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Title: An Antegrade Perfusion Method for Cardiomyocyte Isolation from Mice

Authors and Affiliations: Mariko Omatsu-Kanbe, Ryo Fukunaga, Xinya Mi, and Hiroshi Matsuura

Department of Physiology, Shiga University of Medical Science

Corresponding Author:
Mariko Omatsu-Kanbe
m_omatsu@belle.shiga-med.ac.jp

Co-Authors:
ds111664@g.shiga-med.ac.jp
mi1990@belle.shiga-med.ac.jp
matuurah@belle.shiga-med.ac.jp

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y**, **Nikon SMZ 745T (stereomicroscope for cleaning and clamping the aorta), DIAPHOT 300 (microscope for observing insolated cells)**

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

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

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

Protocol Length
Number of Shots: **51**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Mariko Omatsu-Kanbe**: We developed a simple Langendorff-free method for isolating individual mouse heart cells by an antegrade perfusion technique. This method allows the isolation of heart cells from juvenile and older mice **[1]**.

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

REQUIRED:

1.2. **Xinya Ma**: Langendorff-based retrograde perfusion has been regarded as a gold standard for isolating cardiac myocytes in various experimental animals. However, cannulation of the aorta is technically difficult in mouse due to their small size **[1]**.

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

Ethics Title Card

1.3. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Shiga University of Medical Science.

Protocol

2. Mouse Heart Excision and Aorta Preparation

2.1. For mouse heart harvest, after confirming euthanasia **[1~~-TXT~~]** and shaving the abdomen **[1A]**, open the thoracic cavity quickly to expose the heart **[2]** and use a plastic transfer pipette with the tip cut to approximately the size of the heart **[3A]** to suck the heart into the pipette **[3]**.

2.1.1. WIDE: Talent pinching toe or similar ~~**TEXT: Euthanasia: sodium pentobarbital overdose (>300 mg/kg i.p.) + heparin 8000 unit/kg**~~

2.1.1A. Added shot: Removing hair from mouse (A005_01231354_C116.mov)
Note: Narrative text provided by me, authors did not provide

2.1.2. Cavity being opened

2.1.3.A. Added shot: Tip being cut (A005_01231432_C121)

2.1.3. Shot of tip, then heart being aspirated

2.2. Raise the pipette to create enough space to insert curved scissors **[1]** and use the scissors to excise the heart from the dorsal side, taking care to avoid damaging the atria **[2]**.

2.2.1. Pipette being raised *Videographer: Important step*
2.2.2. Tissue being excised *Videographer: Important step*

2.3. Immediately place the heart into a 30-milliliter glass beaker containing ice-cold CIB-EGTA (C-eye-B-E-G-T-A) for about one minute **[1-TXT]**.

2.3.1. Talent placing heart into beaker, with CIB-EGTA container visible in frame **TEXT: CIB-EGTA: cell isolation buffer supplemented with ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid**

2.4. When the contractions have stopped, place the heart in a 35-milliliter culture dish containing ice cold CIB-EGTA **[1]** and remove the lung and other visible tissues **[2]**.

2.4.1. Talent placing heart into dish, with buffer container visible in frame
2.4.2. Lung and tissue being removed

2.5. Place the roughly cleaned heart onto a heart stand filled with chilled CIB-EGTA apex-side down **[1]** and place the stand under a stereoscopic microscope **[2]**.

2.5.1. Heart being placed onto stand
2.5.2. Talent placing stand under microscope

2.6. Remove the fat and connective tissues from around the aorta **[1]**.

2.6.1. SCOPE: Fat and tissue being removed Note: Clip 9 - 261 take 3 was the most successful take of the under scope mouse surgery; assuming this take covers shots 2.6.1., 2.7.1., 2.7.2., 2.8.1., and 2.8.2., but the videographers notes didn't say

2.7. If the length of the cut aorta is too long, trim the aorta just under the brachiocephalic artery **[1-TXT]** and orient that heart so that the anterior surface is facing forward **[2]**.

2.7.1. SCOPE: Aorta being cut **TEXT: Exclude brachiocephalic artery, left CCA, and left subclavian artery**
2.7.2. SCOPE: Heart being positioned

2.8. Use tweezers to lift the end of the aorta **[1]** and use a small vascular clamp to clamp the aorta near the atria while gently pushing down on the atria **[2]**.

2.8.1. SCOPE: Aorta being grasped *Videographer: Important step*
2.8.2. SCOPE: Aorta being clamped/pushed down *Videographer: Important step*

2.9. Then place the clamped heart on a perfusion plate with the anterior side facing up **[1]** and hydrate the heart with a few drops of CIB-EGTA **[2]**.

2.9.1. Talent placing heart onto plate under microscope
2.9.2. Talent adding buffer to heart, with buffer container visible in frame

3. Antegrade Mouse Heart Perfusion

3.1. For antegrade perfusion, load a 20-milliliter syringe containing prewarmed CIB-EGTA connected to a flexible extension tube and a marked injection needle onto the infusion pump **[1]** and start the pump at a 0.5 milliliter/minute flow rate **[2]**.

3.1.1. WIDE: Talent loading syringe with buffer, with buffer container visible in frame
3.1.2. Talent starting pump

3.2. When the needle and pump have been filled, place the injection needle on the perfusion plate with the shorter side of the diagonal shape in front **[1]** and slide the needle until it is just touching the apex of the heart **[2]**.

3.2.1. Talent placing needle onto plate

3.2.2. SCOPE: Needle being slid to apex of heart

3.3. Carefully insert the needle near the apex of the left ventricle into the ventricular chamber without twisting or detaching the needle from the plate **[1]**, watching the mark to estimate the depth of the needle insertion **[2]**.

3.3.1. SCOPE: Needle being inserted *Videographer: Important/difficult step*
3.3.2. SCOPE: Shot of red mark at insertion completion *Videographer: Important/difficult step; Video Editor: please emphasize red mark as appropriate*

3.4. When the needle insertion is complete, blood should begin to flow from the coronary artery **[1]**.

3.4.1. SCOPE: Blood flowing from coronary artery *Videographer: Important step; Video Editor: please emphasize blood flowing from coronary artery*

3.5. Use tape to fix the injection needle to the plate **[1]** and increase the pump speed to 1 milliliter/minute **[2]**.

3.5.1. Talent taping needle to plate
3.5.2. Talent increasing pump speed

3.6. If the heart is perfused successfully, the flow of the buffer in the capillary should be visible just under the epicardium **[1]**.

3.6.1. SCOPE: Shot of buffer flowing through capillary *Video Editor: please emphasize buffer in capillary when mentioned as possible*

3.7. After 2-3 milliliters of CIB-EGTA perfusion, replace the perfusion buffer with enzyme mix **[1-TXT]**. After 1-2 milliliters have been perfused **[2-TXT]**, increase the pump speed to 1.5 milliliters/minute **[3]**.

3.7.1. Talent adding enzyme buffer to syringe **TEXT: Caution: Avoid bubbles**
3.7.2. Heart being perfused with enzyme mix **TEXT: Check flow and red mark to confirm adequate perfusion**
3.7.3. Talent increasing pump speed

3.8. Use a pipette to remove the accumulated blood-containing perfusate flowing from the heart as necessary **[1]** and stop the perfusion when the total volume of perfused enzyme reaches 10 milliliters **[2]**.

3.8.1. SCOPE: Perfusate being removed
3.8.2. Talent stopping perfusion

4. Individual Heart Cell Isolation

4.1. At the end of the perfusion, transfer 10 milliliters of enzyme mix from the syringe to a 60-millimeter culture dish on a heater mat **[1]** and add 20 milligrams of BSA (B-S-A) to the dish **[2-TXT]**.

4.1.1. WIDE: Talent adding mix to syringe
4.1.2. Talent adding BSA to dish **TEXT: BSA: bovine serum albumin**

4.2. Gently swirl the dish to dissolve the powder **[1]** and remove the injection needle and clamp from the heart **[2]**.

4.2.1. Dish being swirled
4.2.2. SCOPE: Needle and/or clamp being removed Note: Clip 8 - 422 take 1.mov: It appears that the needle or clamp was not removed here (4.2.2 content), and only the content of shot 4.3.1 is here. Maybe the last portion of the 3rd surgery (filename: Clip 9 - 261 take 3.mov) where the needle and clamp are removed can be used for the 4.2.2 shot content?

4.3. Remove the ventricles and atria from the heart **[1]** and place the tissues into the BSA-supplemented enzyme mix **[2]**.

4.3.1. SCOPE: Ventricle and/or atrium being removed Note: See also note for 4.2.2.
4.3.2. Talent placing tissues into dish

4.4. To isolate the ventricular myocytes, use two pairs of tweezers to grasp the epicardium **[1]** and gently pull the ventricles into small pieces **[2]**.

4.4.1. Epicardium being grasped *Videographer: Important step*
4.4.2. Ventricle(s) being torn into pieces *Videographer: Important step*

4.5. When all of the ventricle fragments have been generated, disperse the cells approximately 30 times with gentle pipetting **[1]** and filter the undigested debris through a 100-micron mesh cell strainer into a 15-milliliter centrifuge tube **[2]**.

4.5.1. Cells being pipetted
4.5.2. Talent filtering tissue solution

4.6. After centrifugation, resuspend the cardiomyocyte pellet **[0]** in prewarmed CIB supplemented with calcium and BSA **[1-TXT]** and incubate the cells for 5 minutes at 37 degrees Celsius **[2]**.

4.6.0. Added shot: CU of pellet (A005_01231717_C139)
4.6.1. Shot of pellet if visible, then buffer being added to tube, with buffer container visible in frame **TEXT: 3 min, 50 x g**
4.6.2. Talent placing tube at 37 °C

4.7. At the end of the incubation, centrifuge the cells again **[1-TXT]** and resuspend the precipitated cardiomyocytes in an appropriate volume of cell resuspension solution for their maintenance at 37 degrees Celsius until downstream analysis **[2]**.

4.7.1. Talent placing tube(s) into centrifuge **TEXT: 3 min, 14 x g**
4.7.2. Shot of pellet if visible, then solution being added to tube, with solution container visible in frame Note: Take 1 of this shot is a CU of pellet in tube, with no additional content (filename: A005_01231812_C145.mov). The additional content can be found in take 2 (filename: A005_01231815_C146.mov)

4.8. To isolate the atrial myocytes, transfer the atria to a container of pre-warmed CIB supplemented with calcium and BSA **[1]** and tear the atria into pieces as demonstrated **[2]**.

4.8.1. Talent adding tissues to dish, with buffer container visible in frame
4.8.2. Atria being fragmented

4.9. Use a 20-microliter pipette set to 10 microliters to disrupt the tissues by pipetting **[1]** and collect the dissociated cells to by centrifugation **[2-TXT]**.

4.9.1. Pieces being pipetted
4.9.2. Talent placing tube(s) into centrifuge **TEXT: 3 min, 14 x g**

4.10. Then resuspend the atrial cell in an appropriate volume of cell resuspension solution **[1]**.

4.10.1. Shot of pellet if visible, then solution being added to cells, with solution container visible in frame

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.2., 2.8., 3.3., 3.4., 4.4.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
3.3. According to the structure of the heart, carefully insert the injection needle into the left ventricle while adjusting the direction and the depth.

Results

5. Results: Representative Isolated Cardiomyocyte Imaging and Analysis

5.1. In this image, freshly isolated ventricular myocytes can be observed **[1]**.

5.1.1. LAB MEDIA: Figure 1A

5.2. This isolation procedure results in a 70-80% yield of rod-shaped quiescent ventricular myocytes **[1]** from 8-10-week-old mice within roughly 5 hours of isolation **[2]**.

5.2.1. LAB MEDIA: Figure 1B
5.2.2. LAB MEDIA: Figure 1C *Video Editor: please emphasize green 5 h data points*

5.3. The ratio of freshly isolated viable cells is lower in mice greater than 2 years of age **[1]**.

5.3.1. LAB MEDIA: Figure 1C *Video Editor: please emphasize black 5 h data points*

5.4. The action potentials recorded in the ventricular **[1]** and atrial myocytes are similar to those measured in cells obtained by the Langendorff-based method **[2]**.

5.4.1. LAB MEDIA: Figure 1D *Video Editor: please emphasize black data line*
5.4.2. LAB MEDIA: Figure 1D *Video Editor: please emphasize red data line*

5.5. Immunostaining analysis can be used to assess the organization of the sarcomeric structure of the ventricular myocytes **[1]** and the transformation of the cardiac fibroblasts into myofibroblasts after subculture **[2]**.

5.5.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize alpha-Actinin images*
5.5.2. LAB MEDIA: Figure 2B *Video Editor: please emphasize alpha-Smooth muscle actin image*

5.6. Western blot analysis is recommended to determine the specific expression of proteins of interest in atria and ventricles after processing **[1]**.

5.6.1. LAB MEDIA: Figure 3

5.7. After perfusion with enzymes, proteins from the atria **[1]** and ventricles can be easily homogenized in lysis buffer with light force for protein extraction **[2]**.

5.7.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize ANP bands in A lanes*
5.7.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize lack of bands in V lanes*

Conclusion

6. Conclusion Interview Statements

6.1. **Ryo Fukunaga**: It is important to control the direction and depth of the needle insertion. When inserting the needle into the left ventricle, take care not to pierce the ventricular septum or to penetrate the valve **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.3.) Note: Second take (A005_01231148_C108.mov) is better

6.2. **Mariko Omatsu-Kanbe**: You can change the composition of the perfusate depending on the purpose of the experiment. For example, an EGTA-supplemented detergent can be used to make a cell-free scaffold of the heart **[1]**.

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