Submission ID #: 61224
Scriptwriter Name: Bridget Colvin
Project Page Link: http://www.jove.com/files_upload.php?src=18673368

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

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Christopher C. Glembotski**: My name is Chris Glembotski and I'm a Professor of Medicine and the University of Arizona College of Medicine in Phoenix, Arizona and the Director for the Translational Cardiovascular Research Institute. Demonstrating this procedure today is the senior postdoctoral fellow in my lab and faculty-in-training, Dr. Erik Blackwood, and my senior research associate, Ms. Alina Bilal **[1]**. NOTE: Authors added this during the shoot

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Erik A. Blackwood**: Current cardiac myocyte isolation protocols limit the quantity and viability of the cells in culture, creating an experimental impasse to furthering our understanding of cardiac physiology and pathophysiology **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.3. **Erik A. Blackwood**: This protocol aims not only to expedite the process of adult murine cardiac cell isolation but to also increase the cell yield and viability of atrial and ventricular cardiac cells simultaneously from a single mouse heart **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.4. **Alina S. Bilal**: This technique has profound implications for therapeutic discovery, in that diseases like atrial fibrillation can be better characterized at a cell-type specific level, allowing for the identification of therapeutic targets **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.5. **Alina S. Bilal**: We recommend that individuals without previous microsurgical experience practice the ascending aortic explantation and cannulation steps extensively before attempting the full isolation protocol **[1]**.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Ethics Title Card

1.6. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at San Diego State University.

Protocol

2. Surgical Procedure

2.1. Begin by using scissors to make a midline skin incision to quickly open the chest of an anesthetized, 10-week-old, C57BL6/J (C-fifty-seven black six J) mouse from mid-abdomen to the jaw **[1-TXT]**.

2.1.1. WIDE: Talent making incision *Videographer: More Talent than mouse in shot*
TEXT: Anesthesia: 2% isoflurane

2.2. Enter the peritoneum **[1]** and clear the diaphragm by blunt dissection **[2]**.

2.2.1. Peritoneum being opened

2.2.2. Diaphragm being cleared/dissected

2.3. Cut away the rib cage with incisions along the chest wall on the lateral aspect of both sides **[1]** and snip away the fibrous connections between the heart and chest wall **[2]**.

2.3.1. Rib cage being cut away

2.3.2. Connections being cut

2.4. After complete removal of the rib cage **[1]**, use small forceps and scissors to gently lift the heart by the apex, exposing the posterior aspect of the heart **[2]**.

2.4.1. Ribs being cut

2.4.2. Heart being lifted

2.5. To explant the heart, dissect immediately inferior to the innominate artery on the ascending aorta **[1]** and immediately place the heart in ice-cold heart perfusion medium **[2]**.

2.5.1. Heart being dissected

2.5.2. Talent placing heart into solution

2.6. Quickly dissect away the remaining tissue to expose the ascending aorta **[1]** and use micro-dissecting forceps and scissors to clear the area surrounding the aorta of excess tissue **[2]**.

2.6.1. Tissue being removed *Videographer: Important/difficult step*

2.6.2. Tissue around aorta being cleaned *Videographer: Important/difficult step*

2.7. Use fine-tipped forceps to position the cleaned aorta 2 millimeters onto a perfusion cannula **[1]** and secure the cannula with a 5-0 silk suture **[2]**.

2.7.1. Aorta being positioned onto cannula *Videographer: Important/difficult step* **TEXT: Optional: Mark 2 mm on cannula to ensure sufficient cannulation**

2.7.2. Suture being tied *Videographer: Important/difficult step*

2.8. Flush the cannulated heart with heart perfusion medium at a 3 milliliter/minute flow rate for 4 minutes **[1]** before switching to digestion buffer for 15-17.5 minutes **[2-TXT]**.

2.8.1. Heart being perfused, with perfusion medium container visible in frame

2.8.2. Heart being perfused with digestion buffer, with digestion buffer container visible in frame **TEXT: See text for all buffer and medium preparation details**

2.9. During the final minutes of the perfusion, collect 8 milliliters of the digestion buffer flow **[1]** and transfer the heart from the cannula into a plastic, 60-millimeter, culture dish **[2]**.

2.9.1. Buffer being collected

2.9.2. Talent placing heart into dish

2.10. Then remove the excess tissue **[1]** and submerge the heart in 2.5 milliliters of the digestion buffer **[2]**.

2.10.1. Tissue being removed *Videographer: Important step*

2.10.2. Heart being submerged *Videographer: Important step*

3. Atrial Cell Isolation and Culture

3.1. For atrial cell isolation, dissect the atria away from the heart **[1-TXT]** and place the atria into a plastic, 30-millimeter, culture dish containing 750 microliters of digestion buffer **[2]**.

3.1.1. WIDE: Talent dissect atria **TEXT: Leave ventricles in 60-mm dish until single cell isolation and processing**

3.1.2. Talent placing atria into dish, with buffer container visible in frame

3.2. Using fine-tip surgical scissors, mince and tease the atria apart **[1]** before further dissecting the tissue with fine forceps without agitating or pulling apart the muscle fibers **[2]**.

3.2.1. Atria being minced with scissors

~~3.2.2. Atria being minced with forceps~~

3.3. Using a sterile transfer pipette tip, continue to gently mix and dissociate the tissue for 15 minutes **[1]**.

3.3.1. Tissue being mixed and dissociated

3.4. Every 5 minutes, observe the atrial myocyte disassociation under the 10x objective of a brightfield microscope **[1]**.

3.4.1. Talent at microscope, checking dissociation

3.5. As the tissue becomes further digested, continue gently mixing and dissociating the tissue using a sterile transfer pipette tip with a smaller pore size **[1]** before transferring the cell suspension into a sterile, 2-milliliter micro-centrifuge tube **[2]**.

3.5.1. Tissue being mixed

3.5.2. Talent adding tissue to tube

3.6. Rinse the 30-millimeter plate with 750 microliters of 37-degree Celsius myocyte stopping buffer 1 **[1]** and combine the buffer with the cell suspension **[2]**.

3.6.1. Talent rinsing plate, with buffer container visible in frame

3.6.2. Talent adding buffer to cells

3.7. Allow the atrial myocytes to sediment by gravity and gentle agitation for 10 minutes at room temperature **[1]**.

3.7.1. Talent gently agitating cells

3.8. When a visible pellet has formed **[1]**, centrifuge the cell suspension **[2-TXT]** and transfer the non-myocyte-containing supernatant into a 15-milliliter, polypropylene conical tube without disturbing the atrial myocyte pellet **[3]**.

3.8.1. Shot of pellet

3.8.2. Talent placing tube(s) into centrifuge **TEXT: 5 min, 20 x g, RT**

3.8.3. Talent adding supernatant to tube

3.9. Centrifuge the non-myocyte fraction **[1-TXT]** and resuspend the non-myocyte pellet in 10 milliliters of DMEM (D-M-E-M) supplemented with 10% fetal calf serum **[2-TXT]**.

3.9.1. Talent placing tube(s) into centrifuge **TEXT: 5 min, 20,000 x g, RT**

3.9.2. Talent adding medium to pellet, with medium container visible in frame **TEXT: DMEM: Dulbecco's modified Eagle medium**

3.10. After counting, place the non-myocyte cells at the appropriate experimental density for downstream analysis **[1]**.

3.10.1. Talent adding medium to well(s), with medium container visible in frame

3.11. Then resuspend the isolated atrial myocyte pellet at the appropriate experimental concentration for seeding onto laminin-coated culture plates **[1]**.

3.11.1. Talent adding medium to pellet, with medium container and culture plate visible in frame

4. Ventricular Cell Isolation and Culture

4.1. For ventricular cell isolation, mince the ventricular heart tissue as just demonstrated for the atrial tissue sample **[1-TXT]** and transfer the resulting cell suspension into a 15-milliliter polypropylene conical tube containing 2.5 milliliters of 37-degree Celsius myocyte stopping buffer 1 **[2]**.

4.1.1. WIDE: Talent mincing tissue **TEXT: Atrial and ventricle isolation typically performed concurrently by two separate individuals**

4.1.2. Talent adding tissue to tube, with buffer container visible in frame

4.2. Rinse the dissection plate with 2.5 milliliters of 37-degree Celsius myocyte stopping buffer 1 **[1]** and combine the wash with the cell suspension **[2]**.

4.2.1. Talent rinsing plate, with buffer container visible in frame

4.2.2. Talent adding wash to cell suspension

4.3. Using a sterile transfer pipette, continue to gently mix and dissociate the tissue for 4 minutes **[1]**.

4.3.1. Talent mixing tissue

4.4. At the end of the incubation, use a 10-microliter aliquot of the cells to check for the presence of rod-shaped myocytes **[1]**.

4.4.1. Talent at microscope, looking at cells *Videographer: Important step*

4.5. Pass the cell suspension through a sterile, 100-micrometer nylon filter into a 50-milliliter polypropylene conical tube **[1]** and use 2 milliliters of the previously collected digestion buffer to wash any remaining cells from the sterile nylon filter **[2]**.

4.5.1. Talent pouring cells through filter into tube

4.5.2. Talent washing filter

4.6. Allow the ventricular myocytes to sediment by gravity for 6 minutes with gentle agitation **[1]** until a visible pellet is formed at the bottom of the tube **[2]**.

4.6.1. Talent gently agitating tube *Videographer: Important step*

4.6.2. Shot of visible pellet *Videographer: Important step*

4.7. Using a sterile pipette tip, transfer the non-myocyte-containing supernatant to a 50-milliliter, polypropylene conical without disturbing the ventricular myocyte pellet **[1]** and centrifuge the non-myocyte fraction **[2-TXT]**.

4.7.1. Talent aspirating supernatant

4.7.2. Talent placing tube(s) into centrifuge **TEXT: 5 min, 20,000 x g, RT**

4.8. Resuspend the non-myocyte pellet in 10 milliliters of DMEM supplemented with 10% fetal calf serum for counting **[1]** and plate the cells at the appropriate concentration for their downstream analysis **[2]**.

4.8.1. Talent adding medium to cells, with medium container and cell counter visible in frame

4.8.2. Talent adding cells to plate

4.9. Then resuspend the isolated ventricular myocytes in 2 milliliters of myocyte stopping buffer 2 for counting **[1]**.

4.9.1. Talent adding buffer to cells, with buffer 2 container and cell counter visible in frame

5. Stepwise Paradigm Calcium Reintroduction

5.1. To set up a stepwise paradigm calcium reintroduction, add 50 microliters of 10-millimolar calcium chloride to the ventricular myocyte cell suspension with thorough mixing **[1-TXT]** for a 4-minute incubation at room temperature **[2]**.

5.1.1. WIDE: Talent adding calcium chloride to cells, with calcium chloride container visible in frame. Talent setting time, with tube visible in frame. **TEXT: Calcium reintroduction will cause atrial myocyte necrosis**

5.1.2. ~~Talent setting timer, with tube visible in frame~~ NOTE: This was shot as part of 5.1.1. above

5.2. At the end of the incubation, add an additional 50 microliters of 10-millimolar calcium chloride to the cells with mixing for another 4-minute incubation at room temperature **[1]**.

5.2.1. Talent adding calcium chloride to cells, with calcium chloride container and timer visible in frame

5.3. Next, treat the cells with one 4-minute incubation with 100 microliters of 10-millimolar calcium chloride **[1]** and one 4-minute incubation with 80 microliters of 10-millimolar calcium chloride **[2]**.

5.3.1. Talent adding calcium chloride to tube, with calcium chloride container visible in frame

5.3.2. Talent adding calcium chloride to tube, with calcium chloride container visible in frame

5.4. After the last incubation, resuspend the calcium-treated ventricular myocytes in an appropriate volume of ventricular myocyte plating medium according to their planned downstream analysis **[1]** and seed the cells on laminin-coated culture plates **[2]**.

5.4.1. Talent adding medium to tube, with medium container visible in frame

5.4.2. Talent adding cells to plate

5.5. After 1 hour, replace the supernatant with ventricular myocyte maintaining medium supplemented with 25-micromolar blebbistatin **[1]**.

5.5.1. Talent adding medium to plate, with medium and blebbistatin containers visible in frame

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

2.6., 2.7., 2.10., 4.4., 4.6.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

2.6., 2.7. The most technically demanding step of this protocol is the rapid explantation and cannulation of the mouse ascending aorta. It is recommended that individuals without previous microsurgical experience practice this process extensively with the use of a microscope before attempting the full isolation protocol. Furthermore, an experienced surgeon will ensure success by consistent station set up and a clean workspace so as to limit time associated with movement during the cannulation process. Additionally, it may prove helpful to mark the canula at 2 mm from the tip to indicate depth required for cannulation without detrimental perforation of the aortic semilunar valve.

Results

6. Results: Representative Adult Mouse Heart Atrial and Ventricular Myocyte and Non-Myocyte Characterization

6.1. Cardiac muscle troponin T is a marker of cardiac myocytes **[1]** and is robustly expressed in both atrial **[2]** and ventricular cardiac myocyte cultures **[3]**.

6.1.1. LAB MEDIA: Figure 3A
6.1.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize red data bar*
6.1.3. LAB MEDIA: Figure 3A *Video Editor: please emphasize blue bar*

6.2. In contrast, atrial natriuretic peptide **[1]** and myosin light chain 2 **[2]** are robustly and specifically expressed in atrial and ventricular cardiac myocyte cultures, respectively **[3]**.

6.2.1. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize red data bars*
6.2.2. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize blue data bar in Figure 3C*
6.2.3. LAB MEDIA: Figures 3B and 3C

6.3. Fibroblast markers are exclusively expressed in non-myocyte cultures isolated from both atrial and ventricular chambers **[1]**.

6.3.1. LAB MEDIA: Figures 3D-3F *Video Editor: please emphasize black and grey data bars*

6.4. Immunostaining of adult mouse atrial and adult mouse ventricular myocytes for the t-tubule marker dihydropyridine **[1]** and the ryanodine receptor demonstrates intact t-tubules throughout the isolation and long-term culture **[2]**.

6.4.1. LAB MEDIA: Figures 4A and 4B *Video Editor: please emphasize green signal in images*
6.4.2. LAB MEDIA: Figures 4A and 4B *Video Editor: please emphasize pink signal in images*

6.5. The sarcomeric striation pattern can be used to assess the purity and viability of isolated cardiac myocytes **[1]** in conjunction with a rod-shaped morphology and nuclear staining with TOPRO-3 (toh-pro-three) **[2]**.

6.5.1. LAB MEDIA: Figures 4C and 4D *Video Editor: please emphasize striation in cells in images*

6.5.2. LAB MEDIA: Figures 4C and 4D *Video Editor: please emphasize red signal in images*

6.6. As expected, ventricular cardiac myocytes are large, exhibiting an average length of approximately 150 micrometers **[1]**, whereas atrial cardiac myocytes average approximately 75 micrometers **[2]**.

6.6.1. LAB MEDIA: Figures 4C and 4D *Video Editor: please outline/indicate size of cells in Figure 4D images*

6.6.2. LAB MEDIA: Figures 4C and 4D *Video Editor: please emphasize outline/indicate size of cells in Figure 4C images*

6.7. Furthermore, upon immunostaining analysis, atrial cardiac myocytes exhibit a robust expression of atrial natriuretic peptide in a staining pattern that is characteristic of localization to the endoplasmic reticulum and secretory granules **[1]**.

6.7.1. LAB MEDIA: Figures 4C and 4D *Video Editor: please emphasize green signal in Figure 4C*

6.8. While atrial myocytes secrete atrial natriuretic peptide under basal conditions **[1]**, secretion increases in response to secretagogues **[2]**.

6.8.1. LAB MEDIA: Figure 4E *Video Editor: please emphasize ANP bands in Vehicle lanes*

6.8.2. LAB MEDIA: Figure 4E *Video Editor: please emphasize ANP bands in PE lanes*

6.9. Moreover, atrial cardiac myocytes secrete atrial natriuretic peptide and co-secretionally process a portion of the hormone from its precursor state to the product peptide **[1]**.

6.9.1. LAB MEDIA Figure 4E *Video Editor: please emphasize Pro-ANP bands in both Vehicle and PE lanes*

Conclusion

7. Conclusion Interview Statements

7.1. **Erik A. Blackwood**: The most common avoidable errors include not keeping the animal calm prior to sacrifice, not evacuating air bubbles from the perfusion system and, the surgeon not keeping calm **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (Steps 2.5.-2.8.)

7.2. **Erik A. Blackwood**: Subsequent to this procedure, any common experimental procedure can be performed, including assaying electrophysiological and calcium handling parameters, immunocytochemistry, hypertrophic response and signaling studies, and simulated ischemia-reperfusion studies **[1]**.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera