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Corresponding Author:	Matthew Barajas Vanderbilt University Medical Center Nashville, TN UNITED STATES
Corresponding Author's Institution:	Vanderbilt University Medical Center
Corresponding Author E-Mail:	matthew.b.barajas@vumc.org
Order of Authors:	Matthew Barajas
	Richard Levy
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TITLE:

Modified Technique for the Use of Neonatal Murine Hearts in the Langendorff Preparation

AUTHORS AND AFFILIATIONS:

Matthew B. Barajas[1]*, Richard J. Levy[2]

[1]Department of Anesthesiology, Vanderbilt University Medical Center, Nashville, TN, USA
[2]Department of Anesthesiology, Columbia University Medical Center, New York, NY, USA

Email addresses of the authors:
Matthew Barajas (Matthew.b.barajas@vumc.org)
Richard Levy (rl2740@cumc.columbia.edu)

*Email address of the corresponding author:
Matthew Barajas (Matthew.b.barajas@vumc.org)

KEYWORDS:

Aorta, cannulation, *ex-vivo*, force, heart, ischemia-reperfusion, isolated, Langendorff, mouse, newborn, retrograde perfusion, ventricular

SUMMARY:

The present protocol describes aortic cannulation and retrograde perfusion of the *ex-vivo* neonatal murine heart. A two-person strategy, using a dissecting microscope and a blunted small gauge needle, permits reliable cannulation. Quantification of longitudinal contractile tension is achieved using a force transducer connected to the apex of the left ventricle.

ABSTRACT:

The use of the *ex-vivo* retrograde perfused heart has long been a cornerstone of ischemia-reperfusion investigation since its development by Oskar Langendorff over a century ago. Although this technique has been applied to mice over the last 25 years, its use in this species has been limited to adult animals. Development of a successful method to consistently cannulate the neonatal murine aorta would allow for the systematic study of the isolated retrograde perfused heart during a critical period of cardiac development in a genetically modifiable and low-cost species. Modification of the Langendorff preparation enables cannulation and establishment of reperfusion in the neonatal murine heart while minimizing ischemic time. Optimization requires a two-person technique to permit successful cannulation of the newborn mouse aorta using a dissecting microscope and a modified commercially available needle. The use of this approach will reliably establish retrograde perfusion within 3 min. Because the fragility of the neonatal mouse heart and ventricular cavity size prevents direct measurement of intraventricular pressure generated using a balloon, use of a force transducer connected by a suture to the apex of the left ventricle to quantify longitudinal contractile tension is necessary. This method allows investigators to successfully establish an isolated constant-flow retrograde-perfused newborn murine heart preparation, permitting the study of developmental cardiac biology in an *ex-vivo* manner. Importantly, this model will be a powerful tool to investigate the

physiological and pharmacological responses to ischemia-reperfusion in the neonatal heart.

INTRODUCTION:

Ex-vivo heart preparations have been a staple of physiologic, pathophysiologic, and pharmacologic studies for over a century. Stemming from the work of Elias Cyon in the 1860s, Oskar Langendorff adapted the isolated frog model for retrograde perfusion, pressurizing the aortic root to provide coronary flow with an oxygenated perfusate[1]. Using his adaptation, Langendorff was able to demonstrate a correlation between coronary circulation and mechanical function[2]. The *ex-vivo* retrograde perfused heart, later eponymously dubbed the Langendorff technique, has remained a cornerstone of physiologic investigation, leveraging its simplicity to powerfully study the isolated heart in the absence of potential confounders. The Langendorff preparation has been modified further to permit the heart to eject (the so-called "working heart") and allow the perfusate to recirculate[3]. However, the primary physiologic endpoints of interest have remained unchanged. Such endpoints include measures of contractile function, electrical conduction, cardiac metabolism, and coronary resistance[4].

To evaluate cardiac function in his original frog heart preparation, Langendorff measured the tension generated by ventricular contraction in the longitudinal axis using a suture connected between the heart's apex and a force transducer.[5] Isometric contraction was quantified in this manner with basal tension applied to the heart in the absence of ventricular filling. Refinement of the approach has led to fluid-filled balloons placed into the left ventricle *via* the left atrium to evaluate myocardial performance during isovolumic contraction[6]. To assess cardiac rhythm and the heart rate, surface leads can be placed on the poles of the heart to enable investigators to record the electrocardiogram. However, relative bradycardia can be expected, given the obligatory denervation. Extrinsic pacing may serve to overcome this and eliminate heart rate variability between experiments[1]. Another outcome measure, myocardial metabolism, can be assessed by measuring the oxygen and metabolic substrate content in the coronary perfusate and effluent and calculating the difference between them[7]. Lactate quantification in the coronary effluent can aid in characterizing periods of anaerobic metabolism as is seen with hypoxia, hypoperfusion, ischemia-reperfusion, or metabolic perturbations[7].

Langendorff's original work enabled the study of the *ex-vivo* mammalian heart, using cats as the primary subject[5]. Evaluation of the isolated rat heart gained popularity in the mid-1900s with Howard Morgan, who detailed the 'working heart' rat model in 1967[5]. The use of mice began only 25 years ago due to the technical complexity, tissue fragility, and relatively small murine heart size. Despite the challenges associated with mice study, the lower costs and ease of genetic manipulation have increased the appeal and demand of such murine *ex-vivo* preparations. Unfortunately, the application of the technique has been limited to adult animals, with juvenile 4-week-old mice being the youngest subjects utilized for *ex-vivo* study until quite recently[8,9]. While juvenile mice are "relatively immature" compared with adults, their utility as subjects for developmental biology studies is limited because they have, by and large, weaned from their birth dam and will soon begin puberty[10]. Adolescence occurs well beyond the postnatal transition in myocardial substrate utilization from glucose and lactate to fatty acids[11]. Thus, most information about the metabolic changes in the neonatal heart has historically resulted from *ex-*

vivo work in larger species such as rabbits and guinea pig[11].

Indeed, alternative approaches to the Langendorff preparation exist. These include *in vitro* experimentation, which lacks the whole organ functional data and context, or *in vivo* studies. This can be technically challenging and complicated by confounding variables such as the cardiovascular and respiratory effects of a requisite anesthetic agent, the influence of neurohumoral input, the consequences of core temperature, the nutritional status of the animal, and substrate availability[12,13]. Because the Langendorff approach permits the study of the isolated-perfused heart in an *ex-vivo* manner in a more controlled manner in the absence of such confounders, it has been and continues to be considered a powerful investigational tool. Therefore, the technique presented here gives researchers an experimental approach for the *ex-vivo* study of the newborn murine heart and limits time to reperfusion.

Investigating the heart during periods of development is an important consideration given the wide-ranging biochemical, physiologic, and anatomical transitions that occur during myocardial maturation. Shifts from anaerobic metabolism to oxidative phosphorylation, changes in substrate utilization, and progression from cell proliferation to hypertrophy are dynamic processes that uniquely occur in the immature heart[11,14]. Another critical aspect of the developing heart is that stressors encountered during necessary periods may produce heightened responses in the newborn heart and alter future susceptibility to insults in adulthood[15]. Although prior work has utilized newborn rats, lambs, and rabbits to study the Langendorff-perfused neonatal heart, advances permitting mice use are necessary given the importance of this species to developmental biology research[16]. To address this need, the first murine Langendorff-perfused newborn heart model using 10-day old animals was recently established[6]. Presented here is a method to enable successful aortic cannulation and establish retrograde perfusion of the isolated newborn murine heart. This approach may be utilized for pharmacology, ischemia-reperfusion, or metabolism studies focusing on whole organ function or can be adapted for the isolation of cardiomyocytes.

PROTOCOL:

Institutional Animal Care and Use Committee of Columbia University Medical Center's approvals were obtained for all methods described. Wild-type C57Bl/6 male postnatal day 10 mice were used for the study.

1. Preparation of Langendorff apparatus

1.1. To minimize complexity, use non-recirculating oxygenated perfusate within the Langendorff apparatus (see **Table of Materials**) *via* constant flow or constant pressure.

1.1.1. Use Krebs-Henseleit buffer (KHB), containing 120 mmol/L of NaCl, 4.7 mmol/L of KCl, 1.2 mmol/L of $MgSO_4$, 1.2 mmol/L of KH_2PO_4, 1.25 mmol/L of $CaCl_2$, 25 mmol/L of $NaHCO_3$, and 11 mmol/L of glucose at pH 7.4 (see **Table of Materials**), equilibrate with 95% of O_2 and 5% of CO_2 within the Langendorff apparatus and maintain at 37 °C.

1.2. For the constant flow approach, maintain a continuous flow rate at ~2.5 mL·min^{-1}.

NOTE: This flow rate will approximate coronary flow of ~75-80 mL/g·min, given that the average weight of a 10 day old (P10) mouse heart is ~30 mg[17,18].

2. Fabrication of aortic cannula

2.1. Fabricate the newborn mouse aortic cannula from a 26 G stainless steel needle (see **Table of Materials**). Using sharp scissors, cut off the tip of the needle to blunt the end. Take care not to crimp or restrict the diameter of the needle lumen. Smooth the cut edge and remove any burs by gently scraping the blunted end on the laboratory benchtop using a to-and-fro motion.

NOTE: Microscopic burs and sharp edges must be removed because they can tear the newborn mouse aorta and damage the aortic valve. Alternatively, use fine-grit sandpaper.

2.2. Attach the fabricated cannula to the Langendorff apparatus and assess flow and resistance. Measure flow rates through the cannula by collecting and measuring buffer quantity over a known time period. Ensure actual flow is equal to the set flow rate of 2.5 mL min^{-1}.

2.3. Quantify the pressure differential across the cannula with KHB flowing by following the steps below.

2.3.1. Measure pressure in the system with and without the fabricated cannula attached.

2.3.2. Divide pressure differential across cannula by the flow rate to obtain cannula resistance as per Ohm's law[15].

2.3.3. Ensure that the fabricated cannula resistance is ~16.0 ± 1.9 mmHg·min·mL^{-1} of the resistance[6]. Excessive resistance suggests a potentially compromised cannula lumen.

NOTE: Sample calculation: $P_{\text{with cannula}} - P_{\text{without cannula}} = \Delta P$. If $P_{\text{with}} = 48$ and $P_{\text{without}} = 8$ then $\Delta P = 40$. At a flow rate (Q) of 2.5 mL min^{-1} and ΔP of 40 cannula resistance equals 16 mmHg·min·mL^{-1} using $R = \Delta P/Q$ = 40 / 2.5 = 16.

2.4. Remove the 26 G cannula and attach the high-pressure tubing (see **Table of Materials**) to the cannulation site on the Langendorff apparatus. Attach the aortic cannula to the distal end of the tubing. De-air the tubing and the cannula with oxygenated buffer, ensuring that all bubbles are removed.

NOTE: The use of high-pressure tubing in this manner permits the cannula to be extended to a more remote position. This is necessary to allow aortic cannulation with a dissecting microscope adjacent to the setup (**Figure 1**).

3. Organ harvesting

3.1. Anticoagulate mice *via* intraperitoneal (IP) injection of heparin (10 kU/kg) (see **Table of Materials**) to prevent the formation of coronary microthrombi using a 26 G needle on 1 mL syringe. Allow several minutes for heparin to circulate before proceeding with the injection of any anesthetic.

3.2. Anesthetize the animal with an IP injection using a 26 G needle on 1 mL syringe.

NOTE: It is essential to carefully monitor the animal after anesthetic injection to avoid apnea and subsequent hypoxia. Pentobarbital (70 mg/kg) is a reliable choice of anesthetic, as it allows for rapid onset of sedation without inducing apnea[19,20]. Other anesthetic agents can be utilized, provided that the doses used do not cause apnea[21]. Investigators should consider the effects of alternative sedative-hypnotics on cardiac function[22,23]. Cervical dislocation as a primary mode of euthanasia may prolong pre-cannulation hypoxia and ischemia.

3.3. Place the mouse in the supine position and secure limbs immediately upon loss of consciousness. Begin harvesting as soon as the animal is unresponsive to toe pinch; the animal should breathe spontaneously during the initial dissection.

3.4. Make a transverse subxiphoid incision across the animal's width to expose the abdominal cavity using straight dissecting scissors (see **Table of Materials**).

NOTE: Sterile technique is not necessary given that the procedure represents nonsurvival surgery.

3.4.1. Identify the diaphragm superiorly and incise the anterior portion completely. Cut the ribcage bilaterally along the mid-axillary line in a cephalad direction. Ask an assistant to grasp the xiphoid process with forceps and reflect the sternum and ribs cranially to expose the thoracic organs.

3.5. Identify the infra-diaphragmatic inferior vena cava (IVC) above the liver. Transect the IVC with a curved iris scissor while maintaining slight anterior and cephalad tension on the proximal segment with iris forceps (see **Table of Materials**).

3.5.1. Cut posteriorly along the anterior surface of the spine using curved iris scissors while pulling the IVC up and out of the thoracic cavity. As the heart is mobilized, angle the scissors anteriorly and severe the great vessels superiorly to completely remove the heart and lungs.

NOTE: This method permits rapid explantation of the heart and lungs *en bloc.*

3.6. Immediately submerge the specimen in ice-cold KHB or saline. The heart should stop beating within seconds.

4. Cannulation

4.1. Cut a piece of paper towel and place it at the bottom of a shallow Petri dish to provide friction to stabilize the heart during cannulation. Moisten with ice-cold KHB to prevent the heart from adhering to it.

4.1.1. Place the prepared Petri dish under the dissecting microscope and adjust the focus. Place the aortic cannula attached to the high-pressure extension tubing under the dissecting microscope along with a 5-0 silk suture loosely tied around its hub (see **Table of Materials**).

NOTE: Care must be taken to limit the amount of fluid in the Petri dish because the air-filled lungs can float and cause the excised organs to move.

4.2. Place the excised thoracic organs in the Petri dish. Under the microscope, identify the thymus by its white sheen and two lobes and orient the specimen such that the thymus is anterior and superior[24]. This will ensure proper orientation of the heart.

4.3. Using forceps, bluntly separate the lobes of the thymus to expose the great vessels. Identify the aorta by locating distinguishing branching features of the aortic arch.

NOTE: A dark purple hue often demarcates the right ventricle and the pulmonary artery. The ascending aorta is located between the main pulmonary artery and the right atrium.

4.4. Transect the aorta with fine sharp scissors (see **Table of Materials**) just proximal to the subclavian artery takeoff.

NOTE: If the aorta is transected too close to the aortic valve, there will not be enough aortic tissue to enable the cannula to be secured. Alternatively, if the aorta is transected too high, perfusate can leak out of one or more aortic branches (such as the subclavian artery).

4.5. Gently grasp the transected aorta using jeweler-style fine curved forceps (see **Table of Materials**). Carefully cannulate the aorta with a 26 G blunt needle, taking care not to damage the aortic valve. Hold in place by grasping the aorta with the fine curved forceps around the cannula. Once control of the aorta is established, initiate retrograde perfusion to limit the ischemic time.

NOTE: The heart should begin to beat and will become pale as blood is drained from the myocardium and KHB perfuses the coronary arteries. Failure to spontaneously beat, presence of ventricular engorgement, or lack of color change of the heart indicates a malpositioned cannula.

4.6. Ask the assistant to grasp the ends of the loosely tied suture and carefully ensnare the aorta around the cannula. Cinch the suture above or below the curved fine forceps (holding the cannula in place), depending on the amount of aortic tissue and anatomical considerations. Tighten the suture and confirm the adequacy of coronary flow.

4.7. Disconnect the high-pressure tubing from the Langendorff apparatus. Grasp the hub of

the cannula and disconnect the blunt needle from the high-pressure extension tubing. Rapidly attach the hub of the cannula to the apparatus.

NOTE: Care must be taken not to dislodge the heart or entrain air into the cannula.

4.8. Once the heart is hung on the Langendorff apparatus in the usual position, and adequate perfusion is confirmed, carefully trim off lung, thymus, and excess tissue. Incise the right atrium to permit coronary sinus effluent to drip freely.

5. Functional measurement

5.1. Make a small knot at the end of a 5-0 silk suture (attached to a curved needle). Pierce a small piece of paraffin film (2-3 mm x 2-3 mm) with the needle and slide the paraffin to the knotted end. Carefully pass the needle through the apex of the ventricle and pull the suture through the heart until the paraffin film is snug against the lateral wall of the ventricle.

NOTE: The paraffin film helps to prevent the knot from tearing the heart and pulling through the ventricle.

5.2. Pass the needle through the opening of the water-filled warming jacket of the Langendorff apparatus. The heart can now be encased and warmed.

5.3. Attach the needle to the force transducer (see **Table of Materials**) in such a manner that avoids the coronary sinus drip. Adjust the suture to apply 1-2 g of basal tension, as indicated by the diastolic tension or nadir in tension tracing.

NOTE: Avoid pulling the heart off the cannula or twisting the aorta, thereby compromising coronary perfusion.

5.4. Place surface electrodes on the superior and inferior poles of the heart to record the electrocardiogram.

NOTE: Use pediatric temporary epicardial pacing wire with the needle removed for flexible surface electrode connected to Bio Amp (see **Table of Materials**).

5.5. Sample the coronary sinus effluent for analysis using a 24 G IV catheter (see **Table of Materials**).

5.6. Subtract the cannula resistance from the total system resistance to obtain coronary resistance per Kirchhoff's law[25].

REPRESENTATIVE RESULTS:

P10 mice were used to model for a timepoint in human infancy[26,27]. Fifteen isolated C57Bl/6 newborn mouse hearts were harvested and cannulated successfully. Hearts were perfused with

a continuous flow of 2.5 mL min^{-1} of warmed oxygenated KHB. Metabolic parameters, including glucose extraction, oxygen consumption, lactate production, and physiological parameters such as heart rate, perfusion pressure, and coronary resistance, were measured. Surface electrodes were used to record a continuous electrocardiogram, which allowed determining intrinsic rate and rhythm (**Figure 2**). Contractile force in the longitudinal axis was determined using the method described by Langendorff [28].

The metabolic assessment was performed to assess for adequacy of perfusion. Percent oxygen extraction was calculated by subtracting oxygen content in coronary effluent from the perfusate. Myocardial oxygen consumption was determined by multiplying coronary flow rate by the difference in oxygen content between the perfusate and coronary effluent multiplied by the solubility of oxygen (assuming 24 µL/mL of H_2O at 37 °C and 760 mmHg)[29,30]. Using these calculations, it was determined that this perfusion strategy met the metabolic needs of the newborn mouse heart, given the negligible lactate production and low percent oxygen extraction and glucose consumption (**Table 1**).

All hearts beat spontaneously in sinus rhythm (**Figure 2**). As expected, however, the mean denervated intrinsic heart rate was slower than newborn murine heart rates reported *in vivo*[31]. Mean observed aortic perfusion pressures correlated well with the mean arterial pressures described in neonatal mice[32]. Other physiologic variable means were recorded and calculated (**Table 2**).

Based upon the observational data, exclusionary criteria to ensure consistency of the neonatal preparation needs to be considered (**Table 3**). A factor that is critical to the robustness of the preparation is the time required to initiate reperfusion. Cannulation is by far and away the most challenging step of the procedure, given the minuscule size and fragility of the neonatal mouse aorta. A prolonged delay in establishing cannulation or initiating reperfusion will injure the healthy heart or even precondition the myocardium[1]. Thus, minimizing the ischemic time to under 4 min is suggested (consistent with guidelines for the adult rodent heart)[1]. Following successful cannulation, assessment of the adequacy of perfusion is paramount. Signs of inadequate myocardial perfusion include prolonged arrhythmias, heart rate extremes, or aortic perfusion pressure extremes.

FIGURE AND TABLE LEGENDS:

Figure 1: Aortic cannulation setup. (**A**) The proximal end of high-pressure tubing is attached to the "usual" cannula position site (shown in B). The cannula is attached to the distal end of the tubing (magnified in C). (**B**) "Usual" cannula position in apparatus. (**C**) The cannula is attached to the "slip-tip" end of the high-pressure tubing for ease of removal.

Figure 2: The e*x-vivo* retrograde-perfused neonatal mouse heart. Image of a 10-day postnatal mouse heart after successful aortic cannulation with a 26 G blunt needle. Coronary effluent can be seen dripping from the heart through an incision in the right atrium. Stainless steel surface electrodes were placed at the poles to measure electrocardiogram continuously. Representative

ECG tracing is displayed on the right in green, demonstrating a sinus rhythm and rate of 194 beats min^{-1}. Not pictured is the suture connected between the apex of the heart and force transducer, allowing measurement of ventricular contractile force (waveform depicted in red on the right). Adapted with permission from Reference[6].

Table 1: Metabolic parameters of isolated perfused newborn murine hearts. Values are means ± SE. Affluent and coronary effluent was sampled, and PO_2 (partial pressure of oxygen), glucose, and lactate were measured. Glucose uptake, oxygen extraction, and consumption were calculated. The difference in affluent and effluent determines extraction. Consumption is calculated as coronary flow x (PaO_2 – PvO_2) x O_2 solubility at 760 mmHg (assuming 24 µL/mL of H_2O at 37 °C and 760 mmHg).

Table 2: Physiologic parameters of isolated perfused newborn murine hearts. Values are means ± SE. Coronary resistance was calculated based on the coronary flow rate of 2.5 mL min^{-1} and aortic pressure using Ohm's law. According to Kirchoff's law of resistance in series, aortic pressure was calculated as pressure above baseline resistance in the system. Heart rate was measured *via* the surface electrode, and contractile force was measured *via* a suture connecting the apex of the heart to a force transducer. This table has been reprinted with permission from Reference[6].

Table 3: Proposed exclusion criteria for neonatal murine heart Langendorff preparations. This table has been reprinted with permission from Reference[6].

DISCUSSION:

The present work describes successful aortic cannulation and retrograde perfusion in the isolated newborn mouse heart. Importantly, it allows researchers to overcome the barriers that young murine age and small heart size previously presented[8]. While not complex in design, the approach does require a significant degree of technical skill. Key steps that will inevitably challenge even the most technically proficient investigators will be cannulation of the aorta and securing the cannula in place. Difficulty with neonatal cannulation is not due solely to the small size of the aortic lumen. The relatively short length of the ascending aorta (aortic tissue between the aortic valve and right subclavian takeoff) may challenge investigators to precisely control the aortic cannula and necessitate careful coordination between teammates. Failure to appropriately position and secure the cannula within this region can ruin the preparation. For example, advancing the cannula too deep can damage the aortic valve or result in intraventricular cannulation. Placing the cannula too shallow within the aortic arch can lead to perfusate leakage out of one of the branches, such as the subclavian artery. Furthermore, forceful cannulation can tear the aorta. Such consequences of inartful cannulation will manifest with high flow rates or low perfusion pressures[1]. Alternatively, low flow rates or high perfusion pressures can indicate the presence of thrombi, air emboli, cannula occlusion, or coronary obstruction[1]. Arrhythmias, bradycardia, or tachycardia are all signs of inadequate perfusion regardless of etiology[1,33].

A common and straightforward perfusion strategy should initially be chosen; constant flow using buffered crystalloid perfusate with glucose as a substrate in a spontaneously beating heart[1]. Adaptations to this approach will need to be assessed in future work and should include an

assessment of the effect of different perfusion approaches and alternative perfusate and substrate strategies. While myocardial perfusion in this preparation was shown to be adequate for P10 hearts, the chosen flow rate might exceed the needs of the newborn heart. This is because the cardiac output in 10-day old mice is approximately 5.3 $mL.min^{-1}$ [31]. Thus, future work should investigate the effect of different flow rates and assess constant pressure strategies.

Constant pressure approaches may involve actual time flow adjustment mechanisms or a pop-off valve to limit maximal pressure[5]. This may be particularly important when studying ischemia-reperfusion injury, given the importance of evaluating coronary autoregulation in this context[5]. In addition, while intrinsic heart rate can be used as a biomarker for the adequacy of perfusion, pacing strategies are likely to be feasible and should be investigated in the future. Finally, future work should also assess alternative energy substrates in the oxygenated perfusate. This is because the newborn heart transitions from using glucose and lactate to consuming fatty acids in the neonatal period[11,14]. Thus, alternative metabolic substrates may be more physiologically relevant in this critical period of development.

Methodologic advances for evaluating murine cardiac function continue to emerge. Although the total number of research studies using the Langendorff preparation has remained consistent each year since the 1990s, the percentage of work utilizing murine-specific *ex-vivo* practices has steadily risen[5]. Thus, the importance of the isolated murine heart as a scientific model has increased over time. Innovations, such as the method described here, now permit the field to broaden the approach to the newborn mouse heart. In addition to its utility in ischemia-reperfusion research, such a method could also serve as an adjunct to other types of research techniques. For example, successful cannulation of the newborn mouse heart could facilitate cardiomyocyte isolation. To date, only 'chunk' digestion methods with lower yields have been available for isolating newborn mouse cardiomyocytes[34]. Therefore, the use of the neonatal Langendorff preparation with a retrograde infusion of enzymatic agents can improve the yield and quality of isolated cardiomyocytes[35].

The neonatal response to ischemic injury is not equal to that of the adult, and the immature heart undergoes several transitions during the newborn period [15,36]. However, a better understanding of the developmental biology of the neonatal heart in health and disease is necessary. The differential effects of hypoxia and ischemia and reperfusion between neonatal and hearts have been investigated since the 1970s. However, these prior works have been limited to the use of animal species larger than the mouse[37]. The ability to generate transgenic mutants to study specific pathways and proteins of interest necessitates establishing a newborn murine *ex-vivo* preparation. The method detailed here enables successful aortic cannulation to establish retrograde perfusion of the isolated newborn murine heart. Using this approach, investigators will be able to study ischemia-reperfusion as it relates to the neonatal mouse. Such research will help us better understand the neonatal-specific protective mechanisms during ischemia, the newborn response to hypoxia, and the anatomic and metabolic developmental changes in the immature heart during health and disease states[36,38,39]. Therefore, the isolated perfused newborn heart model will prove to be a powerful tool for developmental cardiac biology research.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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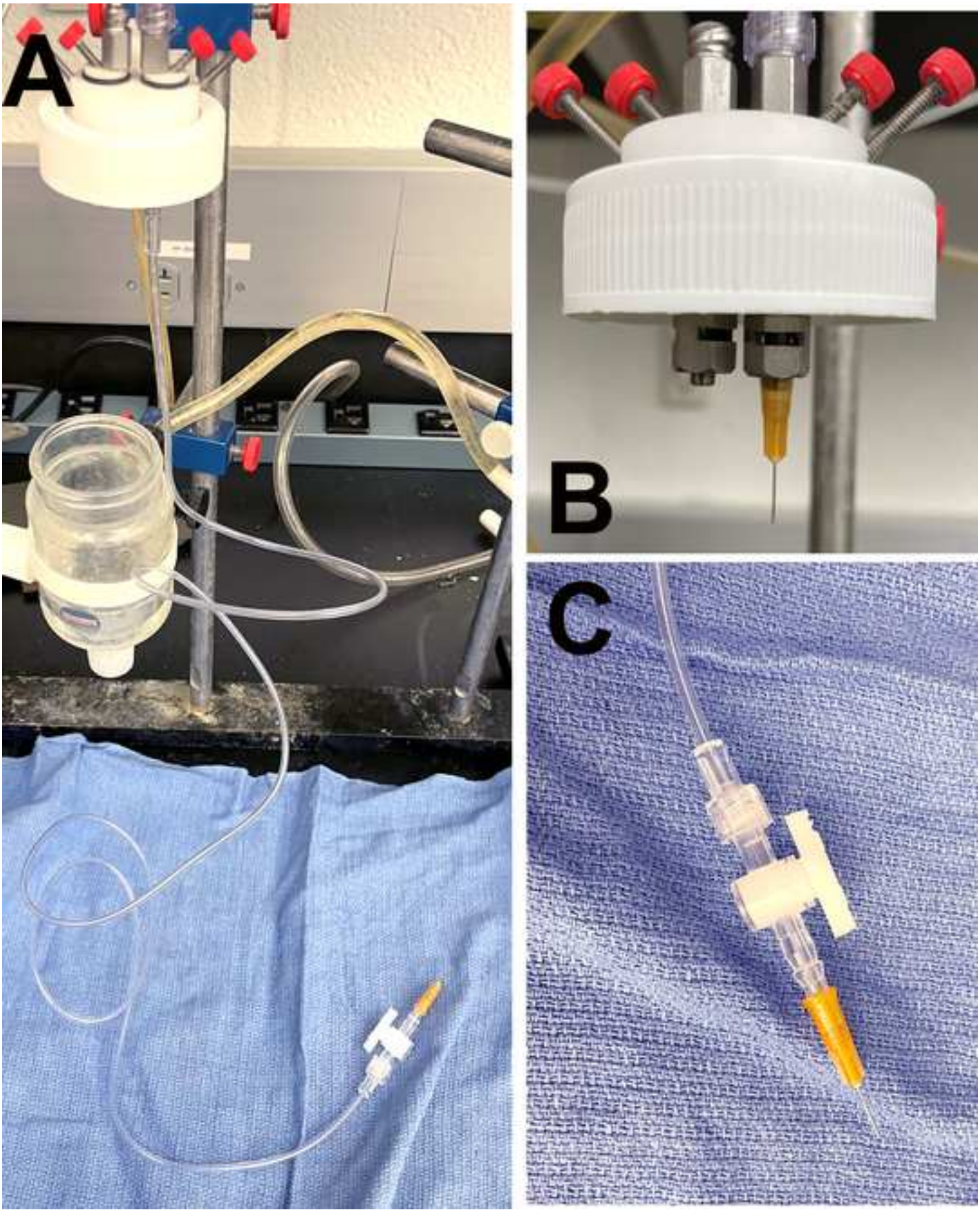

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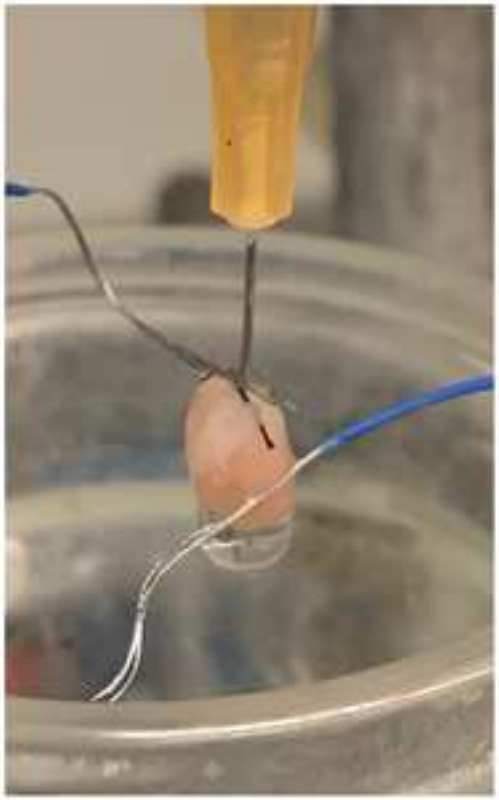

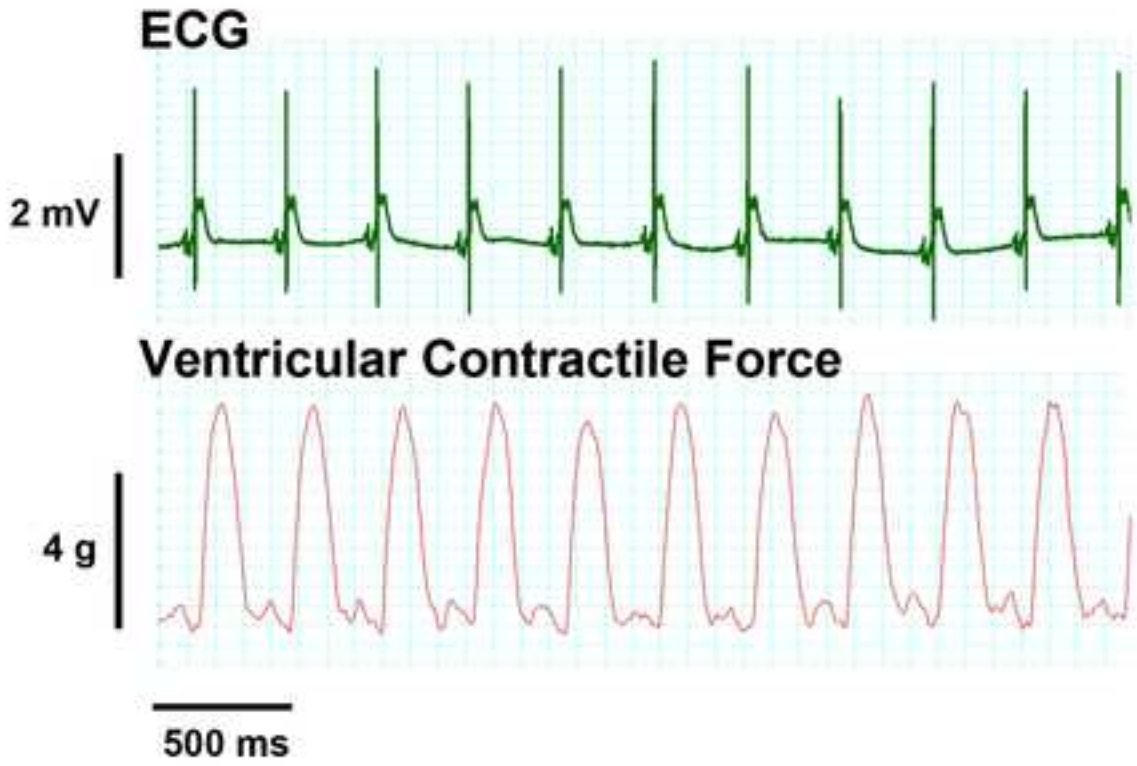

Table 1

Metabolic parameter	Affluent	Effluent	Consumption
Glucose, $mg \cdot dL^{-1}$	194.3 ± 3.8	193.0 ± 5.5	
Lactate, $mmol \cdot L^{-1}$	< 0.3 ± 0.0	< 0.3 ± 0.0	
$P0_2$, mmHg	641 ± 7.9	295 ± 18.4	28.2 ± 1.3 $\mu L \cdot min^{-1}$

Extraction

1.4 ± 0.8 mg·dL^{-1}

$55.7 \pm 2.3\%$

Physiologic Parameter
Aortic perfusion pressure, mmHg
Coronary resistance, $mmHg \cdot min \cdot mL^{-1}$
Heart rate, $beats \cdot min^{-1}$
Ventricular contractile force, g

Mean

47.9 ± 6.9

19.2 ± 2.8

226 ± 8.9

7.2 ± 1.2

Table 3

Physiologic Parameter	**Exclusionary Threshold**
Time to reperfusion, min	>4
Aortic perfusion pressure, mmHg	<20 or >75
Heart rate, beats·min^{-1}	<150 or >300
Arrhythmia duration, min	>3

Rebuttal Document:

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4. Lines 65-67 and 74-78: Please provide citations, if available. Completed
5. Please revise the Introduction to also include the following:
a) The advantages over alternative techniques with applicable references to previous studies. Lines 9387-10094 added to discuss alternatives.
b) A description of the context of the technique in the wider body of literature. Lines 934-10097 added to discuss alternatives to this approach used in literature.
c) Information to help readers to determine whether the method is appropriate for their application ~~Lines 110-112 added.~~This method is appropriate for those who wish to study the developing heart of post-natal day10 and older mice in an ex-vivo fashion.
6. Please number headings of the protocol as well and adjust the numbering accordingly. 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 or dashes. completed
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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 (instructions, extraneous details, remarks, etc.) that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. completed
8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. completed
9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. completed
a. Lines 133-134: Please provide more details on how the pressure differential across the cannula was quantified? Expanded into sections 2.2 and 2.3 and 5.4.
b. 3.1, 3.2: please provide details of the syringes and needles used to inject. 26G needle 1ml syringe combo
c. 3.2: how is proper anesthetization confirmed? With loss of toe pinch reflex.
d. 3.2: Does IP injection prevent induction of apnea and subsequent hypoxia? Please clarify. Pentobarbital can induce apnea and hypoxia with large does (such as 150 mg/kg). These doses are commonly used for euthanasia. However, the reduced dose we have employed induces sedation and unconsciousness without apnea. ~~does not prevent these things.~~ Minimizing delay in ~~in beginning~~ harvesting the heart by starting the procedure as soon as the mouse loses consciousness minimizes the risk of apnea and hypoxia~~through close monitoring of anesthetic plane limits degree of hypoxia~~.
e. 3.3, 3.4: please provide details of surgical tools used and specify the use of sterile conditions if any. Tool detail added. Sterility not necessary given this is nonsurvival surgery~~, no sterile conditions used~~.
f. 3.4: please also mention about how the heart and lungs were excised out of mice. Expanded for clarity.
g. 4.6: please provide a figure showing the apparatus and the attachment of aortic cannula to the apparatus. Figure 1 was added to step 2.4
h. Line 228: how is the applied tension (i.e., 1-2 g) estimated/measured? Tension is adjusted using a calibrated force-transducer. T~~T~~ension should be monitored continuously and applied to~~, and basal or diastolic tension adjusted to reach~~ achieve 1-2g of basal tension on the ventricle.
10. Please highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript,

and therefore will still be available to the reader. Highlights completed.
11. Please discuss Table 3 in Representative Results. Moved prior discussion to Representative results section.
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13. As we are a methods journal, please ensure that the Discussion explicitly covers the following as well in 3-6 paragraphs with citations:
a) Any modifications and troubleshooting of the technique. added
b) Any limitations of the technique ~~added lines 332-334~~Major limitations include lowest age attempted p10, simple perfusion strategy and inability to use balloon for assessment of cardiac function.
c) The significance with respect to existing methods While Langendorff is an established methodology the significance here is the new utility in neonatal mice allowing the study of the developmental period.~~added lines 331-332~~
d) Any future applications of the technique. Discussion of cardiomyocyte isolation, and constant pressure based applications are now discussed.
14. Please remove the embedded Table of Materials. removed.
15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names. We have ensured that the references are in the proper format.

Manuscript Summary:
In this manuscript, Drs. Barajas and Levy describe a technique for performing langendorff perfusions of neonatal murine hearts. This manuscript describes the adaptations/modifications to standard technique used for ex vivo mouse heart perfusions as applicable to neonatal hearts. The authors have also shown representative results in the form of ECG and contractile force along with various physiological parameters.

Minor concerns:

1. Line 75: The authors have mentioned "oxygen and glucose content". I suggest making this statement a more generic one since, in ex vivo perfusions, researchers are not limited to using only glucose in the perfusate. We appreciate the comment and have changed the term g~~G~~lucose ~~changed~~ to metabolic substrate.

2. Line 110: It would nice to note that fetal/neonatal hearts tend to prefer glucose while adult hearts predominantly use fatty acid for energy generation. We agree and~~e bring up~~mention this point in the discussion~~,~~. We highlight that this nuance needs to be considered in ~~as potential for~~ future ~~study~~studies. ~~As we have not performed experiments with fatty acids in p10 hearts yet, I prefer to leave this as future work than to state glucose is preferred.~~

3. Lines 128-130: "… burs and sharp edges must be removed … " - Please include suggestions for effective removal of burs. Is a fine grit sand paper a good option for this purpose? We found utilization of the benchtop as a scraping surface was effective. We did not ~~require additional materials such as sandpaperbut that likely also will work.~~
attempt other methods.

4. Lines 133 - 137: Please include example calculations for these measures. These calculations may or may not common place in many research labs and would be extremely valuable to include. ~~Descriptive walkthrough of the~~Exemplary calculations ~~was~~ have now been added. ~~Note this section was split, with part in what is now section 5.4~~

5. Lines 142 - 143: If the setup will not covered in the video, a figure containing the image, particularly of the relative position of cannula and the high pressure tubing. Location of microscope setup and cannula will

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be covered in the video. Additionally, we have added a photo of the high-pressure tubing and cannula in what is now figure 1.

6. Line 154: Is pentobarbital IP instead of cervical dislocation in order to avoid hypoxia and/or apnea? It may be good to explicitly state to avoid cervical dislocation. Use of IP ~~anesthetization~~ pentobarbital permitted ongoing respiration to avoid hypoxia and apnea as stated by the reviewer. Agree that cervical dislocation may prolong hypoxic time to cannulation, and as such lines 177-178 added. ~~allowed for continued breathing during positioning and initial dissection limiting ischemic time. If performed after dislocation, additional ischemic time would ensue. Lines 170-175~~

7. Line 168: Is there a specific reason to use ice-cold Krebs-Henseleit buffer instead of ice-cold saline that is commonly used with adult hearts? If yes, it would be good to include the reason. No, either would be fine. That option was added to the text.

Reviewer #2:
Manuscript Summary:
This is a very well written manuscript for ex vivo retrograde neonatal heart perfusion. The authors provide a detailed protocol for Langendorff preparation and cannulation of the neonatal aorta to establish perfusion. This will be a great contribution to the study of neonatal cardiomyocyte biology.

Major Concerns:
The methodology is very clear and outlines the process in detail. Since this is a very difficult and sophisticated procedure that requires a skilled operator, I suggest including a trouble shooting section to highlight the fine details of the procedure.

Areas of common failure and resultant physiologic changes in the prep are included. We have expanded to include potential alternatives for difficulty in cannulation and aortic cannula securing steps. Lines. 344~~51~~-348~~56~~

I assume that this procedure can also be used for isolation of neonatal heart cardiomyocytes if the appropriate digestive enzymes are administered during langendorff perfusion. This needs to be addressed at least in the discussion. If the authors have used this methodology for cardiomyocyte isolation, it would be great to include that data, if not then at least a discussion as to whether this procedure can be used for this purpose.
We have not used it for this purpose to date. However, our technique could be used for such purposes. We have added this potential utility ~~in~~ to our discussion, lines 379~~6~~-383~~0~~

Reviewer #3:
This manuscript describes a protocol for a Langendorff-perfused constant flow mouse neonatal heart in which ECG and contractile force can be measured. This model will aid researchers in studying neonatal heart function.
Comments:

1. In the abstract, please indicate that the Langendorff model described in the protocol is with constant retrograde flow. Thank you. We have now a~~A~~dded this in line 47.

2. Can the authors make some comments or recommendations for those who would like to develop the model with constant pressure? Yes. We have now a~~A~~ddressed this in discussion. Line 362~~4~~-365~~6~~.

3. Item 3.1 indicates that heparin needs to circulate before the investigators proceed with the protocol. Just to clarify, do the authors mean to proceed with animal anesthesia? Or is the heparin administered after anesthesia and before tissue collection? To ensure adequate circulation we recommend ~~performing~~ injecting heparin prior to ~~and allowing circulation time prior to~~ anesthetizing the animal. This was clarified in the text, lines 167~~4~~-168~~5~~.

4. Can the authors comment on the use of any other type of anesthesia? Appropriate alternatives may exist. The goal is to maintain respiratory ~~status~~ effort and oxygenation during positioning and initial dissection ~~(up until diaphragm is cut),~~ in order to minimize hypoxic time to the heart. ~~Ketamine-xylazine added as a potential alternative.~~ Lines 170-178~~3~~.

5. To illustrate what is described in item 4.2, it may be good to provide a picture with annotations of the different anatomical parts. Agreed, our intention is that this will be depicted in the video.

6. Can the authors comment on the potential use of a pace maker, as sometimes done in adult heart Langendorff? Here we sought to ~~establish baseline markers, and evaluate heart rate as a potential marker for suboptimal flow~~quantify the physiological response of the denervated newborn heart to the ex vivo prep. Pacing strategies, as is done in older mice, ~~-~~should be a reasonable approach, yet evaluated in the future~~,~~. ~~and t~~This ~~idea was~~has now been added to our discussion, lines 368-369.

7. Please note the typo in Figure 1 " Contractile Force" Thank you. C~~c~~orrected in what is now figure 2.

8. Table 1: "Consumption" and "Extraction". Can those two items in the table be specified, to make the table easier to understand? Extraction is determined by difference in affluent and effluent. Consumption takes into account extraction based on the coronary flow rate. Consumption is~~is~~ calculated as coronary flow x (PaO2 – PvO2) x O2 solubility at 760 mmHg (assuming 24 µl/ml H_2O at 37 °C and 760 mmHg).

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