculate the respiratory control ratios (RCRs) for each tissue. Mitochondrial quality was further assessed by calculating the 1-1/RCR values as characterized by P-L control efficiency standards. Bar graphs to the right of each tracing denote male (blue) and female (green) average RCR and 1-1/RCR values ± SD for a given tissue. A, B, and C refer to data collected using guinea pig, Sprague Dawley (SD) rat, and Friend leukemia virus B (FVB) mouse hearts, respectively. Panels D and E detail results from SD rat liver and kidney, while F refers to HEK293 cells. The three larger liver lobes were collected, while both kidneys were used for isolation.

Table 1. Buffer recipes. Cardioplegia buffer (CB), isolation buffer (IB), and respiration buffer (RB) used throughout the isolation process are to be prepared in advance according to the instructions listed. CB can be stored for up to a month at 4 °C, while IB and RB can be kept for 4 months at -80 °C.

Table 2. Respiratory control ratio analysis. Mitochondrial isolation for functional analyses were conducted across a wide range of species and tissue sources in both male and female rodents as well as HEK293 cells. The sample size of each sex, tissue source, and species is detailed along with the average RCR and 1-1/RCR values ± SD.

Table 3. Recommended modulations to mitochondrial isolation protocol for differing tissues to increase protein yield. Changes in amounts of protease as well as centrifugations are displayed according to the tissue source. Average quantities of total protein (mg) were calculated from the results of the BSA protein assay and the resuspension volume of the final mitochondrial pellet. Values are reported as the mean ± SD. Bolded instructions included in the centrifugation column advise on whether to discard, retain, or pool the supernatant. Discarding refers to disposing of the supernatant in a biological waste container, while retention and pooling refer to transferring supernatant to the following centrifugation tube or collecting for the final spin respectively. Further details concerning alterations to the protocol can be found in the discussion section.

DISCUSSION:

Adhering to the methods concisely described in this protocol will ensure isolation of well-coupled mitochondria from cardiac tissue of small rodents, in addition to other tissue types and sources.

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Overall, the process should take a total of 3 to 3.5 hours, during which, all animal tissue, samples, and isolates should remain on ice at 4 °C as much as possible to limit degradation. This procedure is robust and can be altered in several ways to better fit experimental goals and models utilized. One modulation that can be made during the tissue dissection process is the exclusion of heparin. Heparin is administered to prevent the formation of blood clots[31] but is not necessary if the heart is cannulated and perfused quick enough (within 1.5 minutes from decapitation). Furthermore, perfusion of the heart using CB is recommended for larger rodents, so when working with mouse hearts or other organs it is advised to include IB washes before mincing and initial homogenization. This step allows for blood carried over from the dissection process to be discarded. Other changes include alterations to the homogenization and centrifugation speeds to increase the mitochondrial protein yield. Those outlined above are for isolating cardiac mitochondria from guinea pigs and rats. Importantly, this protocol can be adapted to isolate mitochondria from rodent liver, kidneys, mouse hearts, and cells. Recommended alterations to volume of protease, homogenization, and centrifugation based on specific tissue types and animals are further detailed in Table 3.

After the formation of the purified mitochondrial pellet from the final centrifugation step, mitochondria are to be resuspended in pre-chilled IB. The volume of added IB is dependent on the size of the mitochondrial pellet but is about 80 µL for guinea pigs and rats. If isolating from mouse hearts, 60 µL of IB is added for resuspension. When first isolating mitochondria, it is advised to add smaller volumes of IB to not dilute the stock solution. Due to the consistency of the mitochondrial pellets formed after purifying samples from the liver and kidneys, much less IB (20 µL) is to be added for resuspension. Other methods suggest the use of mechanical resuspension via scraping that can be abrasive to mitochondrial membranes and decrease overall integrity[32,33]. When using this protocol, it is advised to gently wash the pellet with IB to improve quality of the mitochondria. During this process, be careful to avoid producing bubbles or disturbing the pellet with the tip of the pipette as this can lead to membrane rupturing and protein misfolding[34]. Only pushing to the first stop of the pipette can help reduce the likelihood of forming bubbles. Gentle pipette washing is to be done until the entirety of the pellet is in suspension. The total resuspension yield should be 150 – 200 µL for cardiac mitochondria from guinea pigs and rats and appear light brown in color. More concentrated samples will be a darker shade of brown and can be diluted to fit desired working concentration range after quantification of mitochondrial protein.

Standard protein assays using BSA are optimal for mitochondrial protein quantification ~~(PMID: 942051)~~[35]. Protein assays should be delayed and incubated for the recommended durations and temperatures as defined by the manufacturer's protocol. For isolated mitochondria, incubating ~~delaying for 30 minutes and incubating~~ at 37~~40~~ °C for 30 minutes allows for well-spread color development and accurate protein quantification. While quantifying the total amount of protein, it is recommended at first to dilute the resuspended mitochondria and IB at ratios of 1:50, 1:100, and 1:200 to ensure that the protein assay results will be within the calibration range. Further details regarding how to conduct protein assays using BSA as a standard are provided per the manufacturer's kit, so the recommendations listed herein may not be applicable. A CS assay should also be performed to determine the mitochondrial content in each sample. This assay is

well-established and allows for further normalization if studying biological differences between mitochondrial subtypes[36].

Following protein determination, the mitochondrial stock should be diluted to achieve the desired final working concentration for respirometry assays. Mitochondria isolated from guinea pig and rat hearts are diluted to 40 mg/mL and 5 µL of this stock is added to the respiratory chamber to result in a working concentration of 0.1 mg/mL. If isolating from single mouse hearts or from kidneys and liver, dilution of the mitochondrial stock may not be necessary. Larger volumes of mitochondria can also be added to obtain desired concentrations. Rates of oxygen consumption during State 2 that are between 35 – 55 pmol/mL/sec are acceptable for most respirometry analyses ~~(PMID: 29091971)~~[28]. Details pertaining to how RCRs are conducted and analyzed are explained in Figure 1 and the representative results section; however, it is important to note that respiration is fueled by pyruvate and malate. Other substrate conditions such as succinate and rotenone will result in different RCR values since the P/O ratio and other bioenergetic variables are different[37]. The use of pyruvate and malate as respiratory fuels achieves near maximal TCA cycle turnover and production of reducing equivalents; however, maximum TCA cycle activity and coupled ETS function is obtained with 5 mM pyruvate, 1 mM L-malate, and 20 mM succinate. When stimulating oxidative phosphorylation to quantify rates of oxygen consumption during State 3, ADP is added at concentrations at least 10 times the estimated K_D for ADP of the adenine nucleotide translocator[38]. This can be achieved by boluses greater than 350 µM ADP and is why 500 µM was used in all experimental assays. If the duration of State 3 is too short, lower mitochondrial concentrations can be used to prolong it. For further analysis of respiration, modulations can be made to the concentration of ADP that is introduced to the system to better fit experimental parameters[39] ~~(PMID: 21694779)~~. When first developing this protocol, a cytochrome c assay was used to assess outer membrane integrity of the mitochondrial isolates[40] If the RCR values are below expected ranges, perform the cytochrome c test to assess if outer membrane damage is significant. To do this, add 10 µM of cytochrome c to the respiratory chamber and confirm that the increase in respiration is below 5 or 10% of the State 4 rate. The expected ranges are found from prior published studies and are species, tissue, and substrate specific. If the addition of cytochrome c stimulates State 4 respiration above 10%, the last 8,000 x g spin can be repeated to remove damaged mitochondria. That said, outer membrane damage may be a part of a disease phenotype and thus the cytochrome c test should be interpreted with that in mind[41] ~~(PMID: 38521844)~~ ~~(Zhou, 2024 #46)~~. Once consistently high RCRs values with low (< 10%) cytochrome c stimulated effects are obtained, this test only becomes necessary and advised if RCR values lie outside acceptable ranges. If the cytochrome c test is < 10% and RCR values lie outside of the expected range as detailed in Table 2, repeat the respirometry assay with new RB after washing with distilled water 10 times. If decreased rates are still observed, the fresh reagents (pyruvate, malate, EGTA, and ADP) need to be made to diagnose the problem. Additionally, cytochrome c assays can be conducted ~~spectrofluormetrically~~ by way of ELISAs and use of mitochondrial dyes such as TMRE ~~(PMID: 16697956; 25866954)~~[33,42]. Depending on the tissue type and source, these options may be better suited for determining outer membrane integrity.

While there are no major limitations of this protocol if being used to isolated cardiac

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mitochondria, it is important to note that certain consideration should be made when utilizing these methods. The quality of mitochondrial isolates is greatly affected by temperature and the time taken to both perfuse the heart and resuspend the purified pellet. Thus, familiarity with these processes may be required to obtain RCRs comparable to the ones reported here. Additionally, the composition of the buffers and solutions used during the isolation process is important as it directly affects mitochondrial integrity and function[43]. Buffers listed in Table 1 are provided as references and have allowed for the isolation of well-coupled mitochondria across a variety of tissue sources~~.~~, but changes can be made to limit the amount of chloride in respiration analyses as this can interfere with adenine nucleotide translocation and ETS function[28]. Buffer composition can also be altered to better isolate liver mitochondria. As the liver is high in fatty acid concentrations, it is advised that the organ and minced tissue be washed with a buffer containing elevated concentrations of BSA if RCRs outside of the expected range are observed. Although the quality of mitochondrial isolates obtained from liver sections are well-coupled and consistent, this alteration could result in improved organelle function. It should also be recognized that isolation of mitochondria from cells utilizing these methods requires a large quantity of cultured cells, which poses a potential limitation. Furthermore, this protocol is not specifically designed for cellular isolates, but has proven successful when implemented. Therefore, targeted isolation methods for cultured cells may be of better use. Alternatively, to assess mitochondrial quality, researchers may opt for fluorescent probes to calculate RCRs. Spectrofluorometric methods are a popular choice, especially if lower quantities of protein are being extracted ~~(PMID: 17406510; 37776463)~~[44,45].

Overall, this protocol can be used to consistently isolate well-coupled cardiac mitochondria from small animals such as guinea pigs and rats. It can be easily modified to increase protein yields by changing the homogenization speeds, centrifugation times, and number of spins to allow for mitochondrial isolation from mouse hearts, liver, kidneys, and cells. Moreover, this protocol is general and robust enough that it has been used to investigate mitochondrial function from non-mammalian species such as sea lamprey[46], as well as perform structural analysis using classic and cryo-electron microscopy[40,47]. While many recent studies focus on exploration of mitochondrial behavior in intact cell and tissue systems, the breadth and depth of information extracted from isolated mitochondria using these methods reveal impacts on metabolomics, oxidative stress, and ATP production that will never be without merit. Isolation of well-coupled mitochondria allows researchers to investigate key aspects of disease development and progression not otherwise possible in whole cell models. Due to the versatility of this protocol, changes in mitochondrial energetics observed in pathologies such as cardiovascular disease, diabetes, and neurological disorders can be explored using the methodology described herein.

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DISCLOSURES:

Authors declare that there is nothing to disclose.

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REFERENCES:

1 Hogeboom, G. H., Schneider, W. C., Pallade, G. E. Cytochemical studies of mammalian tissues; isolation of intact mitochondria from rat liver; some biochemical properties of mitochondria and submicroscopic particulate material. *J Biol Chem.* **172** (2), 619-635 (1948).

2 Claude, A. Fractionation of mammalian liver cells by differential centrifugation : Ii. Experimental procedures and results. *J Exp Med.* **84** (1), 61-89 (1946).

3 Ernster, L. Schatz, G. Mitochondria: A historical review. *J Cell Biol.* **91** (3 Pt 2), 227s-255s (1981).

4 Martinez-Reyes, I. Chandel, N. S. Mitochondrial tca cycle metabolites control physiology and disease. *Nat Commun.* **11** (1), 102 (2020).

5 Vercellino, I. Sazanov, L. A. The assembly, regulation and function of the mitochondrial respiratory chain. *Nat Rev Mol Cell Biol.* **23** (2), 141-161 (2022).

6 Fernie, A. R., Carrari, F., Sweetlove, L. J. Respiratory metabolism: Glycolysis, the tca cycle and mitochondrial electron transport. *Curr Opin Plant Biol.* **7** (3), 254-261 (2004).

7 Kalpage, H. A. et al. Cytochrome c phosphorylation: Control of mitochondrial electron transport chain flux and apoptosis. *Int J Biochem Cell Biol.* **121** 105704 (2020).

8 Sousa, J. S., D'imprima, E., Vonck, J. Mitochondrial respiratory chain complexes. *Subcell Biochem.* **87** 167-227 (2018).

9 Mitchell, P. Moyle, J. Chemiosmotic hypothesis of oxidative phosphorylation. *Nature.* **213** (5072), 137-139 (1967).

10 Chance, B. Williams, G. R. Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J Biol Chem.* **217** (1), 383-393 (1955).

11 Li, Z. Graham, B. H. Measurement of mitochondrial oxygen consumption using a clark electrode. *Methods Mol Biol.* **837** 63-72 (2012).

12 Rogers, G. W. et al. High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PLoS One.* **6** (7), e21746 (2011).

13 Bisaccia, G., Ricci, F., Gallina, S., Di Baldassarre, A., Ghinassi, B. Mitochondrial dysfunction and heart disease: Critical appraisal of an overlooked association. *Int J Mol Sci.* **22** (2), (2021).

14 Zhou, B. Tian, R. Mitochondrial dysfunction in pathophysiology of heart failure. *J Clin Invest.* **128** (9), 3716-3726 (2018).

15 Holzem, K. M. et al. Mitochondrial structure and function are not different between nonfailing donor and end-stage failing human hearts. *FASEB J.* **30** (8), 2698-2707 (2016).

16 Cordero-Reyes, A. M. et al. Freshly isolated mitochondria from failing human hearts exhibit preserved respiratory function. *J Mol Cell Cardiol.* **68** 98-105 (2014).

17 Norat, P. et al. Mitochondrial dysfunction in neurological disorders: Exploring mitochondrial transplantation. *NPJ Regen Med.* **5** (1), 22 (2020).

18 Diaz-Vegas, A. et al. Is mitochondrial dysfunction a common root of noncommunicable chronic diseases? *Endocr Rev.* **41** (3), (2020).

19 Lai, N. et al. Isolation of mitochondrial subpopulations from skeletal muscle: Optimizing recovery and preserving integrity. *Acta Physiol (Oxf).* **225** (2), e13182 (2019).

20 Holmuhamedov, E. L., Oberlin, A., Short, K., Terzic, A., Jahangir, A. Cardiac subsarcolemmal and interfibrillar mitochondria display distinct responsiveness to

protection by diazoxide. *PLoS One.* **7** (9), e44667 (2012).
21 Frezza, C., Cipolat, S., Scorrano, L. Organelle isolation: Functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat Protoc.* **2** (2), 287-295 (2007).
22 Micakovic, T. et al. Isolation of pure mitochondria from rat kidneys and western blot of mitochondrial respiratory chain complexes. *Bio Protoc.* **9** (19), e3379 (2019).
23 Liao, P. C., Bergamini, C., Fato, R., Pon, L. A., Pallotti, F. Isolation of mitochondria from cells and tissues. *Methods Cell Biol.* **155** 3-31 (2020).
24 Hubbard, W. B. et al. Fractionated mitochondrial magnetic separation for isolation of synaptic mitochondria from brain tissue. *Sci Rep.* **9** (1), 9656 (2019).
25 Zhou, D. et al. Protocol for mitochondrial isolation and sub-cellular localization assay for mitochondrial proteins. *STAR Protoc.* **4** (1), 102088 (2023).
26 Hernandez-Camacho, J. D. et al. Isolation of mitochondria from mouse skeletal muscle for respirometric assays. *J Vis Exp.* 10.3791/63336 (180), (2022).
27 Farrugia, G. E. et al. A protocol for rapid and parallel isolation of myocytes and non-myocytes from multiple mouse hearts. *STAR Protoc.* **2** (4), 100866 (2021).
28 Wollenman, L. C., Vander Ploeg, M. R., Miller, M. L., Zhang, Y., Bazil, J. N. The effect of respiration buffer composition on mitochondrial metabolism and function. *PLoS One.* **12** (11), e0187523 (2017).
29 Vinnakota, K. C. et al. Open-loop control of oxidative phosphorylation in skeletal and cardiac muscle mitochondria by ca(2.). *Biophys J.* **110** (4), 954-961 (2016).
30 Vinnakota, K. C., Bazil, J. N., Van Den Bergh, F., Wiseman, R. W., Beard, D. A. Feedback regulation and time hierarchy of oxidative phosphorylation in cardiac mitochondria. *Biophys J.* **110** (4), 972-980 (2016).
31 Warnock, L. B. Huang, D. in *Statpearls*, Treasure Island (FL) ineligible companies. Disclosure: Davis Huang declares no relevant financial relationships with ineligible companies. (2024).
32 Sercel, A. J. et al. Generating stable isolated mitochondrial recipient clones in mammalian cells using mitopunch mitochondrial transfer. *STAR Protoc.* **2** (4), 100850 (2021).
33 Lampl, T., Crum, J. A., Davis, T. A., Milligan, C., Del Gaizo Moore, V. Isolation and functional analysis of mitochondria from cultured cells and mouse tissue. *J Vis Exp.* 10.3791/52076 (97), (2015).
34 Sobolewski, P., Kandel, J., Eckmann, D. M. Air bubble contact with endothelial cells causes a calcium-independent loss in mitochondrial membrane potential. *PLoS One.* **7** (10), e47254 (2012).
35 Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* **72** 248-254 (1976).
36 Wei, P., Liu, Q., Xue, W., Wang, J. A colorimetric assay of citrate synthase activity in drosophila melanogaster. *J Vis Exp.* 10.3791/59454 (155), (2020).
37 Tomar, N. et al. Substrate-dependent differential regulation of mitochondrial bioenergetics in the heart and kidney cortex and outer medulla. *Biochim Biophys Acta Bioenerg.* **1863** (2), 148518 (2022).
38 Ter Veld, F., Jeneson, J. A., Nicolay, K. Mitochondrial affinity for adp is twofold lower in creatine kinase knock-out muscles. Possible role in rescuing cellular energy homeostasis.

FEBS J. **272** (4), 956-965 (2005).
39 Gouspillou, G. et al. Accurate determination of the oxidative phosphorylation affinity for adp in isolated mitochondria. *PLoS One.* **6** (6), e20709 (2011).
40 Strubbe-Rivera, J. O. et al. The mitochondrial permeability transition phenomenon elucidated by cryo-em reveals the genuine impact of calcium overload on mitochondrial structure and function. *Sci Rep.* **11** (1), 1037 (2021).
41 Zhou, Z. et al. Diverse functions of cytochrome c in cell death and disease. *Cell Death Differ.* **31** (4), 387-404 (2024).
42 Certo, M. et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic bcl-2 family members. *Cancer Cell.* **9** (5), 351-365 (2006).
43 Bose, S., French, S., Evans, F. J., Joubert, F., Balaban, R. S. Metabolic network control of oxidative phosphorylation: Multiple roles of inorganic phosphate. *J Biol Chem.* **278** (40), 39155-39165 (2003).
44 Motawe, Z. Y., Abdelmaboud, S. S., Breslin, J. W. Evaluation of glycolysis and mitochondrial function in endothelial cells using the seahorse analyzer. *Methods Mol Biol.* **2711** 241-256 (2024).
45 Will, Y., Hynes, J., Ogurtsov, V. I., Papkovsky, D. B. Analysis of mitochondrial function using phosphorescent oxygen-sensitive probes. *Nat Protoc.* **1** (6), 2563-2572 (2006).
46 Huerta, B. et al. Sea lamprey cardiac mitochondrial bioenergetics after exposure to tfm and its metabolites. *Aquat Toxicol.* **219** 105380 (2020).
47 Malyala, S., Zhang, Y., Strubbe, J. O., Bazil, J. N. Calcium phosphate precipitation inhibits mitochondrial energy metabolism. *PLoS Comput Biol.* **15** (1), e1006719 (2019).