

Video Article

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

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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 ATP¹. 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-myocytes⁹, cardiomyocyte cultures are extremely pure, with minimal contamination of non-myocytes from the heart, such as fibroblasts and endothelial cells¹⁰. 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 cardiomyocytes¹⁰. 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 variability¹⁶. 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 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.
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.
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, filter 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 guidelines¹⁷.
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 hours (**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 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 (1st 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 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)
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).
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).
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).
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 - 30K ($10 - 30 \times 10^3$) 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.

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 CO₂ or O₂ 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 CO₂ or O₂ 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 μ L 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 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 spread out) (**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 marker¹⁸ 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 (1×10^4) 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.

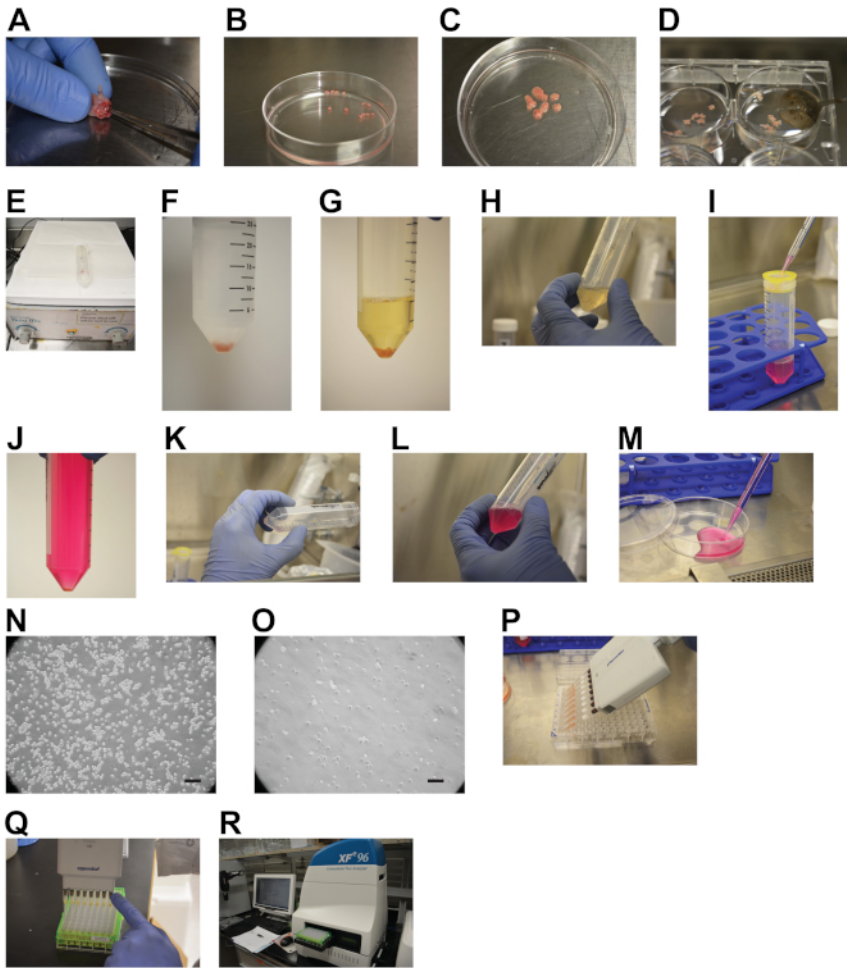


Figure 1. Isolation and culture of neonatal cardiomyocytes from day 0 newborn mice. (A) Neonates are euthanized, and the heart is dissected from the thorax. (B) Hearts are washed in HBSS (without Ca^{2+} , Mg^{2+}). (C) Each heart is cut into 8 pieces. (D) Hearts are moved to a 6-well plate filled with 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. [Please click here to view a larger version of this figure.](#)

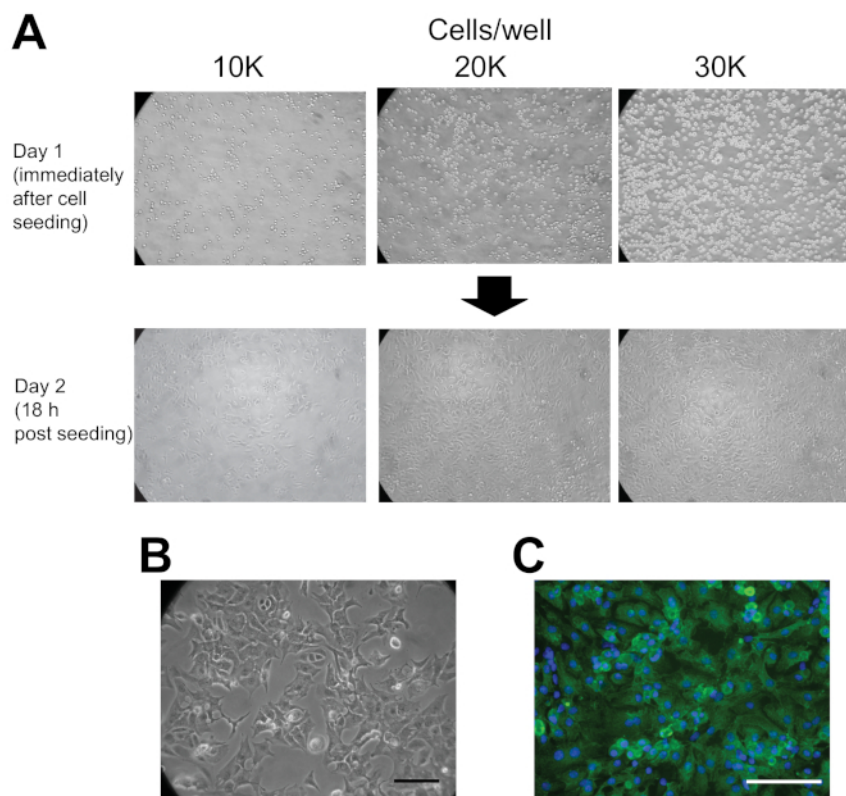


Figure 2. Culture of cardiomyocytes on extracellular flux analyzer 96-well plate. (A) Representative images of cardiomyocytes in 96-well plates with indicated cell densities, immediately after seeding (upper row) or 18 h post seeding (lower row). (B) A higher magnification image of cardiomyocytes 18 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 publication¹⁸. [Please click here to view a larger version of this figure.](#)

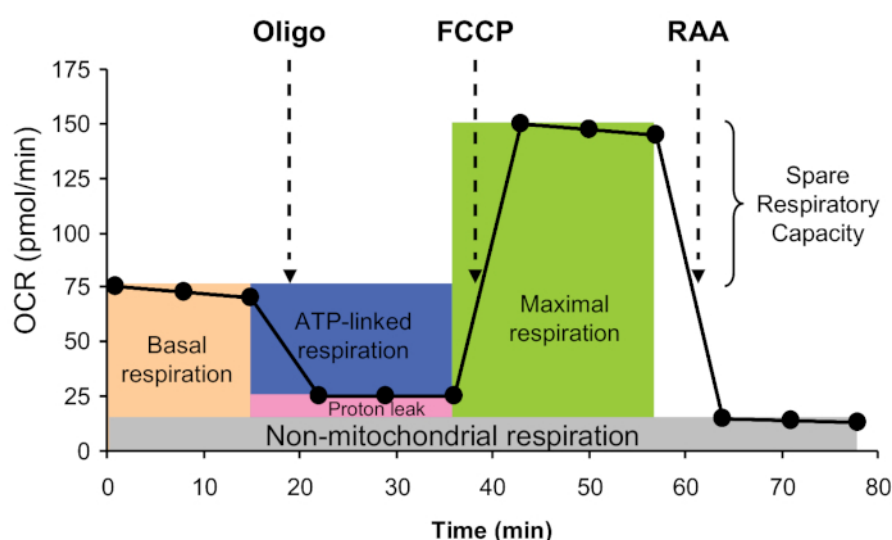


Figure 3. Schematic representation of mitochondrial stress test. Bioenergetic parameters, including Basal, ATP-linked, Maximal, and Non-mitochondrial respiration as well as Spare respiratory capacity and Proton leak, are outlined on the trace with corresponding mitochondrial effectors. [Please click here to view a larger version of this figure.](#)

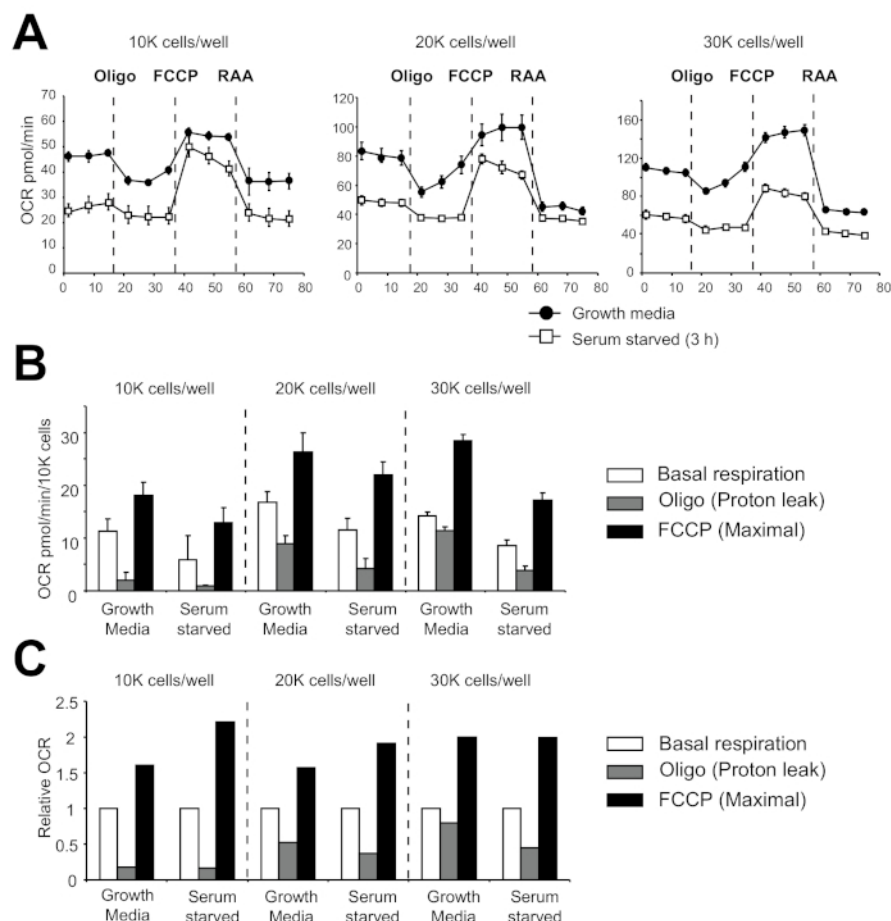


Figure 4. Mitochondrial stress assay. (A) Representative tracing of oxygen consumption rates (OCR, in pMoles/min) of neonatal mouse cardiomyocytes. At the indicated times, oligomycin (Oligo, 1 μ M), FCCP (800 nM), and RAA (rotenone and antimycin A, 1 μ M each) were injected. For each measurement, the 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. [Please click here to view a larger version of this figure.](#)

Name of reagent and solution	Preparation	Notes
Trypsin pre-digestion solution	Dissolve trypsin in HBSS (without Ca ²⁺ and Mg ²⁺). 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 Ca ²⁺ and Mg ²⁺). 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 <u>without</u> 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 1. Reagents and solutions.

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 2. Injection mixtures.

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

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 studies¹⁶. 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 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 consumption²⁷. It was also reported that growth factors enhance overall cellular activity and contraction of cardiomyocytes^{3,28}. Cardiomyocyte contraction requires ATP generation², 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 cardiomyocytes²⁹. Thus, by combining our protocol with genetically manipulated neonatal cardiomyocytes, such as ones that have mitochondrial mutations³⁰, 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 functions³¹, including expression levels of glucose and fatty acid metabolic enzymes³². 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.

Disclosures

The authors declare that they have no competing financial interests.

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