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TITLE:

Simultaneous Isolation and Culture of Atrial Myocytes, Ventricular Myocytes, and Non-Myocytes from an Adult Mouse Heart

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SUMMARY:

A method is described for the simultaneous isolation of myocytes and non-myocytes from both the atria and ventricles of a single adult mouse heart. This protocol results in consistent yields of highly viable cardiac myocytes and non-myocytes and details optimal cell-specific culture conditions for phenotyping and in vitro analysis.

ABSTRACT:

The isolation and culturing of cardiac myocytes from mice has been essential for furthering the understanding of cardiac physiology and pathophysiology. While isolating myocytes from neonatal mouse hearts is relatively straightforward, myocytes from the adult murine heart are preferred. This is because compared to neonatal cells, adult myocytes more accurately recapitulate cell function as it occurs in the adult heart in vivo. However, it is technically difficult to isolate adult mouse cardiac myocytes in the necessary quantities and viability, which contributes to an experimental impasse. Furthermore, published procedures are specific for the isolation of either atrial or ventricular myocytes at the expense of atrial and ventricular non-myocyte cells. Described here is a detailed method for isolating both atrial and ventricular cardiac myocytes, along with atrial and ventricular non-myocytes, simultaneously from a single mouse heart. Also provided are the details for optimal cell-specific culturing methods, which enhance cell viability and function. This protocol aims not only to expedite the process of adult murine cardiac cell isolation, but also to increase the yield and viability of cells for investigations of atrial and ventricular cardiac cells.

INTRODUCTION:

Primary cell culture is an integral resource that offers a controlled environment for detailed mechanistic studies of cardiac myocyte function. Due to their more durable nature and ease of isolation, neonatal rat atrial and ventricular myocytes have been the common source of such cell cultures[1]. However, adult mouse atrial and ventricular myocytes (AMAMs and AMVMs) are highly desirable for in vitro studies, because their molecular and functional characteristics better mimic those of adult heart cells. Thus, they have become relevant for studies related to cardiac pathologies, most of which develop in adults[2].

Furthermore, the availability and use of transgenic and disease mouse models expands the utility of isolated adult cardiac myocytes. Protocols for the isolation and culture of mouse AMVMs for short- and long-term studies have been described in numerous previous publications[2-11]. In comparison, few protocols have been described for the isolation of AMAMs. Furthermore, those that are described are primarily optimized for acute studies of freshly isolated cells, with no long-term culturing protocol described to date[11-13]. As such, AMAM isolation protocols were not designed to provide the utility and versatility of published protocols for the isolation and culture of AMVMs. Furthermore, while the pioneering studies for the isolation of AMAMs and AMVMs have proven resourceful, there are no protocols for optimal concurrent isolation and culture of both AMAMs and AMVMs, which results in efficient use of the whole heart for each preparation.

Until now, published AMAM and AMVM isolation protocols were not designed for simultaneous isolation of both cell types, because most studies on atrial and ventricular function have a chamber-specific focus. For instance, AMAMs are used predominantly to study atrial myocyte electrophysiology, partly because of the interest in atrial fibrillation (AF), the most common cardiac arrhythmia in the U.S. However, AF is not a disease that affects the atria in isolation, and it has been implicated as having a causative role in mild to severe left ventricular dysfunction[14]. Furthermore, electrocardiograms from patients with heart failure with preserved ejection fraction (HFpEF) have illustrated that left atrial size is one of the strongest predictors for susceptibility to heart failure[15].

In addition to its role in electrophysiology and contractility, the atrium is also an endocrine organ, secreting cardiokines (i.e., atrial natriuretic peptide [ANP]) that homeostatically regulate blood pressure and volume[16,17]. Moreover, ANP (presumably from atrial myocytes) has a prominent protective and anti-hypertrophic role in ventricular myocytes[16,17]. While there is a strong implication of neurohormonal communication between atria and ventricles in various disease states, the mechanisms underlying this communication has not been fully explored. This point is further exemplified by the surge in research focusing on 1) the role of non-myocytes (specifically cardiac fibroblasts and immune cells) in the diseased heart and 2) how cardiac remodeling as a function of disease directly affects cardiac myocyte viability and global cardiac function[18-22]. Thus, studying cardiac cells from both atria and ventricles is a necessary approach to gain a more complete picture of their roles in cardiac pathophysiology.

The following protocol describes the simultaneous isolation of atrial and ventricular myocytes and non-myocytes from a single mouse heart under physiological and pathophysiological

conditions. Additionally, this method is the first to describe optimal conditions necessary for maintaining cultures of atrial cardiac myocytes, as conditions for maintaining cultures of ventricular myocytes have already been published.

PROTOCOL:

All research performed on mice reported in this paper has been reviewed and approved by the SDSU Institutional Animal Care and Use Committee and it conforms to the Guide for the Care and Use of Laboratory Animals published by the National Research Council.

1. Preparation of isolation and culture media and plating

1.1. Prepare 1 L of heart perfusion media prior to use by adding Joklik modified minimum essential media (MMEM) to 1 L of sterile water. Adjust the pH to 7.36 with 10 N NaOH, then filter through a 0.2 µm filter and store at 4 °C for up to 2 weeks.

NOTE: Joklik MMEM consists of 112 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 9 mM NaH_2PO_4, and 11.1 mM D-glucose. This is supplemented with 10 mM HEPES (2.38 g/L), 30 mM taurin (3.75 g/L), 2 mM D-l-carnitine (0.4 g/L), 2 mM creatine, and 10 mM butanedione monoxime (1.01 g/L).

1.2. Prepare digestion buffer just prior to perfusion, as follows: supplement 50 mL of heart perfusion media with 6.25 µL of 100 mM $CaCl_2$ (see step 1.3) and collagenase type 2 (enzymatic activity ~310–320 U/mg dw).

NOTE: Weigh the animal prior to sacrifice. The amount of collagenase type 2 supplemented into the digestion buffer is dictated by the weight of the animal (2.25 mg of collagenase type 2 per 1 mg of body weight).

1.3. To prepare 100 mM $CaCl_2$, add 1.47 g of $CaCl_2$ to 100 mL of molecular biology grade water. Stir until dissolved, then pass through a 0.2 µm filter and store at room temperature for up to 2 months.

1.4. Prepare myocyte stopping buffer 1 by supplementing 90 mL of heart perfusion buffer with 10 mL of fetal bovine serum (FBS) and 125 µL of 100 mM $CaCl_2$. Pass through a 0.2 µm filter and store at 4 °C for up to 2 weeks.

1.5. Prepare myocyte stopping buffer 2 by supplementing 114 mL of heart perfusion buffer with 6 mL of FBS and 150 µL of 10 mM $CaCl_2$. Pass through a 0.2 µm filter and store at 4 °C for up to 2 weeks.

1.6. Prepare atrial myocyte plating medium just prior to perfusion by supplementing 95 mL of Dulbecco's modified Eagle medium (DMEM) with 4 mL of FBS, 1 mL of 100x pen/strep-glutamine, 1 mL of 100x insulin-transferrin-selenium, and 1 mL of 10 µM dexamethasone. Pass through a 0.2 µm filter and store at 37 °C until plating.

1.7. Prepare ventricular myocyte plating medium by supplementing 95 mL of minimum essential medium (MEM) with 4 mL of FBS, 1 mL of 100x pen/strep-glutamine, 10 mL of 1 M HEPES solution, 1 mL of 100x insulin-transferrin-selenium, and 0.1 g of butanedione monoxime. Pass through a 0.2 µm filter and store at 4 °C for up to 2 weeks.

1.8. Prepare ventricular myocyte maintaining medium by supplementing 99 mL of MEM with 1 mL of 100x insulin-transferrin-selenium, 0.1 mg/mL bovine serum albumin (BSA), 10 mL of 1 M HEPES solution and 1 mL of 100x pen/strep-glutamine. Pass through a 0.2 µm filter and store at 4 °C for up to 2 weeks.

1.9. To prepare laminin-coated experimental plates and slides, thaw mouse laminin stock solution (1.19 mg/mL). Add 10 µL of laminin stock solution to every 1 mL of DMEM, and mix. Coat experimental plates and slides evenly and store in a 37 °C, 5% CO_2 incubator for at least 1 h prior to perfusion to allow for equilibration.

NOTE: Coated experimental plates and slides can be stored at 4 °C for up to 2 days.

2. Isolation apparatus

2.1. Prior to each isolation, clean the tubing and other components of the system with three complete washes of 70% EtOH by filling the bubble trap to the top (close off the bottom stopcock and keep the top stopcock open).

2.2. Rinse the complete system 3x with sterile H_2O by filling the bubble trap to the top (close off the bottom stopcock and keep the top stopcock open).

2.3. Rinse out the complete system with heart perfusion buffer and fill the bubble trap halfway with media.

2.4. Set the circulating water bath to 37 °C.

2.5. Set a water bath for media to 37 °C.

2.6. Remove any air bubbles in the peristaltic pump tubing.

2.7. Adjust the flow rate of the peristaltic pump to 3 mL/min.

3. Surgical procedure (non-survival)

3.1. Soak surgical instruments in 70% EtOH for at least 5 min.

3.2. Place the heart perfusion media, myocyte stopping buffers, and digestion buffer in the 37 °C water bath.

3.3. Place the atrial myocyte plating medium, ventricular myocyte plating medium, and ventricular myocyte maintaining medium in a 37 °C, 5% CO_2 incubator 1 h prior to use and loosen the caps to allow equilibration.

3.4. Inject 10-week-old male or female C57b6/j mice intraperitoneally (i.p.) with 0.35 mL of heparin, diluted in phosphate buffered saline (PBS) to 100 IU/mL. Allow the drug to take effect for about 10 min.

NOTE: If two hearts are to be subjected to this isolation procedure, the second mouse can be anesthetized and administered heparin at this point in the first procedure. To minimize stress on the animal, light anesthesia can be administered using 2% isoflurane/oxygen mixture in a hermetically sealed induction chamber.

3.5. Anesthetize the animal with a 2% isoflurane/oxygen mixture and inject i.p. with pentobarbital (0.3 mL from 10 mg/mL stock), then prepare the chest by swabbing with 70% EtOH.

3.6. Prepare a tied 5-0 silk suture to be ready to ligate the heart to the perfusion cannula. Mount cannula right next to surgical microscope.

3.7. Quickly open the chest by first making a midline skin incision, from mid-abdomen to the jaw, then entering the peritoneum with the large scissors, clearing the diaphragm away by blunt dissection. Then, cut away the rib cage using the scissors with cuts up the chest wall on the lateral aspect of both sides.

3.8. Snip away fibrous connections between the heart and chest wall (including thymus). Then, cut away the rib cage all together. Using the small forceps and scissors, gently lift the heart by the apex and expose the posterior aspect of the heart.

3.9. Explant the heart by dissecting immediately inferior to the innominate artery on the ascending aorta and immediately place the heart in ice-cold PBS or cold heart perfusion media. Subsequently, quickly dissect away remaining tissue from the explanted heart in ice-cold heart perfusion media, exposing the ascending aorta.

NOTE: It is useful to explant the heart with the thymus intact to use as an anatomical landmark.

3.10. Clean the area surrounding the aorta of excess tissue using micro-dissecting forceps and scissors. Position the aorta onto the cannula using fine-tipped forceps and secure with a 5-0 silk suture.

NOTE: The best placement usually occurs with the aorta extending about 2 mm up on the cannula.

3.11. Perfuse the cannulated heart with heart perfusion media at a flow rate of 3 mL/min for 4

min. Then switch the media from heart perfusion media to digestion buffer for 15.0–17.5 min of perfusion (**Figure 1A**).

NOTE: Coronary flow rate will likely increase, indicating effective tissue digestion (i.e., pale, swollen heart).

3.12. Collect 8 mL of digestion buffer flow through during the final minutes of perfusion for later use in step 5.4.

3.13. Remove the heart from the cannula and place it on a 60 mm plastic culture dish. Remove excess tissue (i.e., aorta, veins) and submerge the heart in 2.5 mL of digestion buffer in preparation for mechanical separation.

NOTE: At this point, the atria are dissected away, and one experimenter should conduct the atrial cell isolation protocol, while a second experimenter should conduct the ventricular cell isolation.

4. Atrial cell isolation and culture

4.1. Dissect the atria away from the heart and place it into a 30 mm plastic culture dish. Submerge it in 0.75 mL of digestion buffer for mechanical separation. Keep the ventricles in the 60 mm dish (step 3.12) and carry out the separate isolation methods simultaneously (section 5, **Figure 1B**).

NOTE: At this point, the atria and ventricles can undergo further separation should there be a need to isolate the left- and right-side cells.

4.2. Begin to mince and tease the atria apart, initially with fine-tip surgical scissors and followed by fine forceps for further mincing. Avoid agitating the tissue, and do not rapidly pull apart muscle fibers.

4.3. Using a sterile transfer pipette tip, continue to gently mix and dissociate the tissue for 15 min. Every 5 min, observe atrial myocyte disassociation from tissue under a 10x objective brightfield microscope. As tissue becomes further digested, continue gently mixing and dissociating tissue using a sterile transfer pipette tip with a smaller pore size.

4.4. Transfer cell suspension to a 2 mL sterile micro-centrifuge tube. Rinse the 30 mm plate with 0.75 mL of 37 °C myocyte stopping buffer 1 and combine with the cell suspension (end volume = 1.5 mL).

4.5. Allow the atrial myocytes to sediment by gravity for 10 min at room temperature. Gently agitate the cell suspension to allow for myocytes to sediment to the bottom of the conical tube, forming a visible pellet.

4.6. Centrifuge the cell suspension for 5 min at 20 x *g*. Carefully remove the supernatant, which contains the non-myocytes, and transfer to a 15 mL polypropylene conical tube using a sterile

pipette tip without disturbing the pellet of atrial myocytes.

4.7. Centrifuge the non-myocyte fraction for 5 min at 20,000 x *g*. Aspirate the supernatant and resuspend the non-myocyte pellet in 10 mL of DMEM supplemented with 10% fetal calf serum (FCS).

4.8. Count non-myocytes using a hemocytometer or other method, then plate as per experimental needs, or further isolate into individual specific cell populations via fluorescence-activated cell sorting (**Figure 1C**).

4.9. Resuspend the pellet of isolated atrial myocytes from step 4.6 in 1 mL of atrial myocyte plating medium and apply 10 µL of this suspension onto a hemocytometer. Perform a cell count of rod-shaped myocytes per field.

4.10. Aspirate laminin from precoated experimental plates/slides and resuspend the isolated atrial myocytes in the appropriate volume of atrial myocyte plating medium supplemented with 25 µM blebbistatin. Plate at the desired density per experimental needs (**Figure 1C**).

NOTE: Typical plating density for the long-term culturing described here is 5 x 10^5 cells/chamber on four-chamber (1.7 cm^2) glass slides.

5. Ventricular cell isolation and culture

5.1. Begin to mince and tease heart apart ventricular tissue, first with fine-tip surgical scissors and followed by fine forceps for further mincing (**Figure 1B**). Avoid agitating the tissue by rapidly pulling apart muscle fibers.

5.2. Transfer the cell suspension to a 15 mL polypropylene conical tube. Rinse the plate with 2.5 mL of 37 °C myocyte stopping buffer 1 and combine with the cell suspension (end volume = 5 mL).

5.3. Using a sterile transfer pipette tip, continue to gently mix and dissociate the tissue for 4 min. Apply 10 µL of this cell suspension onto the slide and visualize the presence of rod-shaped myocytes to ensure the quality of isolation.

5.4. Pass the cell suspension through a 100 µm sterile nylon filter into a 50 mL polypropylene conical tube. Use 2 mL of digestion buffer collected in step 3.12 to wash any remaining cells off the sterile nylon filter.

5.5. Allow the ventricular myocytes to sediment by gravity for 6 min at room temperature. Gently agitate the filtered cell suspension to allow for myocytes to sediment at the bottom of the conical, forming a visible pellet.

5.6. Without disturbing the pellet of ventricular myocytes, carefully remove the supernatant

(non-myocytes) and transfer to a 50 mL polypropylene conical tube using a sterile pipette tip. Centrifuge the non-myocyte fraction for 5 min at 20,000 x *g*. Aspirate the supernatant and resuspend the non-myocyte pellet in 10 mL of DMEM supplemented with 10% FCS.

5.7. Count non-myocytes using a hemocytometer and resuspend in an appropriate volume of DMEM supplemented with 10% FCS. Then, plate according to experimental needs, or further isolate into individual specific cell populations via fluorescence-activated cell sorting (**Figure 1C**).

5.8. Resuspend the isolated ventricular myocytes in 2 mL of myocyte stopping buffer 2 and apply 10 µL of cell suspension onto hemocytometer. Perform a cell count of rod-shaped myocytes per field.

5.9. Reintroduction of Ca^{2+} using a stepwise paradigm

NOTE: Calcium reintroduction steps are specific for ventricular myocytes and should not be performed for atrial myocytes, as this will result in cell death.

5.9.1. Add 50 µL of 10 mM $CaCl_2$ to the ventricular myocyte cell suspension. Mix well and incubate for 4 min at room temperature.

5.9.2. Add an additional 50 µL of 10 mM $CaCl_2$ to the ventricular myocyte cell suspension. Mix well and incubate for 4 min at room temperature.

5.9.3. Add an additional 100 µL of 10 mM $CaCl_2$ to the ventricular myocyte cell suspension. Mix well and incubate for 4 min at room temperature.

5.9.4. Add 80 µL of 100 mM $CaCl_2$ to the ventricular myocyte cell suspension. Mix well and incubate for 4 min at room temperature.

5.10. Remove laminin coating from plates or slides and resuspend the isolated ventricular myocytes in an appropriate volume of ventricular myocyte plating medium according to experimental needs (**Figure 1C**).

NOTE: Typical plating density for the long-term culturing described here is 5 x 10^5 cells/chamber on four-chamber (1.7 cm^2) glass slides. Avoid plating at a density of greater than 75% confluency, as cellular over-densification promotes cell clumping, thereby inhibiting attachment to the plate. Furthermore, large numbers of cells will be lost during the first medium change. Plate cells quickly after isolation, as extracellular Ca^{2+} promotes hypercontraction and loss of viable myocytes.

5.11. Allow the ventricular myocytes to settle and adhere for at least 1 h. Subsequently change the media to ventricular myocyte maintaining medium supplemented with 25 µM blebbistatin.

NOTE: Ventricular myocytes can be cultured up to 96 h after plating in ventricular myocyte maintaining medium supplemented with 25 µM blebbistatin. Experiments should be conducted

in ventricular myocyte maintaining medium in the absence of blebbistatin.

REPRESENTATIVE RESULTS:

A wildtype 10-week-old C57b6/j mouse heart typically results in between 75,000−150,000 atrial myocytes and 1.0−1.5 x 10^6 ventricular myocytes, equating to an approximate yield of 30%−50% for atrial and ventricular myocytes[18,19]. During and immediately after isolations, viable cardiac myocytes should appear rod-shaped and non-contracting. A majority of isolated cardiac myocytes should adapt this morphology, which is an indication of effective perfusion. The rod-shape morphology can also be a predictor of viability. The protocol aims to enhance the yield and viability of myocytes and non-myocytes isolated from a diseased mouse heart. Furthermore, it has been tested in a model of pressure overload-induced heart failure (data not shown).

To confirm adequate and replicable isolation of myocytes and non-myocytes from atrial and ventricular tissue, cells were observed and photographed at various days in culture (**Figure 2**). Additionally, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed to measure the levels of transcripts that were cell type-specific. Cardiac muscle troponin T (*Tnnt2*) is a marker of cardiac myocytes and was robustly expressed in both atrial and ventricular cardiac myocyte cultures (**Figure 3A**). In contrast, atrial natriuretic peptide (*Nppa*, which is typically expressed exclusively in adult atrial cardiac myocytes under physiological conditions) and myosin light chain 2 (*Myl2*, which is a ventricular myocyte specific gene) were robustly and specifically expressed in atrial and ventricular cardiac myocyte cultures, respectively (**Figure 3B,C**).

Fibroblast markers, transcription factor 21 (*Tcf21*), platelet-derived growth factor receptor A (*Pdgfra*), and monocyte-derived cell marker cluster of differentiation 68 (*Cd68*) were exclusively expressed in non-myocyte cultures isolated from both atrial and ventricular chambers (**Figure 3D–F**). It is estimated that non-myocytes compromise ~65% of all heart cells and that a majority of these originate from a fibroblast or monocyte-derived lineage[18,19,23,24]. Thus, markers for these two lineages were chosen to be representative, given the interest in these cellular populations in studies of various models and etiologies of cardiac pathology.

Immunostaining of AMAMs and AMVMs for the t-tubule marker dihydropyridine (DHPR, which is a voltage-dependent (L)-type calcium channel) as well as the ryanodine receptor (RYR2) demonstrated intact t-tubules throughout isolation and long-term culture (**Figure 4A,B**). The abundance of DHPR and localization that was characteristic and unique to atrial and ventricular myocytes indicated the presences of t-tubules. Moreover, colocalization of DHPR with RYR2 immunostaining was an indicator of intact diad structures. Immunostaining for the sarcomeric protein alpha-actinin in atrial and ventricular cardiac myocytes resulted in the expected sarcomeric striation pattern. The sarcomeric striation pattern was used to assess the purity and viability of isolated cardiac myocytes in conjunction with rod-shaped morphological shape and nuclear staining with TOPRO-3 (**Figure 4C,D**; purple and red). As expected, ventricular cardiac myocytes were large, exhibiting an average length of ~150 μm, whereas atrial cardiac myocytes averaged ~75 μm. Furthermore, upon immunostaining analysis, atrial cardiac myocytes (but not ventricular cardiac myocytes) exhibited robust expression of atrial natriuretic peptide (ANP) in a

staining pattern that was characteristic of localization to the endoplasmic reticulum and secretory granules (**Figure 4C,D**; green).

A characteristic unique to atrial cardiac myocytes is its classification as an endocrine cell in addition to contractile cell. While atrial myocytes secrete ANP under basal conditions, secretion increases in response to secretagogues (i.e., the alpha-adrenergic agonist, phenylephrine [PE]). Moreover, atrial cardiac myocytes secrete ANP and co-secretionally process a portion of the hormone from its precursor state (Pro-ANP, 15 kD) to the product peptide (ANP 3kD)[16,17]. This secretory ability can be quantified via immunoblot detection of ANP in the media of isolated atrial cardiac myocytes in response to acute PE treatment (**Figure 4E**). This secretory and processing ability of the atrial cardiac myocyte was found to be sensitive to culturing conditions. Thus, it is imperative that the atrial myocyte plating medium is supplemented with dexamethasone, insulin, transferrin, and selenium.

FIGURE LEGENDS:

Figure 1: Schematic overview of retrograde heart perfusion, digestion, and cell isolation. Shown are the main steps involved in cell isolation from both atrial and ventricular chambers simultaneously from a single mouse heart. (**A**) A single mouse heart is rapidly cannulated via the ascending aorta and perfused in a retrograde manner. (**B**) The heart is separated into atrial and ventricular tissues for further digestion and physical separation. (**C**) Following adequate digestion, the cells are separated via gravity filtration into a total of four cellular fractions that are cultured for subsequent experimentation.

Figure 2: Morphological analysis of isolated atrial and ventricular cardiac myocytes and non-myocytes in culture. (**A**) Isolated adult mouse atrial myocytes (AMAMs), (**B**) adult mouse atrial non-myocytes (AMANMs), (**C**) adult mouse ventricular myocytes (AMVMs), or (**D**) adult mouse ventricular non-myocytes (AMVNMs) were plated at 5 x 10^5 cells/chamber on four-chamber (1.7 cm^2) glass slides in respective plated media. Phase images were obtained at indicated days in culture using a 10x objective under an epifluorescence microscope.

Figure 3: Representative qRT-PCR analysis of isolated cell cultures. RNA was extracted from freshly isolated cardiac myocytes and non-myocytes, and mRNA levels for cell-specific gene markers were determined by qRT-PCR[4]. (**A**) *Tnnt2*, cardiac muscle troponin T (cardiac myocyte marker); (**B**) *Nppa*, atrial natriuretic peptide (atrial myocyte marker); (**C**) *Myl2*, myosin light chain 2 (ventricular myocyte marker); (**D**) *Tcf21*, transcription factor 21 (fibroblast marker); (**E**) *Pdgfra*, platelet-derived growth factor receptor A (fibroblast marker); (**F**) *Cd68*, cluster of differentiation 68 (monocyte-derived cell marker). Data represent mean ± SEM (*$p \leq 0.05$ different from all other values, as determined by ANOVA followed by Newman Keul's post-hoc analysis).

Figure 4: Representative morphological and functional analysis of isolated atrial and ventricular cardiac myoyctes. (**A**) AMAMs or (**B**) AMVMs were plated at 5 x 10^5 cells/chamber on four-chamber (1.7 cm^2) glass slides in respective plating media for 1 h to allow for adhesion. This was followed by either refeeding atrial myocyte plating media or changing to ventricular myocyte

maintaining media supplemented with blebbistatin for an additional 16 h. Cultures were subsequently fixed then immunostained for RYR2 (purple), DHPR (green), and nuclear stain TOPRO-3 (red). (**C**) AMAMs or (**D**) AMVMs were isolated and plated, then immunostained for α-actinin (purple), ANP (green), and TOPRO-3 (red). Shown are two representative images for each cell type. (**E**) AMAMs were plated at 5 x 10^5 cells/well on a 12 well culture dish for 16 h in atrial myocyte plating media. AMAMs were subsequently treated for 0.5 h with vehicle or the ANP secretagogue (phenylephrine, 50 μM) before media were collected and subjected to immunoblot analysis for ANP. Prior to immunoblot analysis, media samples were centrifuged at 500 x *g* for 5 min to remove cellular debris and ensure that the observed ANP was the result of active secretion from AMAMs.

DISCUSSION:

The quality of the cells isolated using the procedure described here, as determined by the cell yield and overall health of the cells in culture, depends on numerous controllable factors. Starting with the mouse itself, it has been documented that stress imposed on the animal can negatively affect cell yield and viability in culture, presumably due to excess systemic cortisol, catecholamines, and the hypercontractile state of cardiac tissue[2,5,7]. For these reasons, measures should be taken to avoid alarming the animal prior to sacrifice. Such measures include covering the animal's cage and limiting time outside of vivarium prior to sacrifice. Heparin and many barbiturates commonly used for euthanasia can affect signaling pathways; thus, the optimal method of euthanasia should be customized accordingly. The age of the animal has a considerable impact on the quality and viability of isolated cells, most likely due to progressive accumulation of interstitial fibrosis occurring concurrently with the aging process, which can affect tissue digestion[25]. In data not presented here, while the method described above works in mice of up to 78 weeks old, the quality of the cells was lower in these older animals.

The most critical step in the isolation process described, as well as other protocols featuring a Langendorff apparatus for retrograde perfusion, is the cannulation and initial perfusion of the heart. For optimum results, the time from cardiac explantation to cannulation of the ascending aorta and initiation of perfusion should take no more than 90 s. In addition to time, two additional important factors are the depth of cannula and possibility of introducing air emboli from the perfusion apparatus. Accordingly, the cannula should be advanced into the ascending aorta so as not to enter the aortic root and obstruct the aortic valve, which would impair perfusion of the coronary vessels.

During the digestion process, it is important to regularly test the rigidity of the heart to avoid prolonged exposure to the digestive enzyme collagenase, which reduces cardiac myocyte calcium tolerance. The protocol described above for calcium reintroduction into the isolated ventricular myocyte cultures was designed to limit cardiac myocyte death via inappropriate calcium influx via store operated calcium channels. It should be noted that the stepwise calcium reintroduction should not be performed for isolated atrial myocyte cultures, as this will promote cell death during short- and long-term culture[12]. For further precaution, the perfusion and digestion buffers used here include the cardiac muscle contraction inhibitor butanedione monoxime (BDM) to avoid hypercontraction of isolated myocytes, as well as the calcium paradox, both of which

impact myocyte viability[26]. However, the switch from BDM to blebbistatin should be noted, as it is the preferred anti-contractile agent in maintaining media for isolated cardiac myocytes. In data not shown, blebbistatin confers greater viability for long-term culture of isolated cardiac myocytes.

Immediately after isolation, it is important to consider the ramifications of long-term culture of cardiac cells, especially myocytes. The cardiac non-myocyte isolation and culturing protocol described here is based on common methods that take advantage of the different densities and adhesive properties of different cardiac cells. The benefit of non-myocytes is their high expansion potential in culture; thus, unlike cardiac myocytes, they are amenable to passaging for perpetuation. However, it is known that culturing conditions, including medium supplementation with FBS, can affect cardiac myocyte functionality[27]. The culture media described here were designed to optimize viability and limit functional derangements, especially for the isolated atrial myocytes. While no overt impaired contractile ability was observed in isolated cardiac myocytes after culture in the absence of blebbistatin supplementation, studies that focus on electrophysiology, contractility, and other single-cell in vivo-based molecular signaling should be performed soon after isolation, when the sarcomeric structure and molecular signature still mimics that of the intact heart.

A hallmark feature of the atrial myocyte is its ability to moonlight as an endocrine cell with immense secretory capacity, in addition to their contractile function. Under physiological conditions, atrial myocytes produce large quantities of ANP, which is stored in the endoplasmic reticulum and in large dense-core secretory granules poised for regulated exocytosis upon receiving a stimulus[16,17]. While many isolated atrial myocyte studies focus on their unique electrophysiological properties, this is the first study to design a culture media. This allows for long-term viability as well as promotion of the maintained functions of endocrine and contractile properties of atrial myocytes. This novel method for culturing, as well as simultaneous isolation of all cell types from atrial and ventricular chambers from a single mouse heart, will be useful and efficacious for studies on the physiological and pathophysiological properties of both atrial and ventricular myocytes.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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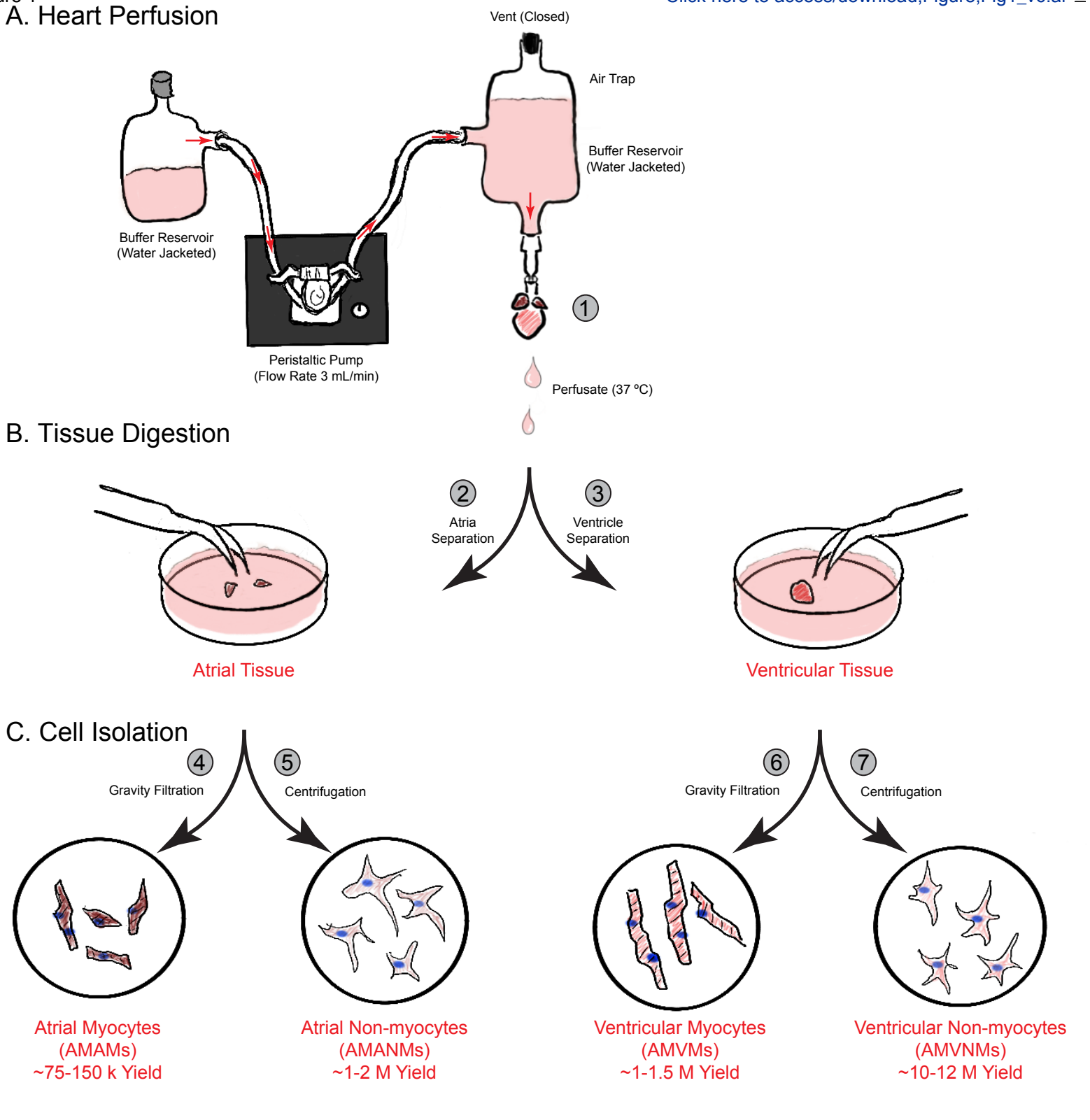


Figure 1

Figure 2

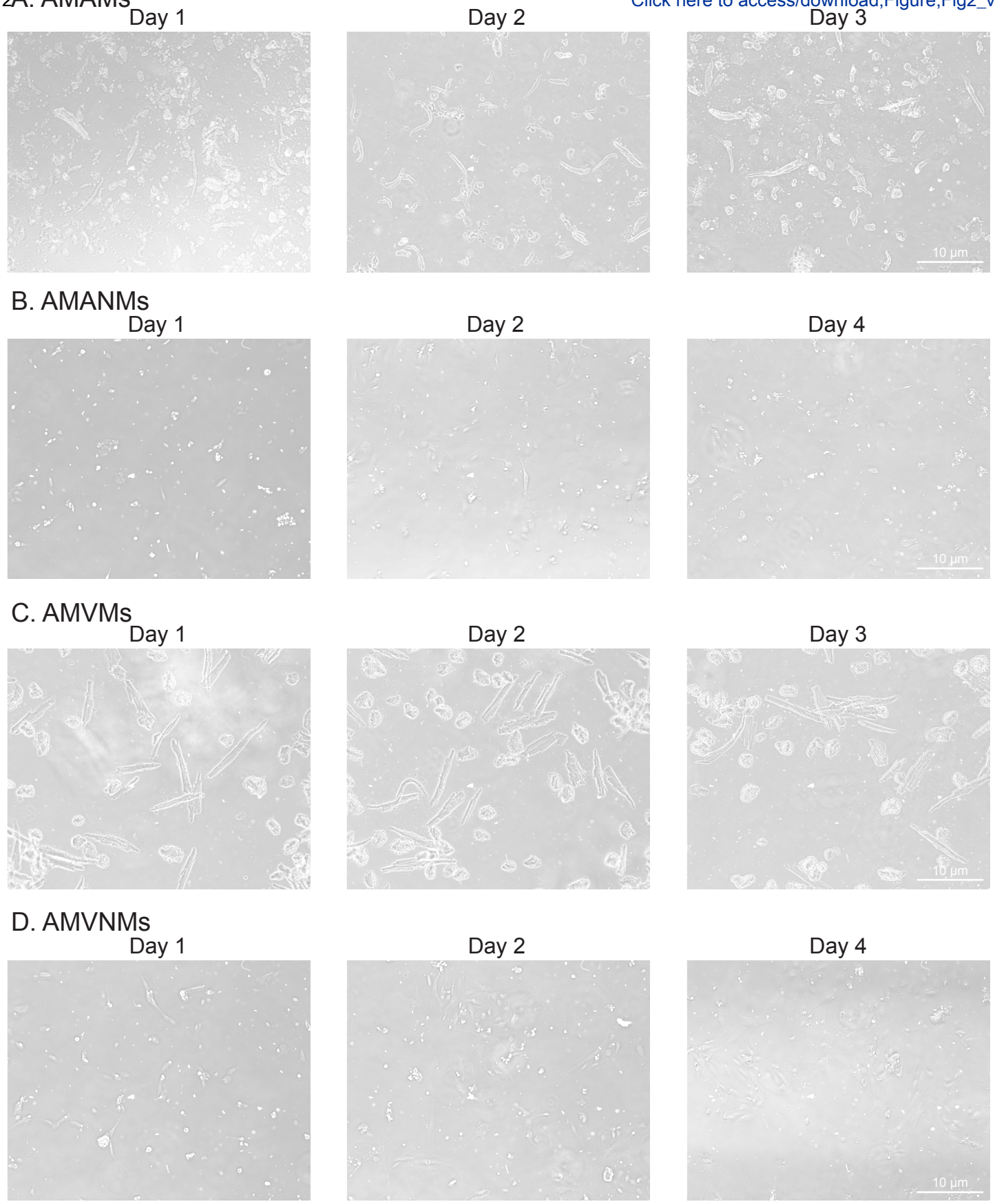


Figure 2

Figure 3

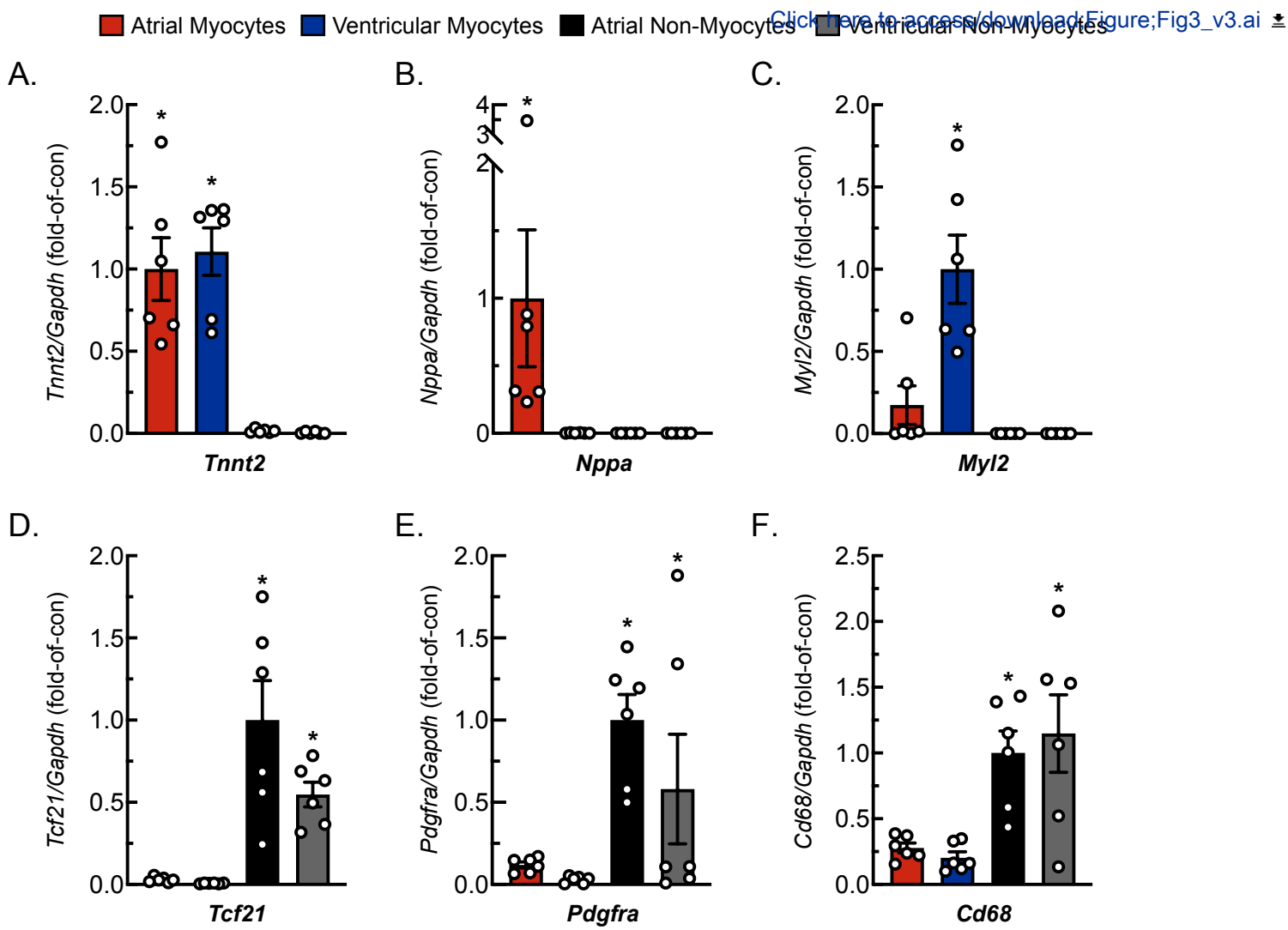
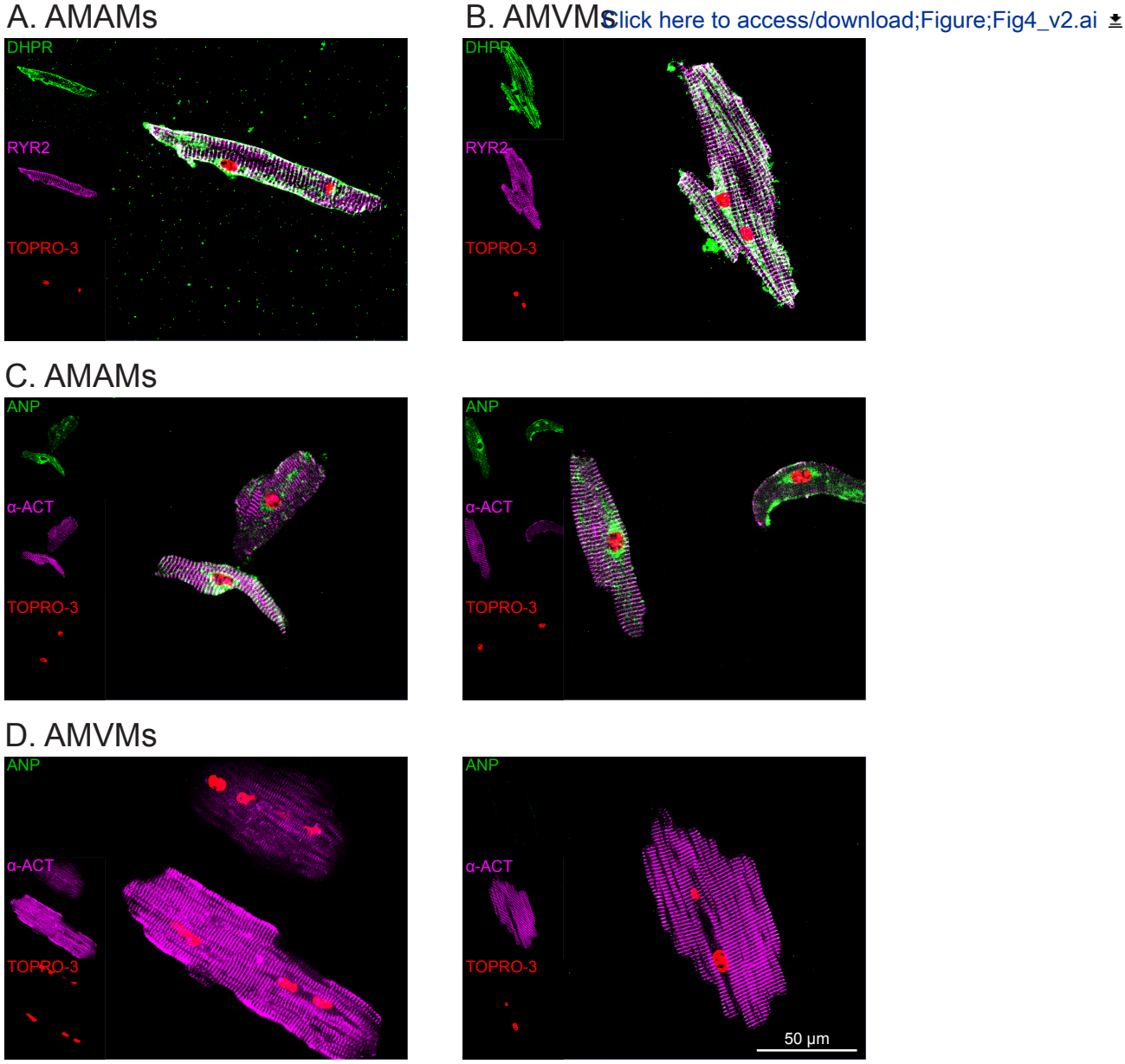


Figure 3

Figure 4



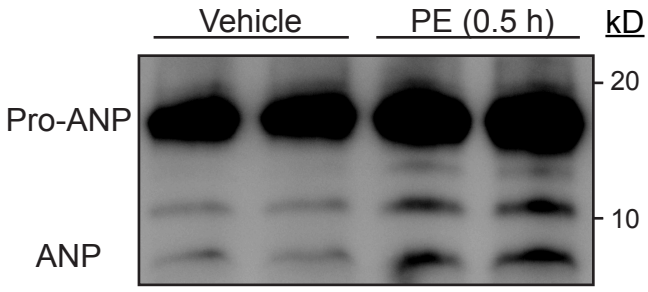


Figure 4

Name of Material/Equipment	Company	Catalog Number	Comments/Description
(-)-Blebbistatin	Sigma-Aldrich	B0560	
1 Liter Water Jacketed Reservoir	Radnoti	120142-1	
2,3-Butanedione monoxime	Sigma-Aldrich	B0753	
5-0 Silk Suture Thread	Fine Science Tools	18020-50	
Adenosine	Sigma-Aldrich	A9251	
Bovine Serum Albumin	Sigma-Aldrich	A6003	
Bubble Trap Compliance Chamber	Radnoti	130149	
Calcium Chloride Anhydrous	Fisher Scientific	C614-500	
Carnitine hydrochloride	Sigma-Aldrich	C9500	
Collagenase type 2	Worthington Biochemical Corporation	LS004176	
Creatine	Sigma-Aldrich	C0780	
Dexamethasone	Sigma-Aldrich	D2915	
DMEM/F12 (1:1; 1X)	Gibco	11330-032	
Dumont #7 - Fine Forceps	Fine Science Tools	11274-20	
Epifluorescent micropscpe	Olympus X70	IX70	
Fetal Bovine Serum (Heat Inactivated)	Omega Scientific	FB-12	Lot# 206018
Fine Scissors - Sharp	Fine Science Tools	14060-09	
Graefe Forceps	Fine Science Tools	11051-10	
Headband Magnifiers	Fine Science Tools	28030-04	
Hemacytometer (Bright-Line)	Hausser Scientific	1475	
HEPES (1M)	Gibco	15630-080	
Inosine	Sigma-Aldrich	I4125	
Insulin-Transferrin-Selenium-X	Gibco	51500-056	
Isotemp 105 Water Bath	Fisher Scientific	NC0858659	

Isotemp 3006	Fisher Scientific	13-874-182	
Joklik Modified Minimum Essential Media	Sigma-Aldrich	M-0518	
Laminin (Natural, Mouse)	Gibco	1795024	Lot# 1735572
L-Glutamine	Sigma-Aldrich	G8540	
Masterflex C/L Single-Channel Variable-Speed Compact Pump	Cole-Palmer	EW-77122-24	
Minimum Essential Medium (MEM 1X)	Gibco	12350-039	
Molecular Biology Grade Water	Corning	46-000-CM	
Pen Strep Glutamine (100X)	Gibco	10378-016	
Spring Scissors - 6mm Cutting Edge	Fine Science Tools	15020-15	
Taurine	Sigma-Aldrich	T-8691	

Preface:

We thank the *JoVE* editors and reviewers for their time and consideration in reviewing our manuscript. We have performed several new experiments as suggested by the reviewers and have addressed all of their comments. As a result of our responses to the reviews, we believe the manuscript has been improved considerably and hope that it is now suitable for publication.

Here we highlight the new experiments we carried out during this the two-week resubmission deadline from *JoVE*:

1. **New Experiment-** Images of cells in culture: We performed additional isolations of myocytes and non-myocytes from atria and ventricles using this dual isolation technique and took phase images of all cells in culture (new Figure 2). It is important to note that the non-myocytes are nearly transparent and the contrast has been adjusted to better visualize the growth and proliferation of these cells as a function of time in culture. This experiment directly addresses the comment made by Reviewer #2 who stated, "*Please provide the pictures of cells (AMVM, AMAM, and non-myocytes) from the day of isolation to different time points during the culture.*"

2. **New Experiment-** Imaging of t-tubule proteins: We performed an additional isolation and after fixation, immunostained AMAMs and AMVMs for the voltage-dependent (L)-type calcium channel, dihydropyridine receptor (DHPR), which is localized to t-tubules. The cells were counterstained for the ryanodine receptor, RYR2 (new Figure 4A,B), which should be co-localized with DHPRs in the t-tubules. This experiment should resonate with Reviewer #2 who stated, "*The author should provide T-tubule density as T-tubule loss results in a reduction in cell surface area, reflected as a measured decrease in cell capacitance.*" The abundance of DHPR and localization characteristic and unique to atrial and ventricular myocytes indicates the presence of t-tubules and overlay with RYR2 is an indicator of intact diad structures [1-2].

Detailed Responses to Editor and Reviewer Comments:

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

This has been completed.

2. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3

sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

This has been completed.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sigma, Worthington, Invitrogen, etc.

This has been completed.

4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

This has been completed.

5. 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."

This has been completed.

6. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

This has been completed.

7. Line 169: Please specify the age, gender and strain of mice used.

This has been completed.

8. Line 173: Please specify the concentration of isoflurane used. Please mention how proper anesthetization is confirmed.

This has been completed.

9. Please include single line spacing between each numbered step or note in the protocol.

This has been completed.

10. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Please do not highlight any steps describing anesthetization and euthanasia.

This has been completed.

11. References: Please do not abbreviate journal titles; use full journal name.

This has been completed.

12. Figure 1 and Figure 3: Please include a space between all numbers and the corresponding unit (3 mL/min, 37 °C, 50 µm, 0.5 h).

This has been completed.

13. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

This has been completed.

14. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

This has been completed.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript by Blackwood et al. showed an efficient method for isolating and culturing atrial and ventricular cardiac myocytes, along with atrial and ventricular non-myocytes, simultaneously from a single mouse heart. The authors achieved a protocol designed to increase the yield and viability of each cardiac cell in the isolation step. Although this protocol

would be an efficient process, I have several major and minor concerns for the authors to address to improve their manuscript.

Major Concerns:
(1) The authors should show their experimental results for the contraction of the isolated atrial and ventricular cardiomyocytes during electrical stimulation, as they discussed the retention of the contractile function of an isolated myocyte in paragraph 4 of page 9 in the Discussion section.

We thank the reviewer for this suggested experiment. However, we do not currently have access to the instrumentation that is necessary to conduct electrical stimulation and contraction assays in isolated cells. Accordingly, we have amended the Discussion to better reflect the observable characteristics of the myocytes as being capable of contracting in the absence of blebbistatin.

(2) The authors should discuss the non-myocyte protocol in the Discussion section.

We thank the reviewer for this suggestion and have added further details pertaining to the non-myocyte protocol in the Discussion section.

Minor Concerns:
(1) The authors should state the enzyme activity, as they used collagenase type 2 during the isolation protocol, the activities of which were different in every lot, as presented on page 2, lines 112-113.

Thank you for this technically important point. As the reviewer mentions, type 2 collagenase enzymatic activity does vary amongst lots and we are careful use lots with activities between 310-320 u/mg dw. This enzymatic activity has been added to the manuscript.

(2) The authors did not use the antibiotic in the Atrial Myocyte Plating Medium, although they used it in the Ventricular Myocyte Plating Medium. I would like the authors to describe the reason why one medium had antibiotics and the other did not.

We thank the reviewer for pointing this out as it alerted us to a mistake. The Atrial Myocyte Plating Medium is indeed supplemented with antibiotics and this has been corrected in the manuscript.

(3) The authors used the Newman-Keul test as a post hoc test in the statistical analysis. I would like the authors to describe why this test was used instead of the Tukey test.

It is our understanding that the the Newman-Keuls and Tukey tests differ, in that the Newman-Keuls test is a sequential test that by design can have more power than the Tukey test.

Moreover, the Newman–Keuls test is often used whenever a significant difference between three or more sample means has been revealed by an analysis of variance. [3].

Reviewer #2:
Manuscript Summary:
The manuscript Simultaneous Isolation and Culture of Adult Murine Atrial Myocytes, Ventricular Myocytes and 3 Non-myocytes from a Single Mouse Heart is well written, however, it requires the protocol in details with figures like preparation of the apparatus and cannulation of the heart. Blebbistatin, a myosin II inhibitor, interferes with myosin-actin interaction and microtubule assembly, has been previously used in myocyte's isolation from the mouse in 2008. The protocols are similar other than the separation of AMAM from AMVM and non-myocytes. I have a few questions:

Major Concerns:
Did the author finish the AMAM isolation first and then continued working with the AMVM? If so, then what media were the AMVM seating in while working with the AMAM.

We thank the reviewer for this point of clarification that is necessary for us to clarify in the protocol. This protocol is typically performed with two people. After the Langendorff-mediated enzymatic perfusion, the atria and ventricles are separated and one person carries out the atrial isolation while the other person performs the ventricular isolation. This clarification has been added to the manuscript.

The author should provide T-tubule density as T-tubule loss results in a reduction in cell surface area, reflected as a measured decrease in cell capacitance.

We thank the reviewer for this important comment. Given the limited time frame for revisions, we elected to conduct immunocytofluorescence to detect the localization of the t-tubule protein DHPR and the sarcoplasmic reticulum protein RYR2 to provide sufficient and adequate data to assess the presence and quality of cardiac myocyte t-tubules after isolation and in culture (new Figure 4A,B).

Please provide the pictures of cells (AMVM, AMAM, and non-myocytes) from the day of isolation to different time points during the culture.

We thank the reviewer for this comment, as it is appropriate to include images of all cells in culture to validate our protocol. We have performed additional isolations and provide the suggested images in new Figure 2.

Minor Concerns:
Why the text in the surgical section is highlighted yellow?

Per JoVE instructions to authors, "…please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video."

References:

1. Hong, T.T., Shaw, R.M. Cardiac T-Tubule Microanatomy and Function. *Physiological Reviews*. **97** (1), 227-252 (2017).
2. Hu, J,. et al. RBFox2-miR-34a-Jph2 axis Contributes to Cardiac Decompensation During Heart Failure. *PNAS*. **116** (13), 6172-6180 (2019).
3. Abdi, H., Williams, L.J. *Encyclopedia of Research Design*. doi:10.4135/9781412961288 (2010).