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

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October 26, 2018
Alisha DSouza, Ph.D.
Senior Science Editor
JoVE

Re: JoVE59052
"Analyzing Oxygen Consumption Rate in Primary Cultured Mouse Neonatal Cardiomyocytes Using an Extracellular Flux Analyzer"

Dear Alisha,
Please find the revision of our manuscript. We thank you and the reviewers for their thoughtful comments. We were pleased that the reviewers found the manuscript was well written and acknowledged that we have developed a detailed protocol to culture mouse neonatal cardiomyocytes and assess mitochondrial respiration in these cells using an extracellular flux analyzer. We have carefully considered all of the editorial and reviewers' comments and revised the manuscript to address these concerns. Please find below a summary of the modifications to the manuscript, and a detailed point-by-point response to the editorial and reviewers' comments.

We hope that with these revisions, the reviewers and editors will find our manuscript valuable for the readership of *JoVE*. Should you have any additional questions, please feel free to contact us.

Yours sincerely,

Yoshitake Cho, PhD

TITLE:

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

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KEYWORDS:

Mouse neonatal cardiomyocytes, oxygen consumption, extracellular flux analyzer, respiration, mitochondria, heart

SUMMARY:

The goal of this protocol is to illustrate how to use mouse neonatal cardiomyocytes as a model system to examine how various factors can alter oxygen consumption in the heart.

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.

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 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 hours) 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 for testing 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

1.1. 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 µm 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.

1.2. 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 µm filter and keep on ice until use. Make collagenase digestion solution on the day of experiment.

1.3. 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.

1.4. 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.

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

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

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

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

1.9. 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)

2.1. Autoclave scissors, forceps, and a Moria spoon to sterilize.

2.2. Perform all steps in the cell culture hood for sterility.

2.3. 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.

2.4. Prepare 20 mL of trypsin pre-digestion solution in a 50 mL sterile conical tube. Keep all solutions on ice.

2.5. Quickly dip newborn (day 0) mice in 70% ethanol solution for sterilization.

2.6. 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].

2.7. 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**).

2.8. Remove any residual lung tissue, larger vessels, *etc.* (and atria, if desired). Wash hearts in the HBSS solution using gentle agitation.

2.9. 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**).

2.10. 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.

2.11. 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 hours (**Figure 1E**).

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

3.1. 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)

3.2. 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.

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

3.4. Aspirate any residual coating solution before seeding cardiomyocytes.

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

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

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

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

4.3. 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).

4.4. 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).

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

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

4.7. 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.

4.8. 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.

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

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

4.11. 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.

4.12. Place a sterile cell-strainer (100 µm 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**)

4.13. Centrifuge conical tube containing cardiomyocytes for 5 min at 180 x 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**).

4.14. Gently resuspend the cells and plate cells onto a 10 cm cell culture dish (plastic without any type of coating) and incubate for 1h 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**).

4.15. After the 1 h incubation, gently agitate the plate, wash non-adherent cells (enriched in cardiomyocytes) from the 10 cm culture dish, and re-suspend 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 1h 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**).

4.16. 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)

5.1. Count the cells using a hemocytometer.

5.2. Plate the cells into an extracellular matrix coated 96-well cell culture plate at a density between 10 - 30K (10 - 30 x 10^3) cells/well by using a multi-channel 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 10K, 20K, or 30K 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 16-24 h 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.

6.1. 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.

6.2. 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.

6.3. 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.

6.4. 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**.

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

6.6. 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.

6.7. 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.

6.8. 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.

6.9. 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 x 10^5 cells/pup were obtained, and cardiomyocytes were seeded at densities of 10K, 20K, or 30K 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 30K 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 10K ($1x10^4$) cells. OCR per 10K 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. However, short term (3 h) serum starvation decreases OCR. The information is useful for testing mouse neonatal cardiomyocytes OCR with various experimental conditions.

FIGURE AND TABLE LEGENDS:

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 4h 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. 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 10K cells/well. (C) Cardiomyocytes were stained with anti-sarcomeric α-actinin antibody (green) and DAPI (as a nuclear stain) (blue). 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 10K cells. **(C)** OCR is expressed relative to basal respiration.

Table 1. Reagents and solutions.

Table 2. Injection mixtures.

Table 3: Extracellular flux analyzer program.

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 HBSS, 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[22-24] . 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 10K, 20K, and 30K cells/well, was similar. Given that plating 30K cells/well produced a confluent well within one day after seeding, we recommend splitting cells between 10K to 30K cells/well in a 96-well plate, for the OCR assay. Interestingly we found 3h 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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DISCLOSURES:

The authors declare that they have no competing financial interests.

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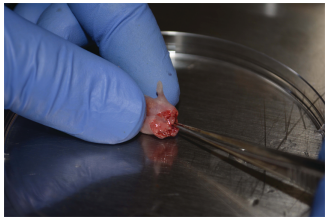

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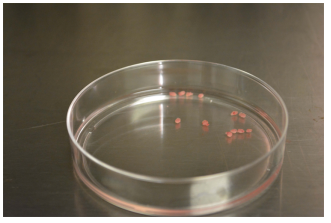

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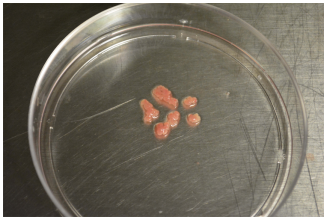

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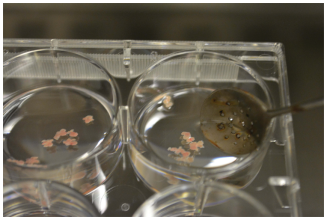

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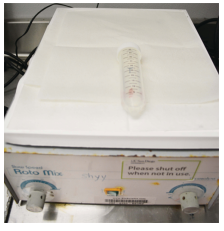

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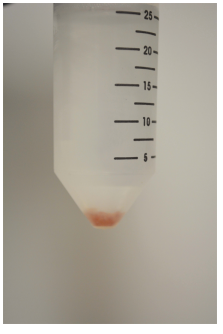

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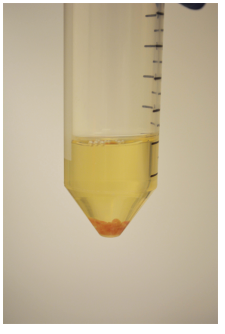

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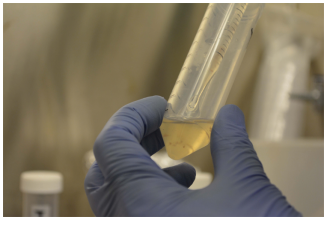

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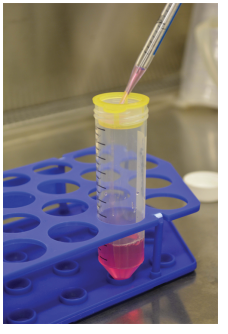

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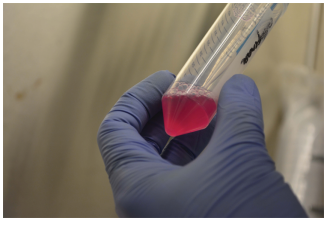

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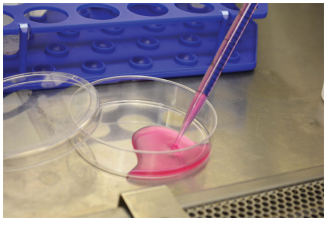

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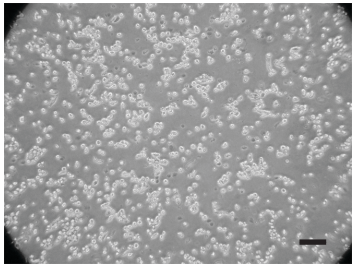

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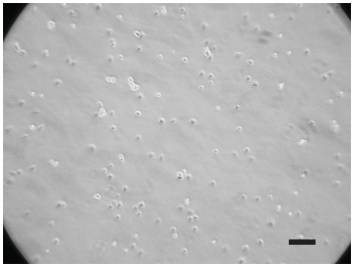

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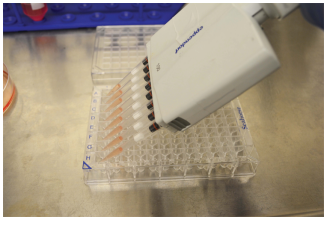

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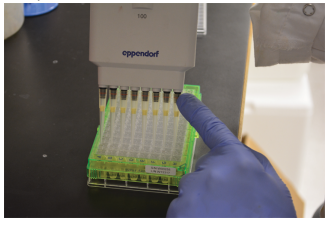

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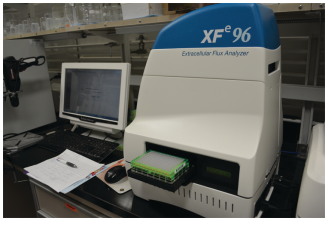
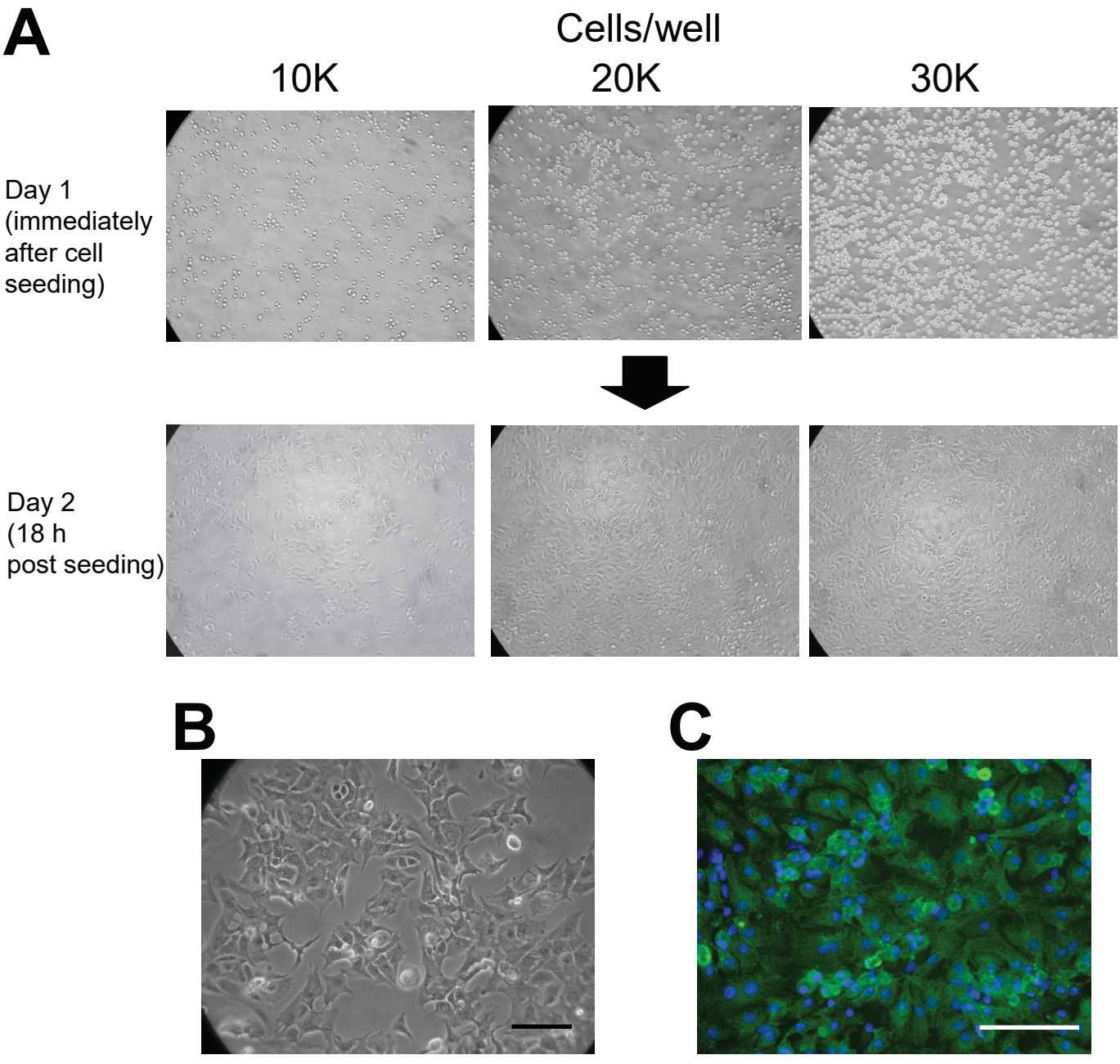
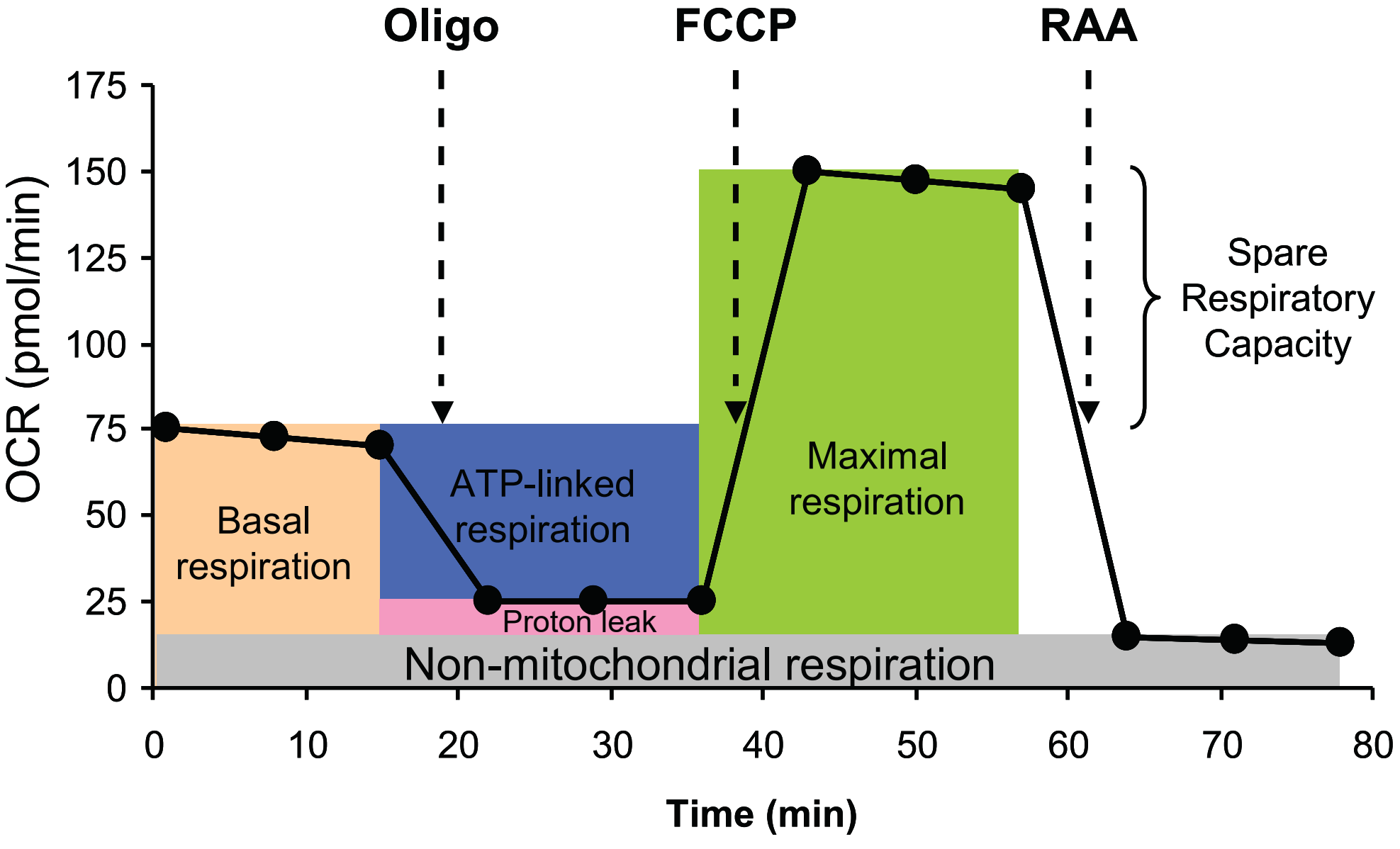



A
Cells/well
10K
20K
30K
Day 1
(immediately
after cell
seeding)
Day 2
(18 h
post seeding)
B
C


Oligo
FCCP
RAA
OCR (pmol/min)
175
150
125
100
75
50
25
0
Basal respiration
ATP-linked respiration
Proton leak
Maximal respiration
Spare Respiratory Capacity
Non-mitochondrial respiration
0
10
20
30
40
50
60
70
80
Time (min)

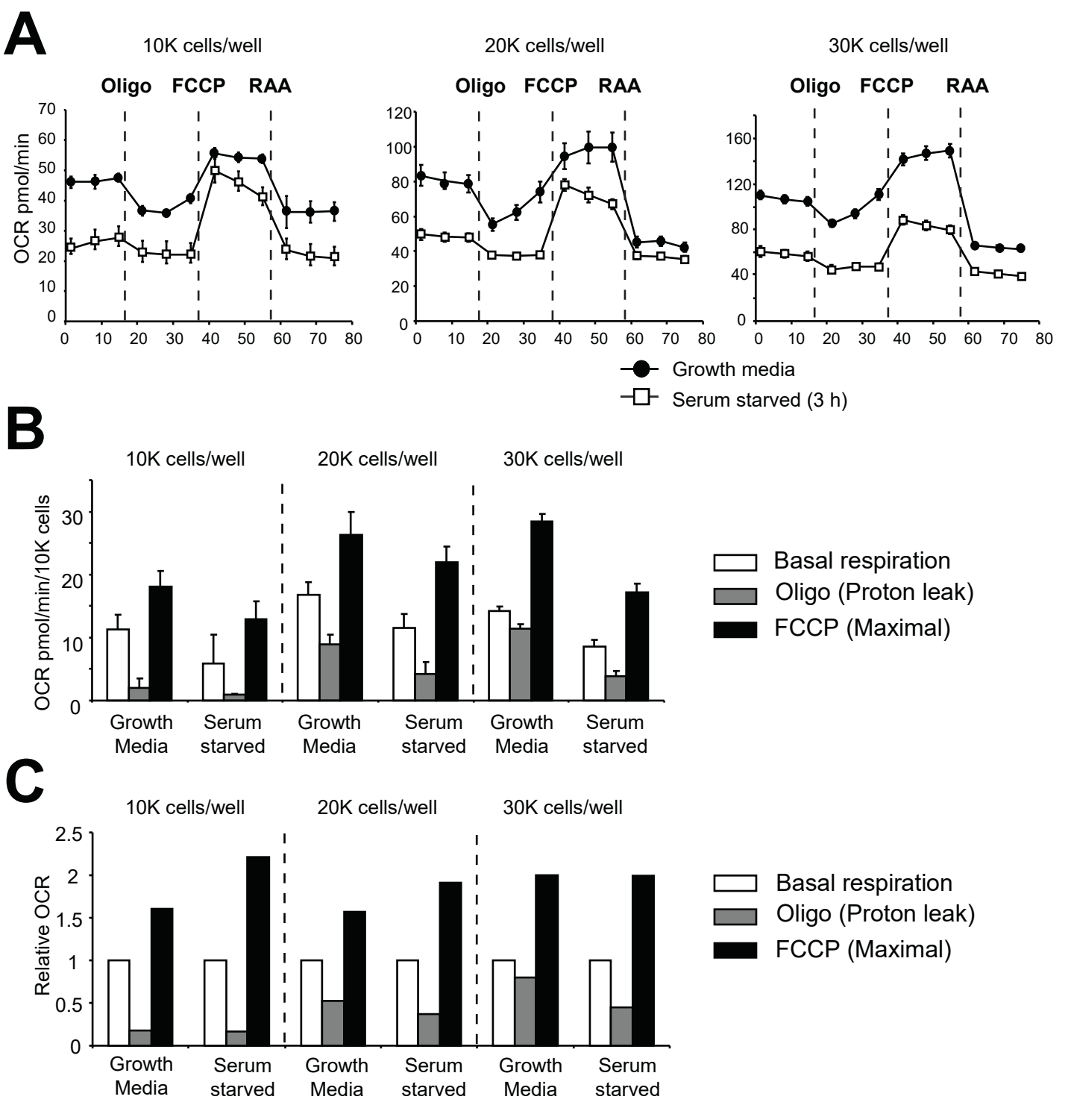

A
10K cells/well
20K cells/well
30K cells/well
Oligo
FCCP
RAA
OCR pmol/min
Growth media
Serum starved (3 h)
B
10K cells/well
20K cells/well
30K cells/well
OCR pmol/min/10K cells
Basal respiration
Oligo (Proton leak)
FCCP (Maximal)
Growth Media
Serum starved
C
10K cells/well
20K cells/well
30K cells/well
Relative OCR
Basal respiration
Oligo (Proton leak)
FCCP (Maximal)
Growth Media
Serum starved

Table 1

Name of reagent and solution	Preparation
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.
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.
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.
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).
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.
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.
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.
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.
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.

Notes
Make pre-digestion solution on the day of experiment.
Make collagenase digestion solution on the day of experiment.
Cardiomyocyte culture media can be made prior to the experiment and be stored at 4 °C for a month.
Final concentration for supplements are following: sodium pyruvate (1 mM), L-glutamine (2 mM), glucose (10 mM), and Hepes (2 mM)
On the day of assay, take one aliquot to use. Avoid many freeze-thaw cycles.
On the day of assay, take one aliquot to use. Avoid many freeze-thaw cycles.
On the day of assay, take one aliquot to use. Avoid many freeze-thaw cycles.
On the day of assay, take one aliquot to use. Avoid many freeze-thaw cycles.
0.5% gelatin solution can be stored at room temperature for up to 2 months.

Table 2

Injection Port	Compounds and concentration	Volume injected during run (µl)
A	16 µM oligomycin	25
B	9 µM FCCP	25
C	20 µM rotenone (Rot) and 20 µM antimycin A (AA)	25

Final Concentration in the assay
2 µM
1 µM
2 µM of each

Table 3 Click here to access/download;Table;Table 3_103018.xlsx

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

Name of Material/ Equipment	Company	Catalog Number
Antimycin A	SIGMA	A8674
Cell strainer 100 µm pores	FALCON	352360
Collagenase type II	Worthington	LS004176
D-Glucose	SIGMA	75351
DMEM high glucose	Life technologies	11965-092
DMEM without NaHCO3, Glucose, pyruvate, glumanine, and Hepes	SIGMA	D5030-10X1L
FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone)	SIGMA	C2920
Fetal bovine serum (FBS)	Life technologies	26140-079
Fibronectin from bovine plasma	SIGMA	F1141-5MG
Fine scissors	Fine Sciences Tools	14060-10
Gelatin from porcine skin	SIGMA	G-1890
HBSS (Hank's balnced salt solution,without Ca2+, Mg2+)	Cellgro	21-022-CV
HEPES (1M)	Fisher scientific	15630080
Horse serum	Life technologies	26050-088
L-Glutamine	SIGMA	G-3126
M-199	Cellgro	10-060-CV
Moria spoon	Fisher scientific	NC9190356
Oligomycin	SIGMA	75351-5MG
RIPA buffer	Fisher scientific	89900
Rotenone	SIGMA	R8875
Seahorse XFe96 Extracellular Flux Analyzer	Agilent	

Seahorse XFe96 FluxPak	Agilent	102601-100
Sodium pyruvate	SIGMA	P2256
Straight scissors	Fine Sciences Tools	91401-12
Syringe filter 0.2 µm size		
Trypsin	USB Corporation	22715 25GM

Comments/Description / Use
Inhibits complex III of the mitochondria
To capture undigested tissue
To make collagenase digestion solution
To make mitochodnrial stress test medium
To make cell culture medium
To make mitochodnrial stress test medium
Uncouples mitochondrial respiration
To make cell culture medium
To make coating solution for tissue culture plates
For dissection of hearts
To make coating solution for tissue culture plates
To wash hearts and make pre-digestion and collagnase digestion solution
To make mitochodnrial stress test medium
To make cell culture medium
To make mitochodnrial stress test medium
To make cell culture medium
To wash hearts
Inhibits mitochondrial ATP synthase
To lyse the cells for protein assay
Inhibits complex I of the mitochondria
Device used to analyze oxygen consumption rate

Package of flux analyzer culture plates, sensor cartridges, and calibrant
To make mitochodnrial stress test medium
For dissection of hearts
For sterile filtration of digestion medium
To make pre-digestion solution

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Author(s): Tachibana S, Chen C, Zhang O, Schurr S, Hill C, Li R, Manso A, Zhang J, Andreyev A, Murphy A, Ross R, Cho Y

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CORRESPONDING AUTHOR:

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Article Title: Analyzing Oxygen Consumption Rate in Primary Cultured Mouse Neonatal cardiomyocytes Using an Extracellular Flux Analyzer

Signature: 

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MS ID: JoVE59052
MS TITLE: Analyzing Oxygen Consumption Rate in Primary Cultured Mouse Neonatal Cardiomyocytes Using an Extracellular Flux Analyzer

We would like to thank editors' thoughtful comments. We have carefully considered all editorial comments, performed some additional analysis address some of the concerns, and revised the manuscript to address the issues that were raised. Please find below a summary of the modifications to the manuscript and a detailed point-by-point response to the editorial comments.

Summary of modifications: (added figures and tables)
1) Added new Table 1, showing how to make reagents and buffers, which are used in the protocol. This information is useful for readers to reproduce the procedure and experiments.

2) Added Figure 2C, presenting positive immunostaining of cardiomyocyte specific maker sarcomeric α-Actinin in isolated neonatal cardiomyocytes. (to respond Reviewer's concern)

3) The protocol is highlighted for identifying the essential steps of the protocol for the video.

Point-by-point response to the editorial comments (editorial comments are in italics):

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
A. We have proofread and corrected all spelling and grammar issues.

2. Please revise lines 88-90, 170-171, 248-249 (Table 2), 282-284 to avoid previously published text.
A. The manuscript has been revised to avoid previously published text in the above mentioned lines. The new lines of the revised manuscript are lines 97-98, 181-182, Table 3, and 299-300.

3. Please provide an email address for each author.
A. We provided email addresses in our manuscript. Lines 21-26.

4. Please replace "XFe96", which is commercial language, with another keyword.
A. We have replaced "XFe96" with "96-well format extracellular flux analyzer".

5. Please expand the Summary to briefly describe the applications of this protocol.
A. The manuscript has been revised as suggested. Please see lines 428-436.

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materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: XFe96, Eppendorf, Wave, etc.
A. We have corrected all commercial language accordingly.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
A. The protocol has been revised as suggested.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
A. The protocol has been revised as suggested.

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
A. The protocol has been revised as suggested.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
A. The protocol has been revised as suggested. Please see the text.

11. Lines 122-128: Listing an approximate volume to prepare would be helpful.
A. An approximate volume is added to the protocol.

12. Line 140: Are the mice anesthetized before this procedure? If so, please mention how they are anesthetized and how proper anesthetization is confirmed.
A. The mice are not anesthetized. According to NIH and American Veterinary Medical Association guidelines, decapitation alone for neonatal mouse pups < 7 days of age, is an acceptable means of euthanasia.

13. Line 148: What is used to cut?
A. The tool has been added to the text.

14. Line 154: What is the concentration of trypsin?
A. The concentration has been added.

15. Line 158: Please list an approximate volume to prepare.
A. A volume has been added to the text.

16. Line 256: Please provide composition of RIPA cell lysis solution. If it is purchased, please cite the Table of Materials.
A. The source of RIPA solution has been added to the text.

17. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
A. The protocol has been revised as suggested.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.
A. The manuscript has been revised as suggested.

19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
A. The essential steps in the protocol are highlighted.

20. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.
A. The protocol has been highlighted as suggested.

21. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
A. The details have been provided as suggested.

22. Figures 2 and 4: Please change "hrs" to "h" for time unit.
A. The manuscript has been revised as suggested.

23. Figure 3: Please define RAA in the figure legend.
A. The manuscript is revised.

24. Table 1: Please change the unit "µl" to "µL".
A. The manuscript has been revised as suggested.

25. Please remove the embedded table(s) from the manuscript. Each table must be accompanied by a title/description after the Representative Results of the manuscript text.
A. The manuscript has been revised as suggested.

26. Discussion: Please discuss any limitations of the technique.
A. The manuscript has been revised as suggested. Please see lines 437-443.

27. References: Please do not abbreviate journal titles.
A. The references have been revised as suggested.

MS ID: JoVE59052
MS TITLE: Analyzing Oxygen Consumption Rate in Primary Cultured Mouse Neonatal Cardiomyocytes Using an Extracellular Flux Analyzer

We were pleased that the reviewers found the manuscript was well written and acknowledged that we have developed a detailed protocol to culture mouse neonatal cardiomyocytes and assess mitochondrial respiration in these cells using an extracellular flux analyzer. We have carefully considered all their comments, performed some additional analysis address some of the concerns, and revised the manuscript to address the issues that were raised. Please find below a summary of the modifications to the manuscript and a detailed point-by-point response to the reviewers' comments.

Summary of modifications: (added figures and tables)
1) Added new Table 1, showing how to make reagents and buffers.

2) Added Figure 2C, presenting positive immunostaining of cardiomyocyte specific maker sarcomeric α-Actinin in isolated neonatal cardiomyocytes.

Point-by-point response to the reviewer's comments (reviewer's comments are in italics):

Reviewer #1:

Manuscript Summary:
The authors have developed a detailed protocol to culture mouse neonatal cardiomyocytes and assess mitochondrial respiration in these cells using an extracellular flux analyzer. They have conducted various experiments to show the potential use of these cells for research purposes

Major Concerns:
1] Authors need to discuss how this protocol is different from already published protocols.
We appreciate reviewer's comments. Please see our revised discussion in lines 383-390.

2] *Authors mention that these cells have characteristics of cardiomyocytes. They need to characterize these cells using cardiomyocyte specific antibodies.*
A. We have immunostained the cells with an antibody against sarcomeric α-Actinin, which is a cardiomyocyte-specific marker. As shown in Figure 2C, almost all cells show positive immunostaining for sarcomeric α-Actinin. These results demonstrate that the majority of isolated cells are cardiomyocytes. Please see lines 288-291, 354-357, and Figure 2C.

Minor Concerns:
3] Authors have used same determinants for maximal and basal respiration in the result section for figure 4. They need to correct that.
A. We would like to thank the reviewer for this important comment. The manuscript has been revised accordingly. Please see line 314.

Reviewer #2:
Manuscript Summary:
The manuscript by Tachibana et al., describes the protocol for rapid isolation of neonatal mouse cardiomyocytes and assess their mitochondrial function via oxygen consumption rate of these cardiomyocytes using an extracellular flux analyzer 96 (XFe96). The manuscript was well written and easy to follow. The reviewer has no concerns on the manuscript.