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Title: Surgery and Sample Processing for Correlative Imaging of the Murine Pulmonary Valve

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   Yes

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

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?

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, 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? **Yes**

   Same building but different floors. The animal dissection room is a little small, however. The wet lab should be able to accommodate everyone without issue.

Current Protocol Length
Number of Steps: 20
Number of Shots: 43

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Yifei Liu:** This protocol is significant because it is designed to answer questions about the structure-function correlate of the murine pulmonary valve, which is generally tricky because of its inherent heterogeneity **[1]**.

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

1.2. **Yifei Liu:** The controlled fixation and correlative imaging were used to capture the structure on multiple length scales. Local, high-resolution images can then be mapped back to the precise anatomical location on the PV **[1]**.

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

OPTIONAL:

1.3. **Yifei Liu:** The protocol presented here is general, and the correlative imaging approach is non-specific **[1]**.

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

Introduction of Demonstrator on Camera

1.4. **Christopher Breuer:** Demonstrating the procedure will be Tai Yi, a Microsurgery Director at the Center for Regenerative Medicine at Nationwide Children's Hospital **[1]**.

NOTE: Details are updated by the authors for 1.4.

1.4.1. The named demonstrator(s) looks up from the workbench or desk, or microscope and acknowledges the camera.

Ethics Title Card

1.5. The use of animals in this study was in accordance with Nationwide Children's Hospital Institutional Animal Care and Use Committee.

Protocol

2. Pulmonary Valve Excision

2.1. Begin by autoclaving the tools needed for the mouse dissection, including fine scissors, micro forceps, microvascular clamps, clamp applying forceps, microneedle holders, spring scissors, and retractors **[1]**.

2.1.1. WIDE: Establishing shot of talent walking to the lab bench with the autoclaved tools placed in a tray

2.2. After euthanizing an adult C57BL/6 *(C-fifty-seven black six)* mouse, place it in a dorsal recumbence position on a tray **[1]**, secure its limbs with tape, and perform the thoracotomy **[2]**.

2.2.1. Talent placing the mouse in an appropriate position

2.2.2. Talent securing the mouse limbs with tape

2.3. Expose the heart by removing any excess adipose tissue and fascia *(fah-shuh)* **[1]**, then remove the right atrium **[2]** and perfuse the left ventricle with room temperature saline solution **[3-TXT]**.

2.3.1. SCOPE: Exposed heart

2.3.2. SCOPE: Talent removing the right atrium

2.3.3. SCOPE: Talent perfusing left ventricle with saline solution **TEXT: Perfusion take approximately 20 mL over 30 s**

NOTE for SCOPE SHOT 2.3.1, 2.3.2, and 2.3.3: Footage should be submitted by the videographer.

2.4. Remove the entire heart by severing the superior vena cava, inferior vena cava, pulmonary artery, and aorta **[1]** and cut approximately 2 millimeters above the ventriculo-arterial junction, which will serve as the conduit for pressurization **[2]**.

2.4.1. SCOPE: Exposed entire heart

2.4.2. SCOPE: Talent cutting across the ventriculo-arterial junction *Videographer: This step is important!*

NOTE for SCOPE SHOT 2.4.1 and 2.4.2: Footage should be submitted by the videographer.

2.5. Remove the left and right ventricles to expose the chambers to atmospheric pressure **[1]**, ensuring that the pulmonary trunk structure is unaffected by removing the ventricles **[2]**. *Videographer: This step is important!*

2.5.1. SCOPE: Talent removing left and right ventricles
2.5.2. SCOPE: Talent checking unaffected/intact pulmonary trunk structure

NOTE for SCOPE SHOT 2.5.1 and 2.5.2: Footage should be submitted by the videographer.

3. Pressure Fixation of Pulmonary Valve

3.1. Anastomose *(ana-stuh-mose)* pressurization tubing with the pulmonary artery, leaving an approximately 1-millimeter distance between the sino-tubular junction and the end of the tubing to accommodate for large movements of the leaflets and pulmonary trunk **[1]**.
3.1.1. Talent connecting pressurization tubing with pulmonary artery

3.2. Elevate the reservoir to an analogous physiological pressure **[1]** and fill it with the saline solution **[2]**. Test the flow-through system to ensure there are no blockages or air bubbles **[3]**.
3.2.1. Talent elevating the reservoir
3.2.2. Talent filling the reservoir with saline solution
3.2.3. Talent checking the flow-through systems for no blockages or air bubbles *Videographer: This step is important!*

3.3. Attach a stopcock to the anastomosed pulmonary valve **[1]** and ensure adequate flow through the tubing by switching the outflow tract **[2]**. Once the flow is sufficient, switch the outflow to the anastomosed pulmonary valve **[3]** and ensure pressurization of the pulmonary trunk, identified by trunk distention **[4]**.
3.3.1. Talent attaching stopcock to the anastomosed pulmonary valve
3.3.2. Talent checking the adequate flow through the tubing
3.3.3. Talent switching the outflow to the anastomosed pulmonary valve
3.3.4. Enlarged pulmonary trunk

3.4. After confirming the pressurization of the pulmonary trunk, gradually incorporate primary fixative solution until 25% of the reservoir capacity of the saline solution is purged **[1-TXT]**.
3.4.1. The primary fixative solution is being incorporated **TEXT: 1.25% glutaraldehyde, 1.0% paraformaldehyde in 0.15 M cacodylate** *Videographer: This step is important!*

3.5. Place a fixative-soaked gauze over the tissue sample to prevent drying **[1]**. Perfuse the fixative for 3 hours, refilling the reservoir to maintain a constant pressure **[2]**.
3.5.1. Talent placing the fixative-soaked gauze over the tissue sample
3.5.2. Shot of fixative incorporation

3.6. After perfusion, store the heart valve in the fixative solution at 4 degrees Celsius until use for up to 1 week **[1]**.
3.6.1. Talent placing the heart valve in the fixative solution

4. En bloc Sample Staining and Embedment

4.1. For staining, wash the fixed heart valve sample three times with cold 0.15 molar cacodylate buffer for 5 minutes **[1]** and completely submerge the heart valve in a solution of 1.5% potassium ferrocyanide, 0.15 molar cacodylate, 2 millimolar calcium chloride, and 2% osmium tetroxide on ice for 1 hour **[2]**.
4.1.1. Talent giving washes to the heart valve sample
4.1.2. Talent placing the heart valve in a solution

4.2. Wash the samples with room temperature double distilled water by placing them in a tube for 5 minutes with slight agitation **[1]**.
4.2.1. Sample in water on a shaker

4.3. After three more washes, place the sample in filtered thiocarbohydrazide solution at room temperature **[1]**. After 20 minutes, wash the sample three times with water as demonstrated earlier **[2]**.
4.3.1. Talent placing the sample in thiocarbohydrazide solution
4.3.2. Talent giving water washes to the samples

4.4. Next, place the sample in 2% osmium tetroxide at room temperature **[1]**. After 30 minutes, wash it with water three times for 5 minutes each and incubate the sample in 1% uranyl acetate overnight at 4 degrees Celsius **[2]**. Meanwhile, prepare a solution of lead nitrate **[3-TXT]**.
4.4.1. Talent placing the sample in osmium tetroxide solution
4.4.2. Talent placing the sample in uranyl acetate solution for overnight incubation
4.4.3. Talent with lead nitrate solution set on the workbench **TEXT: Refer to the text for preparation of lead nitrate solution**

4.5. On the following day, perform the washing step three times as demonstrated earlier **[1]**, then incubate the heart valve tissue in lead aspartate solution at 60 degrees Celsius for 30 minutes **[2]**.
4.5.1. Talent giving water washes to the sample
4.5.2. Talent placing the heart valve for incubation in lead aspartate solution

4.6. Wash the samples three more times, then perform a serial dehydration treatment on the heart valve tissues with freshly prepared 20, 50, 70, and 90% ethanol on ice for 5 minutes each **[1],** followed by two subsequent treatments with 100% ethanol **[2]**.
4.6.1. Talent performing serial dehydration with ethanol
4.6.2. Talent performing dehydration with 100% ethanol

4.7. After dehydration, move the tissue to ice-cold acetone **[1]**, then place it in fresh acetone at room temperature for 10 minutes **[2]**.
   4.7.1. Talent placing the tissue in ice-cold acetone
   4.7.2. Talent placing the tissue in room temperature acetone

4.8. For embedding, make the resin mixture according to the manufacturer's specifications **[1]**. Place tissues in subsequent treatments of 25:75, 50:50, and 75:25 resin: acetone mixture for 2 hours each **[2]**. Finally, place the tissues in 100% resin overnight **[3]**.
   4.8.1. Talent showing the labeled different volume ratios of the resin mixture solutions
   4.8.2. Talent placing the tissues in a mixture of resin: acetone mixture
   4.8.3. Talent placing the tissues in 100% resin

4.9. On the following day, place tissues in fresh 100% resin **[1]**. After 2 hours, transfer the tissues to an embedding capsule **[2]**, add fresh 100% resin **[3]**, and place it in a 60-degree Celsius oven for 48 hours to cure **[4]**.
   4.9.1. Talent placing the tissues in fresh 100% resin
   4.9.2. Talent transferring the tissues to an embedding capsule
   4.9.3. Talent adding fresh resin to the capsule
   4.9.4. Talent placing the capsule in an oven

Results

5. Results: Correlative Imaging of the Murine Pulmonary Valve

5.1. The excised anastomosed pulmonary artery before **[1]** and following the application of hydrostatic pressure is shown here **[2]**. The pulmonary trunk distends radially, indicating that the pulmonary valve leaflets are in a closed configuration **[3]**.
- 5.1.1. LAB MEDIA: Figure 1A
- 5.1.2. LAB MEDIA: Figure 1B
- 5.1.3. LAB MEDIA: Figure 1B *Video Editor: please emphasize on the dotted line*

5.2. Micro-computed tomography confirmed the pulmonary valve conformation **[1]**. The leaflets were closed, and the annulus was circular **[2]** while varying degrees of inadequate pulmonary valve pressurization by either fixation **[3]** or arterial collapse were observed **[4]**.
- 5.2.1. LAB MEDIA: Figure 2
- 5.2.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize on a blue circle*
- 5.2.3. LAB MEDIA: Figure 2B
- 5.2.4. LAB MEDIA: Figure 2C

5.3. The micro-CT volume rendering virtual cross-sections **[1]** was correlated with optical images to confirm the slicing direction and location **[2]**.
- 5.3.1. LAB MEDIA: Figure 3A *Video Editor: please emphasize on red circles for pulmonary valve leaflets*
- 5.3.2. LAB MEDIA: Figure 3B *Video Editor: please emphasize on red circles for pulmonary valve leaflets*

5.4. High-resolution SBF-SEM images were taken at a local region within a leaflet when the specimen block was at the desired location and orientation **[1]**. Correlated images between the micro-CT volume rendering virtual slice **[2]**, low-resolution **[3]**, and high-resolution SBF-SEM images are shown here **[4]**.
- 5.4.1. LAB MEDIA: Figure 4
- 5.4.2. LAB MEDIA: Figure 4A *Video Editor: please emphasize on the red box indicates the imaged region in Figure 4B using SBF-SEM*
- 5.4.3. LAB MEDIA: Figure 4B *Video Editor: please emphasize on the blue box represents the location of Figure 4C*
- 5.4.4. LAB MEDIA: Figure 4C

5.5. In 3D representation of a segmented region of the pulmonary valve, extracellular components like endothelial cells **[1]**, valvular interstitial cells **[2]**, and extracellular fibers can be identified **[3]**.

5.5.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize on green color for endothelial cells*

5.5.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize on blue color for valvular interstitial cells*

5.5.3. LAB MEDIA: Figure 5 *Video Editor: please emphasize on yellow color for extracellular fibers*

Conclusion

6. Conclusion Interview Statements

6.1. **Yifei Liu:** When attempting this protocol, please keep in mind that performing the pressurization is critical and will ensure the yield to be as high as possible **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B role: 2.4.2, 3.1.1, 3.3.4.*