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Authors

Tachibana, Shizuko
Chen, Chao
Zhang, Oliver R
et al.

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Analyzing Oxygen Consumption Rate in Primary Cultured Mouse Neonatal Cardiomyocytes Using an Extracellular Flux Analyzer

Shizuko Tachibana[1], **Chao Chen**[1], **Oliver R. Zhang**[1], **Sarah V. Schurr**[1], **Cameron Hill**[1], **Ruixia Li**[1], **Ana M. Manso**[1], **Jianlin Zhang**[1], **Aleksander Andreyev**[2], **Anne N. Murphy**[2], **Robert S. Ross**[1,3], **Yoshitake Cho**[1]

[1]Division of Cardiology, Department of Medicine, University of California San Diego

[2]Department of Pharmacology, University of California San Diego

[3]Cardiology Section, Department of Medicine, Veterans Administration Healthcare, San Diego

Abstract

Mitochondria and oxidative metabolism are critical for maintaining cardiac muscle function. Research has shown that mitochondrial dysfunction is an important contributing factor to impaired cardiac function found in heart failure. By contrast, restoring defective mitochondrial function may have beneficial effects to improve cardiac function in the failing heart. Therefore, studying the regulatory mechanisms and identifying novel regulators for mitochondrial function could provide insight which could be used to develop new therapeutic targets for treating heart disease. Here, cardiac myocyte mitochondrial respiration is analyzed using a unique cell culture system. First, a protocol has been optimized to rapidly isolate and culture high viability neonatal mouse cardiomyocytes. Then, a 96-well format extracellular flux analyzer is used to assess the oxygen consumption rate of these cardiomyocytes. For this protocol, we optimized seeding conditions and demonstrated that neonatal mouse cardiomyocytes oxygen consumption rate can be easily assessed in an extracellular flux analyzer. Finally, we note that our protocol can be applied to a larger culture size and other studies, such as intracellular signaling and contractile function analysis.

Keywords

Medicine; Issue 144; Mouse neonatal cardiomyocytes; oxygen consumption; extracellular flux analyzer; respiration; mitochondria; heart

Introduction

To sustain a continuous cardiac contractile function, cardiomyocytes must maintain a constant supply of cellular energy primarily in the form of ATP[1]. In the heart, approximately

Correspondence to: Yoshitake Cho at cyoshitake@ucsd.edu.

Video Link

The video component of this article can be found at https://www.jove.com/video/59052/

Disclosures

The authors declare that they have no competing financial interests.

95% of ATP is generated by mitochondria, mainly through oxidative phosphorylation, showing that mitochondria play a crucial bioenergetic role in cardiac function[2,3]. Supporting this notion is that dysregulation of mitochondrial function can lead to cardiomyopathy and heart failure[4,5]. Conversely, restoring mitochondrial function has been shown to improve cardiac function of the failing heart[6,7]. Therefore, studying the mechanism of mitochondrial bioenergetics and identifying novel regulators of mitochondrial function in cardiomyocytes will not only reveal mechanistic insights of cardiac energy production but also could provide insight that will lead to development of new therapeutic targets to treat heart diseases[6,8].

Compared to the whole heart, which contains a mixture of myocytes and non-myocytes[9], cardiomyocyte cultures are extremely pure, with minimal contamination of non-myocytes from the heart, such as fibroblasts and endothelial cells[10]. In addition, isolating cardiomyocytes from neonatal pups enables culturing a large number of cells in a small amount of time, compared to isolating cells from adult hearts[10,11]. Most importantly, primary cultured adult mouse cardiomyocytes have short survival times (e.g. 24 h) and at longer time points de-differentiate. Neonatal mouse cardiomyocytes can survive and be manipulated for upwards of 7 days in culture, making them ideal fortesting the effects of drug compounds and gene manipulation on the function of mitochondria in cardiomyocytes[10]. Of course, there are significant biological differences between the adult and neonatal cells, but the longer duration available for culture of neonatal cells makes them appropriate for many different types of studies, including those of mitochondrial function.

To date, primary cultured neonatal mouse and rat cardiomyocytes have been used as models to study cardiac bioenergetics[12,13]. In recent years, studies used an extracellular flux analyzer to measure oxygen consumption rate (OCR) and evaluate oxidative capacity in mouse and rat neonatal cardiomyocytes[14,15]. While compared to rats, the cell viability of mouse neonatal cardiomyocytes is lower and has greater variability[16]. Also, the ability to study cells from genetically engineered mouse models makes the mouse cell model very important. Given that OCR studies are so sensitive to cell number and seeding density, development of a reproducible, reliable, and simple protocol to achieve consistent cell yield and viability is needed.

Here, we report an optimized protocol that has been developed which uses cultured mouse neonatal cardiomyocytes along with a 96-well-format extracellular flux analyzer for OCR analysis. This protocol greatly increases reproducibility of the assay. In addition, the protocol not only provides a novel and reproducible method for OCR analysis, but also could be adapted to a larger size culture for other experimental purposes, such as that which may be needed to study myofibrillar functions and intracellular signaling pathways.

In particular, this protocol describes a one-day procedure for isolation and culture of neonatal mouse cardiomyocytes in a 96-well cell culture plate. In addition, it describes the procedure to measure oxygen consumption using an extracellular flux analyzer. All solutions used are sterile or sterile filtered. All tools are sterilized by 75% ethanol. We provide a Table of Materials for various parts of the procedure. For culturing cardiomyocytes, all procedures and steps are performed in a standard cell culture hood. This protocol is developed for the

isolation of neonatal mouse hearts from one litter (approximately 8-10 pups). However, the protocol can also be adapted for isolating cardiomyocytes from multiple litters.

Protocol

For work with neonatal mice, please refer to local university/institute guidelines set forth by the animal care programs and adhere to one's institutional and other appropriate regulations. All methods described in this protocol have been approved by the UC San Diego Institutional Animal Care and Use Committee (IACUC) and adhere to federal and state regulations.

1. Preparation of Reagents

1. Prepare 25 mL of **pre-digestion solution**: HBSS (without Ca^{2+} and Mg^{2+}) supplemented with trypsin (0.5 mg/mL). Sterilize the solution using a 0.22 pm filter and keep on ice until use. Make pre-digestion solution on the day of experiment.

   NOTE: It is critical to use HBSS without Ca^{2+} and Mg^{2+}, as Ca^{2+} and Mg^{2+} will cause myocyte contraction and subsequent cell death during isolation.

2. Prepare 30 mL of **collagenase digestion buffer**: collagenase (0.8 mg/mL, approximately 350 U/mL) dissolved in HBSS (without Ca^{2+} and Mg^{2+}) buffer. Sterilize the solution using a 0.22 pm filter and keep on ice until use. Make collagenase digestion solution on the day of experiment.

3. Prepare 500 mL of **cardiomyocyte culture media (growth media)**: Mix 375 mL of DMEM, 125 mL of M-199, 25 mL of Horse serum, and 12.5 mL of FBS. Supplement with 1 % penicillin and 1 % streptomycin solution.

4. Prepare **mitochondrial stress test medium**: Make 200 mL of DMEM based stress test medium (DMEM without $NaHCO_3$, see Table of Materials) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, and 10 mM glucose, and 2 mM Hepes.

   NOTE: Make 1 L of medium with DMEM without sodium pyruvate, L-glutamine, glucose, and Hepes, filer sterile, and store in 4 °C. Prepare the stress test medium on the day of the assay by adding other reagents. Using DMEM medium without $NaHCO_3$ is critical.

5. Adjust pH of mitochondrial stress test media to 7.4 on the day of use. Warm media to 37 °C before use.

6. Prepare **oligomycin**: Prepare 5 mL of a 5 mM stock solution in DMSO, make 250 μL aliquots, and store at −20 °C.

7. Prepare **FCCP**: Prepare 5 mL of a 5 mM stock solution in DMSO, make 250 μL aliquots, and store at −20 °C.

8. Prepare **antimycin A**: Prepare 5 mL of a 5 mM stock solution in DMSO, make 250 μL aliquots, and store at −20 °C.

9. Prepare **rotenone**: Prepare 5 mL of a 5 mM stock solution in DMSO, make 250 μL aliquots, and store at −20 °C.

   NOTE: All reagents and solutions used in this protocol are listed in Table 1.

2. Harvesting and Pre-digestion of Hearts from Neonatal Mice (Day 1)

1. Autoclave scissors, forceps, and a Moria spoon to sterilize.
2. Perform all steps in the cell culture hood for sterility.
3. Aliquot 5 mL of HBSS (without Ca^{2+}, Mg^{2+}) to each well of a 6-well cell culture plate; place on ice. Aliquot 10 mL of HBSS into a 10 cm cell culture dish.
4. Prepare 20 mL of trypsin pre-digestion solution in a 50 mL sterile conical tube. Keep all solutions on ice.
5. Quickly dip newborn (day 0) mice in 70% ethanol solution for sterilization.
6. Decapitate pups using sterile scissors (straight) without anesthesia, and then open chest along the sternum to allow access to the chest cavity and the heart. (Figure 1A)

   NOTE: 1) It is critical to use P0 neonatal mice to achieve high cell viability. 2) This euthanasia method is permitted for neonates in accordance with NIH and American Veterinary Medical Association guidelines [17].
7. Extract hearts from the body with a fine scissors and transfer immediately into the sterile cell culture dish containing HBSS (without Ca^{2+}, Mg^{2+}) (Figure 1B).
8. Remove any residual lung tissue, larger vessels, *etc.* (and atria, if desired). Wash hearts in the HBSS solution using gentle agitation.
9. Cut each heart with a fine scissors into 8 pieces and transfer the all heart tissue with forceps into one well of a 6-well cell culture plate with HBSS (Figure 1C **and** 1D).
10. Wash the hearts by transferring the hearts from well to well in the 6-well plate filled with HBSS, using a Moria spoon (Figure 1D).

    NOTE: Transferring the hearts from well to well is enough to wash out the blood. Since blood interferes with enzymatic digestion it is important to wash the hearts with HBSS and remove blood.
11. Transfer the hearts with a Moria spoon into a conical tube containing 20 mL of trypsin (0.5 mg/mL) and incubate with gentle agitation at 4°C for 4 h (Figure 1E).

3. Prepare a 96-well Culture Plate (Day 1)

1. Prepare 5 mL of coating solution: PBS containing 0.5% gelatin (autoclave before use) and 1% fibronectin solution, (e.g. 5 mL gelatin solution plus 50 μL of fibronectin solution)

2. Aliquot 50 µL of coating solution into each well of the 96-well cell culture plate (see Table of Materials). If bubbles are present, remove them by using a 20 µL pipette to suck bubbles out.

   NOTE: It is important to cover all the surface area of each well with coating solution.

3. Incubate the plate in a 37 °C cell culture incubator for 1 h or more to allow drying of the matrix coating.

4. Aspirate any residual coating solution before seeding cardiomyocytes.

4. Enzymatic Digestion and Plating of Cells (Day 1)

1. Pre-warm collagenase digestion solution in a 37 °C water bath.

   NOTE: This is step is important to achieve efficient enzymatic digestion.

2. Move the conical tube containing hearts and pre-digestion solution from 4 °C to a cell culture hood. (Figure 1F)

3. Let the hearts sink to the bottom of the tube and remove the pre-digestion solution by using a 10 mL serological pipette (1 to 2 mL of the isolation medium may remain in the tube).

4. Add 10 mL of HBSS into the tube. Re-suspend the hearts with HBSS 2-3 times to wash out trypsin using a 10 mL serological pipette. Aspirate HBSS (1 to 2 mL may remain in the tube).

5. Add 10 mL of pre-warmed collagenase digestion solution into the tube with hearts. (Figure 1G)

6. Incubate the tube with hearts in a 37 °C water bath for 10 min without agitation (1 st digestion).

7. After 1st digestion, move the tube to the cell culture hood. Gently triturate hearts by re-suspending the hearts within the tube gently 10 times using a 10 mL serological pipette. This will allow hearts to disperse and cells to be released from heart tissue. (Figure 1H)

   NOTE: Since cardiomyocytes are fragile, gentle trituration is important to achieve high viability.

8. Let the undigested tissue sink, transfer digested solution enriched in cardiomyocytes (approximately 9 −10 mL) to a new conical tube, and immediately add an equal amount of cell culture media to stop the collagenase digestion.

9. Add 10 mL of collagenase digestion solution into the tube containing the remaining undigested heart tissue.

10. Incubate the tube with heart tissue in a 37 °C water bath for 10 min (2nd digestion).

11. Repeat procedure 4.7 and 4.8.

    NOTE: If there is still much undigested tissue, repeat digestion one more time. However, in most cases, two digestions are enough to disperse most of the cells from the heart tissue.

12. Place a sterile cell-strainer (100 pm nylon mesh) in a new sterile 50 mL conical tube. Pre-wet the cell strainer with 2-3 mL of cell culture media and pass cells through the cell-strainer. Rinse the cell-strainer with 2-3 mL of cell culture media. (Figure 1I)

13. Centrifuge conical tube containing cardiomyocytes for 5 min at 180 × *g* (Figure 1J). Aspirate the supernatant (Figure 1K), which will contain cell tissue debris and re-suspend the cell pellet in 10 mL of cell culture media (Figure 1L).

14. Gently resuspend the cells and plate cells onto a 10 cm cell culture dish (plastic without any type of coating) and incubate for 1 h in a cell culture incubator (1st pre-plating) (Figure 1M). This pre-plating step allows non-cardiomyocytes, such as fibroblasts and endothelial cells, to adhere to the uncoated cell-culture dish.

    NOTE: At this point, cardiomyocytes are typically a round shape and appear shiny under the microscope. (Figure 1N).

15. After the 1 h incubation, gently agitate the plate, wash non-adherent cells (enriched in cardiomyocytes) from the 10 cm culture dish, and resuspend cells by repeatedly pipetting the cell culture medium over the dish using a 10 mL serological pipette. Then, transfer non-adherent cells (enriched in cardiomyocytes) into a new 10 cm cell culture dish (plastic without any coating) and incubate for an additional 1 h in a cell culture incubator (2nd pre-plating).

    NOTE: Cells that attach to the non-coated plate are dominantly non-cardiomyocytes: fibroblasts and endothelial cells, that can be visualized under a microscope (Figure 1O).

16. After 2nd pre-plating, gently agitate the plate, wash non-adherent cells (cardiomyocytes) from the 10 cm culture dish, then transfer the cardiomyocytes into a new 50 mL conical tube.

5. Counting Cells and Plating Cells into a 96-well Cell Culture Plate (Day 1)

1. Count the cells using a hemocytometer.

2. Plate the cells into an extracellular matrix coated 96-well cell culture plate at a density between 10-30 × 10^3 cells/well by using a multichannel pipette in a final volume of 200 μL (Figure 1P). Use wells A1, A12, H1, and H12 for background: add 200 μL of culture medium as other wells in these wells (no cells). Incubate the plate in a 37 °C cell culture incubator.

    NOTE: In this study, oxygen consumption was tested by using different cell densities such as 10 × 10^3, 20 × 10^3, or 30 × 10^3 cells/well. (Figure 2A). Also, as above, cardiomyocytes immediately after isolation are typically a round shape

and appear shiny under the microscope. Viable cells will flatten out within 16-24h of culture.

6. Oxygen Consumption Assay using a 96-well-format Extracellular Flux Analyzer (Day 2)

NOTE: Oxygen consumption assay can be carried out one day after plating the cells or later. Neonatal cardiomyocytes cultured using this protocol can survive up to 7 days post isolation.

1. Hydrate a flux analyzer sensor cartridge (see Table of Materials) for at least 3 hours, but ideally for a full day, before the assay. Add 200 µL of Calibrant solution (see Table of Materials) into the each well of the utility plate, put the sensor cartridge back onto the utility plate, and incubate in a 37°C incubator without CO_2 or O_2 supplementation.

2. Change cell culture media to mitochondria stress test medium one hour prior to the assay. Cardiomyocytes are fragile. Therefore, gently remove the cell culture media by using a multi-channel pipette and wash the cells with 200 µL of pre-warmed mitochondrial stress test media twice. After second wash, add 175 µL of pre-warmed mitochondria stress test media and culture the cells in a 37°C incubator without CO_2 or O_2 supplementation.

3. Prepare concentrated test compounds. For mitochondria stress test, prepare 3.0 mL each of 16 µM oligomycin, 9 µM FCCP, and a mixture of 20 µM rotenone and 20 µM antimycin A, all in mitochondrial stress test medium.

   NOTE: Each compound at the concentration described has been tested. However, titrating the concentration of each compound in one's own laboratory is necessary.

4. Load 25 µL of each compound into the injector ports of the sensor cartridge using a multichannel pipette (Figure 1Q). The volume and final concentration are described in Table 2.

5. Set up extracellular flux assay protocol. The program is described in Table 3.

6. Start the program. First, put the sensor cartridge into the machine for calibration (Figure 1R). Replace the calibrant for the assay plate once the calibration step is done.

   NOTE: Using the software provided by manufacturer, indicate groups of wells and each compound and port.

7. If desired, after the assay, carefully discard all assay medium by using a multi-channel pipette and store the cell culture microplate at −20 °C for future cell normalization using protein assay.

8. Measure protein content. Add 50 µL of standard RIPA cell lysis solution (see Table of Materials). Incubate the plate on ice for 30 min to fully lyse cells. Transfer all material to a new clear flat bottom 96 well assay plate.

9. Measure protein concentration by BCA assay according to manufacturer's protocol.

NOTE: Coating the well with extracellular matrix results in a high protein concentration in each well. Therefore, subtract the amount of the background well(s) (no cells) to get actual cell protein concentration. The protein concentration derived from cells is low in a 96-well culture plate. In addition, protein concentration can vary from well-to-well due to extracellular matrix coating. Therefore, using cell number to normalize the OCR is suggested.

Representative Results

By using the protocol described, hearts were isolated from day 0 neonatal pups. 5×10^5 cells/pup were obtained, and cardiomyocytes were seeded at densities of 10×10^3, 20×10^3, or 30×10^3 cells/well, in 96 well plates (Figure 2A). After overnight culture, cardiomyocytes were found well-attached to the coated plastic surface and there were very few unattached cells (the unattached cells will still appear as round and shiny, as compared to the healthy, attached cells which are spreadout) (Figure 2A **and** B). At this point, spontaneously contracting cardiomyocytes were easily visible. A seeding density of 30×10^3 cells/well showed confluence one day after seeding as the cells spread. As the number of round (dead) cells were very low, these results showed that the protocol gives high cell viability of cardiomyocytes. Cardiomyocytes were immunostained with an antibody against sarcomeric α-actinin, a cardiomyocyte specific marker[18] as proof of this statement. As shown in Figure 2C, most of cells showed positive staining of α-actinin showing the high purity of the cardiomyocyte isolation.

A scheme of one typical mitochondrial stress test is shown in Figure 3. The mitochondrial stress test starts with a baseline measurement of the oxygen consumption rate (OCR). This is followed by the injection of oligomycin, which inhibits ATPase. The difference before and after oligomycin injection shows the OCR linked to ATP production. Then, the uncoupling agent FCCP was injected to measure maximum oxygen consumption rate. Spare respiratory capacity can be calculated as the difference between basal and the maximal OCR. Finally, with the injection of two electron transport complex inhibitors (antimycin A and rotenone), mitochondrial respiration completely stops, and OCR decreases to its lowest level. By blocking mitochondrial activity, at this level, oxygen consumption is non-mitochondrial. Basal respiration, proton leak, and maximal respiration can be calculated as the difference between non-mitochondrial respiration (OCR in the presence of antimycin A and rotenone) and baseline measurement, OCR in the presence of oligomycin, and OCR in the presence of FCCP, respectively.

In this study, OCR analysis was performed using a 96-well format extracellular flux analyzer system one day after cell isolation. In addition, to test the effect of serum starvation on OCR, three hours prior to the assay, the cell culture media were changed to regular cell culture growth media with serum, or without the serum. After the run, OCR measurement data 1 till 12 for each well, were exported to a spreadsheet for record keeping and further analysis. Metabolic characteristics were determined as follows:

Non-mitochondrial respiration = average OCR (10, 11, 12)

Basal respiration = average OCR (1,2,3) - average OCR (10, 11, 12)

ATP production = average OCR (1,2, 3) - average OCR (4, 5, 6)

Proton leak = average OCR (4, 5, 6) - average OCR (10, 11, 12)

Maximal respiration = average OCR (7,8,9) - average OCR (10, 11, 12)

As shown in Figure 4A, OCR values were easily analyzed, and the effects of injected compounds were also obvious. Importantly, the variation from well to well was small as shown by the standard error. Increase in cell number resulted in increased baseline measurement, Oligomycin, and FCCP-treated respiration. Compared to serum starved cells, OCR was higher in cells analyzed that had been incubated with growth media. As shown in Figure 4B, OCR is shown as basal respiration, proton leak (Oligo), and Maximal respiration (FCCP) per 1×10^4 cells. OCR per 10×10^3 cells was higher in seeding density of 20K and 30K cells/well than that of 10K cells/well. As shown in Figure 4C, by expressing OCR relative to basal respiration, the relative OCR of Proton leak (Oligo) and Maximal (FCCP) show similar values between growth media and serum starved groups. These results suggest that seeding density does not affect the relative proton leak and maximal oxidative capacity.

Overall, by using this protocol, excellent yields of mouse neonatal cardiomyocytes were successfully isolated and cultured. OCR can be assessed by using these myocytes in an extracellular flux analyzer. The results also show that seeding density does not significantly affect OCR calculated by cell number. Flowever, short term (3 h) serum starvation decreases OCR. The information is useful fortesting mouse neonatal cardiomyocytes OCR with various experimental conditions.

Discussion

In this study, we have established a simple protocol for isolating and culturing mouse neonatal cardiomyocytes. By using these cardiomyocytes, we also optimized the conditions to measure oxygen consumption rate by using an extracellular flux analyzer system. The protocol allows one to use mouse neonatal cardiomyocytes as a model system to examine how various factors can alter oxygen consumption in the principal working cells of the heart, akin to what would be measured in the intact organ. Our protocol is different from previously published protocols[16,18]. First, to achieve high viability, only pups immediately at birth are used (i.e. P0), instead of ones from days zero to three days of age (P0 to P3 pups), which are commonly used in other protocols[16,19]. Second, to minimize the time of the experiment, hearts were only pre-digested with trypsin for 4 h. In addition, to make the procedure simple and achieve reproducible results, we employed a minimum number of steps, as outlined. For example, our protocol uses only two types of buffers, PBS and FIBSS, and only one type of cell culture media, and does not employ agitation during collagenase digestion.

To achieve high viability of neonatal mouse cardiomyocytes, which is important for reproducibility of the OCR assay, there are several critical points. First, using P0 newborn pups and performing trypsin pre-digestion and collagenase digestion on the same day is

critical to achieve high viability. Second, during collagenase digestion, it is not recommended to agitate the heart tissue in the 37 °C water bath. Although agitation is suggested in many protocols, we found this is not necessary as P0 hearts are easy to be digested by collagenase. Finally, gently triturating heart tissue is critical to dissociate the cardiomyocytes after collagenase digestion.

We have also tested myocytes from P1 and P2 pup heart tissue, using the same protocol. However, for these older heart samples, we found it is necessary to perform trypsin pre-digestion overnight to achieve sufficient cell yields (data not shown). In addition, the cell viability for P1 and P2 cardiomyocytes was around 60%, similar to that in other published studies[16]. These results suggest that P0 myocardium has relatively lower amounts of extracellular matrix than older hearts, which enables shorter times for trypsin pre-digestion, resulting in higher cell viability and yields from these younger hearts.

Extracellular flux (XF) analysis has become a major and popular method to measure bioenergetic function in cells and isolated mitochondria[20,21]. To date, a number of studies used rat neonatal cardiomyocytes and measured oxygen consumption, using an Agilent XF24 system[22,23,24]. These studies using rat cells as opposed to mouse-derived cells, were likely performed as the rat cells generally have higher cell viability and assay reproducibility, as compared to the mouse cardiac myocytes studied here. Given that genetically modified animals have mainly been generated in mouse lines, a simple and reproducible protocol for analyzing bioenergetic function in mouse neonatal cardiomyocytes, as we discuss in this manuscript, provides opportunities to study mitochondrial function in mouse cardiac myocytes. This method can be more easily translated to data that may lead to understanding new mechanisms for bioenergetic regulation in the heart, by using new or existing genetically-manipulated mouse lines[25,26].

In this study, we tested the effect of cell number and density on OCR. As shown in Figure 4B, OCR measured in wells with 10×10^3, 20×10^3, and 30×10^3 cells/well, was similar. Given that plating 30×10^3 cells/well produced a confluent well within one day after seeding, we recommend splitting cells between 10×10^3 to 30×10^3 cells/well in a 96-well plate, for the OCR assay. Interestingly we found 3 h of serum starvation decreased OCR. Growth factors are known to activate glycolysis and increase oxygen consumption[27]. It was also reported that growth factors enhance overall cellular activity and contraction of cardiomyocytes[3,28]. Cardiomyocyte contraction requires ATP generation[2], which depends on oxygen consumption. Therefore, these data suggest that serum starvation decreases oxygen consumption through inactivation of cardiomyocytes. We would recommend testing the effect of serum starvation on OCR in one’s own experimental setting.

As mentioned, mitochondria play key roles in heart function. Therefore, identifying novel regulators and pathways regulating oxidative metabolism could provide a means to identify novel therapeutic targets for treating heart failure. Since a 96 well format provides for the ability to test a larger number of testing conditions compared to a 24 well format, this protocol could also be easily adapted to a high throughput screen for identifying novel bioenergetic regulators in cardiomyocytes[29]. Thus, by combining our protocol with genetically manipulated neonatal cardiomyocytes, such as ones that have mitochondrial

mutations[30], could provide interesting studies to screen the effects of chemical compounds or cDNA libraries, on OCR in wild-type vs. metabolically-defective cardiomyocytes.

We are aware that neonatal and adult cardiomyocytes have many different characteristics and metabolic functions[31], including expression levels of glucose and fatty acid metabolic enzymes[32]. Therefore, ideally to use in vitro cultured cardiomyocytes as a model system of the intact heart, it would be important to further develop a protocol to efficiently analyze OCR in adult cardiomyocytes, so that one could compare their oxidative capacity and characteristics with neonatal cardiomyocytes. Future studies will need to be pursued to adapt this methodology to a reproducible system using adult cardiac myocytes.

In summary, we show a simple and reproducible protocol for analyzing oxygen consumption using primary cultured mouse neonatal mouse cardiomyocytes. By using this protocol, we could successfully isolate and culture mouse neonatal cardiomyocytes and perform this OCR assay using a 96-well format extracellular flux analyzer system. Our protocol gives high viability and importantly, consistently reproducible results. Although the current study is mainly focused on oxygen consumption and a mitochondrial stress test, the protocol could be easily adapted to analyze fatty acid oxidation and glycolysis in cardiomyocytes.

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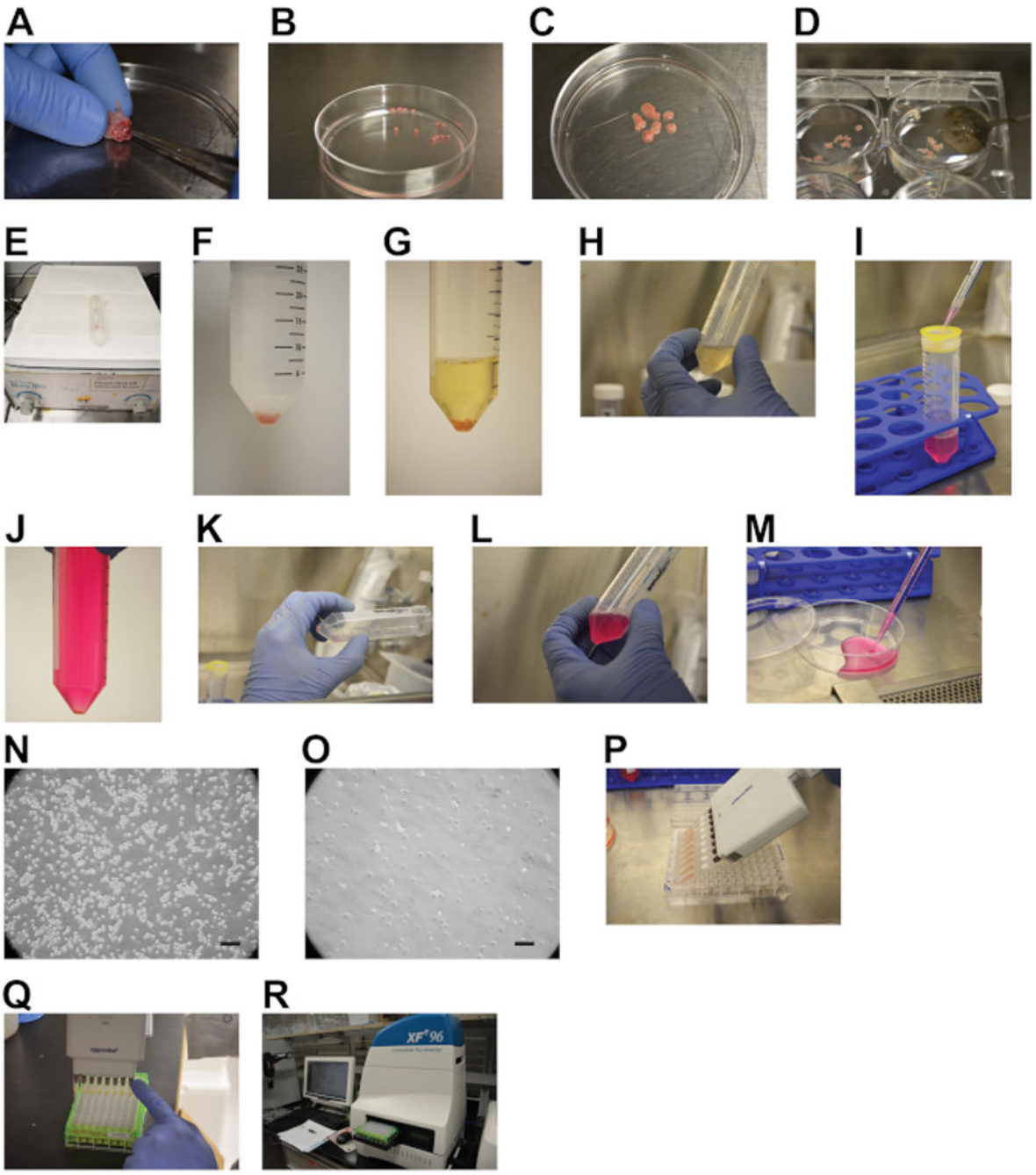

Figure 1: Isolation and culture of neonatal cardiomyocytes from day 0 newborn mice.

(**A**) Neonates are euthanized, and the heart is dissected from the thorax. (**B**) Hearts are washed in HBSS (without Ca^{2+}, Mg^{2+}). (**C**) Each heart is cut into 8 pieces. (**D**) Hearts are moved to a 6-well plate filled with HBSS. Hearts are washed by using a Moria spoon to transfer them from well to well. (**E**) Hearts are pre-digested in Trypsin-HBSS at 4 °C with gentle agitation. (**F**) Predigested heart tissue after 4 h incubation at 4 °C. (**G**) Heart tissues after 10 min collagenase digestion. (**H**) Collagenase digested heart tissues are triturated. (**I**) Isolated cardiomyocytes were filtered through a cell strainer. (**J**) Cardiomyocytes are

pelleted by centrifugation. (**K**) Collagenase and culture media are removed by aspiration. (**L**) Cardiomyocytes are re-suspended in fresh culture medium. (**M**) Cardiomyocytes are cultured in a 10 cm plastic dish for pre-plating. (**N**) A representative image of cardiomyocytes (round and shiny cells) after 1 h of pre-plating. (**O**) After pre-plating and then removal of non-adherent cardiomyocytes, remaining cells that are typically fibroblasts and endothelial cells are visible. (**P**) Plating cardiomyocytes into a 96-well plate. (**Q**) Loading reagents into injection ports of sensor cartridge. (**R**) Putting sensor cartridge into an extracellular flux analyzer machine. Scale bar = 0.1 mm.

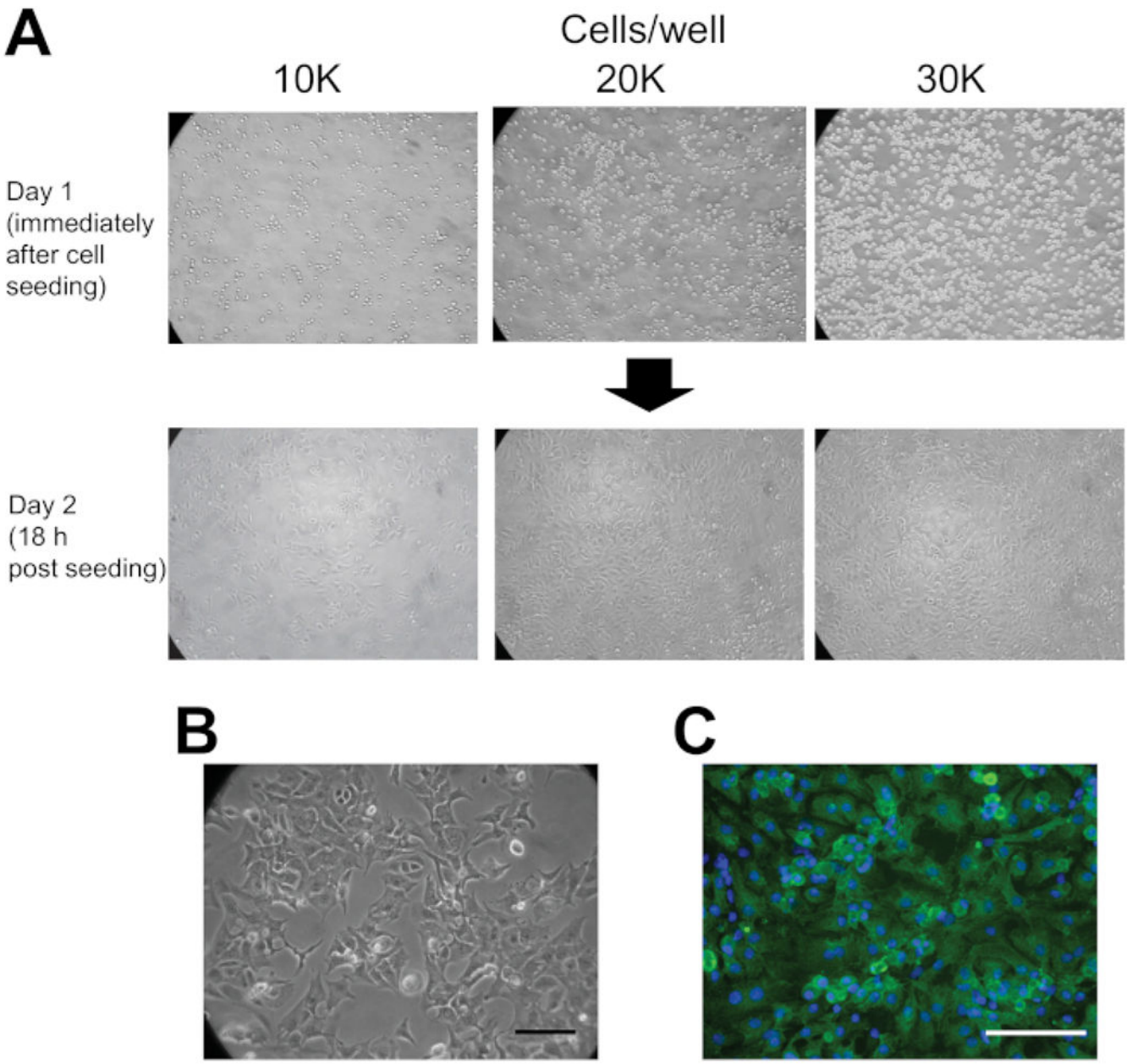

Figure 2: Culture of cardiomyocytes on extracellular flux analyzer 96-well plate.

(**A**) Representative images of cardiomyocytes in 96-well plates with indicated cell densities, immediately after seeding (upper row) or 18 h post seeding (lower row). (**B**) A higher magnification image of cardiomyocytes 18 h post seeding at a cell density of 10×10^3 cells/well. (**C**) Cardiomyocytes were stained with anti-sarcomeric α-actinin antibody (green)and DAPI (as a nuclear stain) (blue). Scale bar = 0.1 mm. The methods used for immunostaining can be found in our previous publication[18].

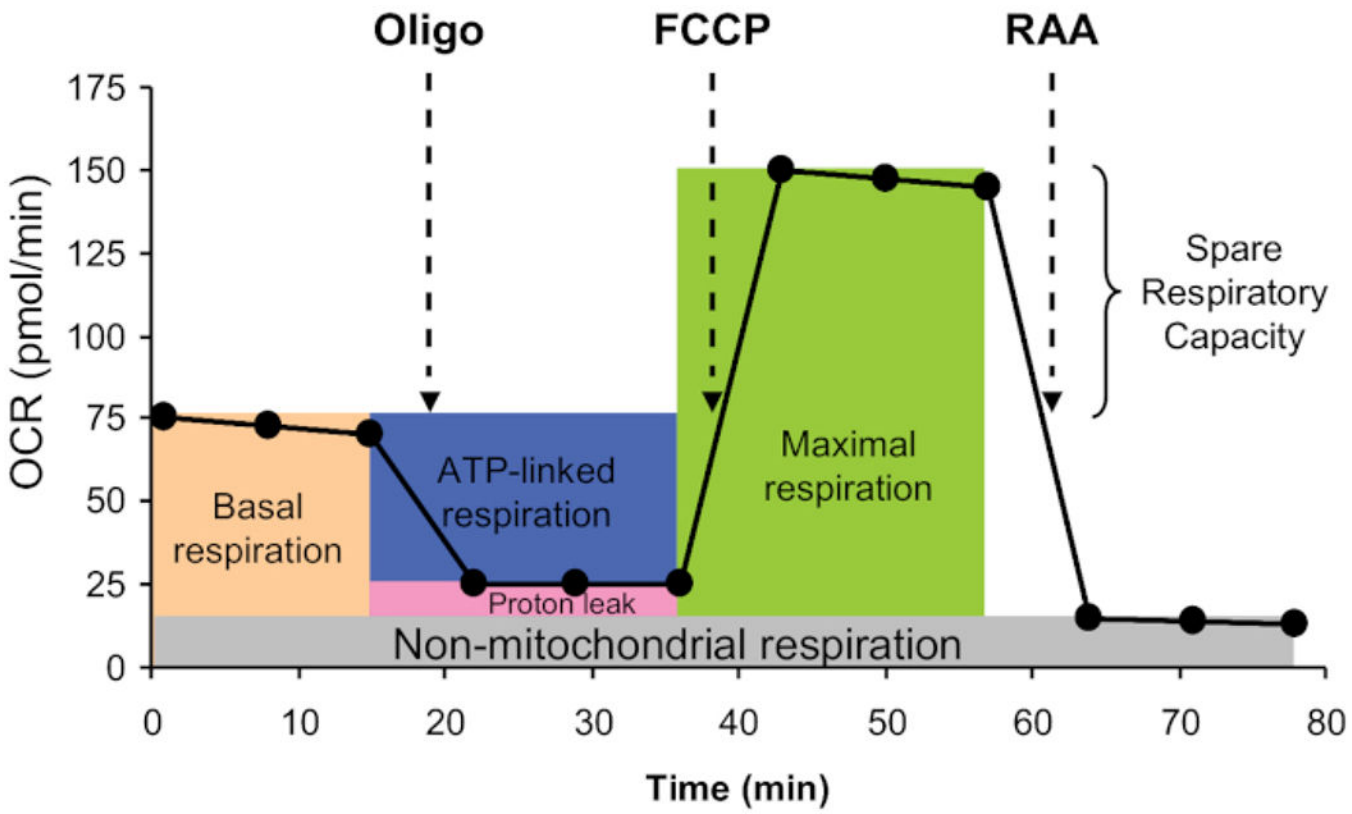

Figure 3. Schematic representation of mitochondrial stress test.

Bioenergetic parameters, including Basal, ATP-linked, Maximal, and Non-mitochondrial respiration as well as Spare respiratory capacity and Proton leak, are outlined on the trace with corresponding mitochondrial effectors.

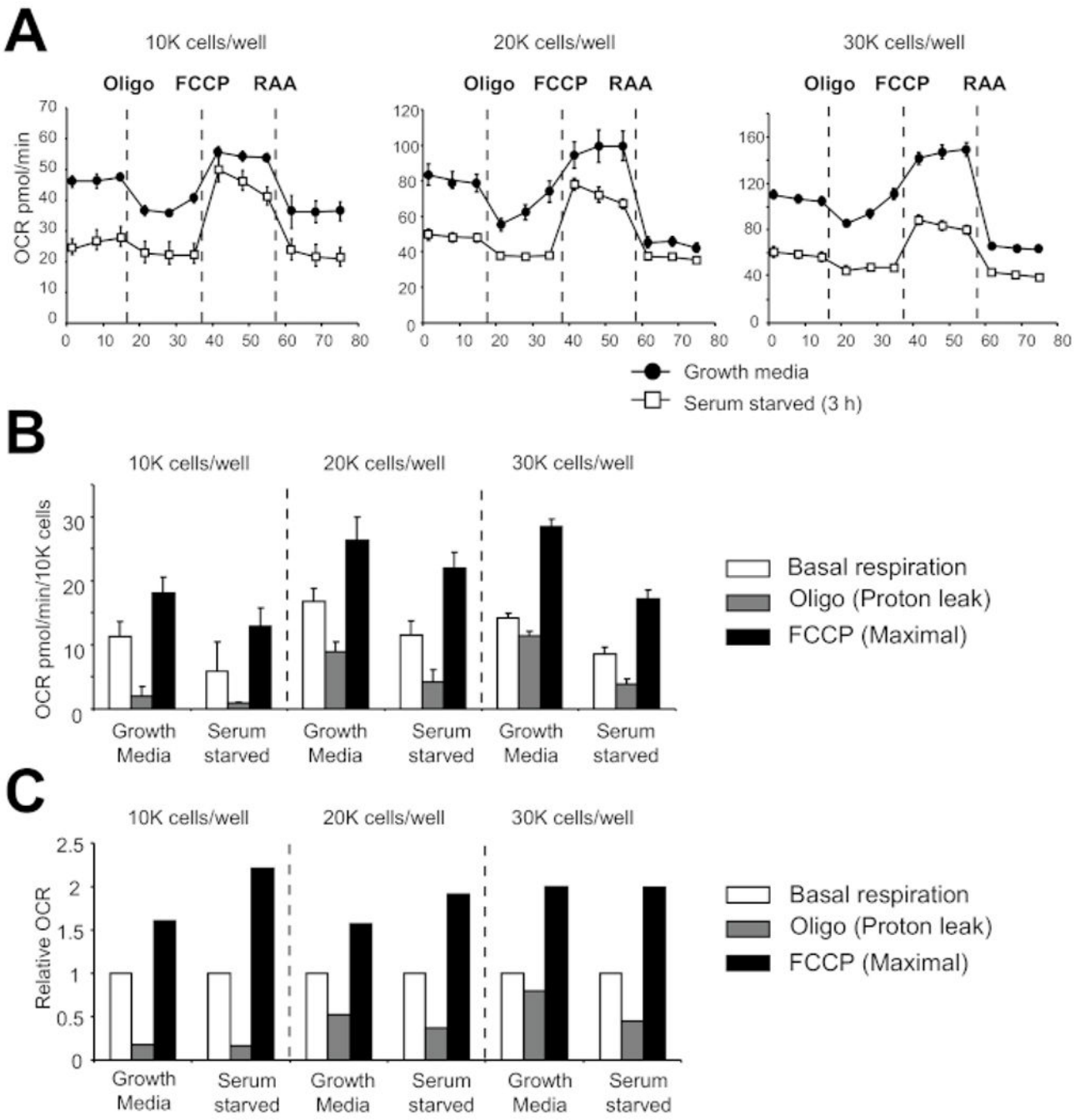

Figure 4: Mitochondrial stress assay.

(**A**) Representative tracing of oxygen consumption rates (OCR, in pMoles/min) of neonatal mouse cardiomyocytes. At the indicated times, oligomycin (Oligo, 1 μM), FCCP (800 nM), and RAA (rotenone and antimycin A, 1 μM each) were injected. For each measurement, the mean and standard error of the mean (SEM) of 10 individual wells is presented. (**B**) OCR is calculated as Basal respiration, Oligo (Proton leak), and FCCP (Maximal respiration) per 10 × 10^3 cells. (**C**) OCR is expressed relative to basal respiration.

Table 1:

Reagents and solutions.

Name of reagent and solution	Preparation	Notes
Trypsin pre-digestion solution	Dissolve trypsin in HBSS (without Ca2+ and Mg2+). Final concentration is 0.5mg/mL. Sterilize the solution using a 0.22 μm filter and keep on ice until use.	Make pre-digestion solution on the day of experiment.
Collagenase digestion solution	Dissolve trypsin in HBSS (without Ca2+ and Mg2+). Final concentration is 0.5mg/mL. Sterilize the solution using a 0.22 μm filter and keep on ice until use.	Make collagenase digestion solution on the day of experiment.
Cardiomyocyte culture media	Mix 375 mL of DMEM, 125 mL of M-199, 25 mL of Horse serum, and 12.5 mL of FBS. Supplement with 1 % penicillin and 1% streptomycin solution.	Cardiomyocyte culture media can be made prior to the experiment and be stored at 4 °C for a month.
Mitochondrial stress test medium	First, Make 1 L of medium with DMEM without any sodium pyruvate, L-glutamine, glucose, and Hepes, filter to sterilize, and store at 4 °C. On the day of assay, add sodium pyruvate, L-glutamine, glucose, and Hepes. Adjust pH to 7.4 before use (sterilization is not required).	Final concentration for supplements are following: sodium pyruvate (1 mM), L-glutamine (2 mM), glucose (10 mM), and Hepes (2 mM)
Oligomycin stock	Dissolve oligomycin in DMSO. First, prepare 5 mL of stock solution. Once oligomycin is completely dissolved in DMSO, make 250 μL aliquots, store at −20 °C.	On the day of assay, take one aliquot to use. Avoid many freeze-thaw cycles.
FCCP stock	Dissolve FCCP in DMSO. First, prepare 5 mL of stock solution. Final concentration is 5 mM. Once FCCP is completely dissolved in DMSO, make 250 μL aliquots, store at −20 °C.	On the day of assay, take one aliquot to use. Avoid many freeze-thaw cycles.
Antimycin A stock	Dissolve antimycin A in DMSO. First, prepare 5 mL of stock solution. Final concentration is 5 mM. Once antimycin A is completely dissolved in DMSO, make 250 μL aliquots, store at −20 °C.	On the day of assay, take one aliquot to use. Avoid many freeze-thaw cycles.
Rotenone stock	Dissolve rotenone in DMSO. First, prepare 5 mL of stock solution. Final concentration is 5 mM. Once rotenone is completely dissolved in DMSO, make 250 μL aliquots, store at −20 °C.	On the day of assay, take one aliquot to use. Avoid many freeze-thaw cycles.
Cell culture plate coating solution	First, make 200 mL of 0.5% gelatin solution. Dissolve gelatin in PBS and autoclave. On the day of experiment, add 50 μL of fibronectin solution in 5 mL gelatin solution to make coating solution.	0.5% gelatin solution can be stored at room temperature for up to 2 months.

Table 2:

Injection mixtures.

Injection Port	Compounds and concentration	Volume injected during run (µl)	Final Concentration in the assay
A	16 µM oligomycin	25	2 µM
B	9 µM FCCP	25	1 µM
C	20 µM rotenone (Rot) and 20 µM antimycin A (AA)	25	2 µM of each

Table 3:

Extracellular flux analyzer program.

Steps and Procedures	Measurements and Loops
Calibration	-
Equilibration	-
Baseline measurement	3 times: Mix 3 min, wait 2 min, measure 3 min
Inject Port A (Oligomycin)	-
Measurements	3 times: Mix 3 min, wait 2 min, measure 3 min
Inject Port B (FCCP)	-
Measurements	3 times: Mix 3 min, wait 2 min, measure 3 min
Inject Port C (RAA)	-
Measurements	3 times: Mix 3 min, wait 2 min, measure 3 min

Materials

Name	Company	Catalog Number	Comments
Antimycin A	SIGMA	A8674	Inhibits complex III of the mitochondria
Cell strainer 100 μm pores	FALCON	352360	To capture undigested tissue
Collagenase type II	Worthington	LS004176	To make collagenase digestion solution
D-Glucose	SIGMA	75351	To make mitochodnrial stress test medium
DMEM high glucose	Life technologies	11965-092	To make cell culture medium
DMEM without NaHCO3, Glucose, pyruvate, glumanine, and Hepes	SIGMA	D5030-10X1L	To make mitochodnrial stress test medium
FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone)	SIGMA	C2920	Uncouples mitochondrial respiration
Fetal bovine serum (FBS)	Life technologies	26140-079	To make cell culture medium
Fibronectin from bovine plasma	SIGMA	F1141-5MG	To make coating solution for tissue culture plates
Fine scissors	Fine Sciences Tools	14060-10	For dissection of hearts
Gelatin from porcine skin	SIGMA	G-1890	To make coating solution for tissue culture plates
HBSS (Hank's balnced salt solution,without Ca2+, Mg2+)	Cellgro	21-022-CV	To wash hearts and make pre-digestion and collagnase digestion solution
HEPES (1M)	Fisher scientific	15630080	To make mitochodnrial stress test medium
Horse serum	Life technologies	26050-088	To make cell culture medium
L-Glutamine	SIGMA	G-3126	To make mitochodnrial stress test medium
M-199	Cellgro	10-060-CV	To make cell culture medium
Moria spoon	Fisher scientific	NC9190356	To wash hearts
Oligomycin	SIGMA	75351-5MG	Inhibits mitochondrial ATP synthase
RIPA buffer	Fisher scientific	89900	To lyse the cells for protein assay
Rotenone	SIGMA	R8875	Inhibits complex I of the mitochondria
Seahorse XFe96 Extracellular Flux Analyzer	Agilent		Device used to analyze oxygen consumption rate
Seahorse XFe96 FluxPak	Agilent	102601-100	Package of flux analyzer culture plates, sensor cartridges, and calibrant
Sodium pyruvate	SIGMA	P2256	To make mitochodnrial stress test medium
Straight scissors	Fine Sciences Tools	91401-12	For dissection of hearts
Syringe filter 0.2 μm size			For sterile filtration of digestion medium
Trypsin	USB Corporation	22715 25GM	To make pre-digestion solution