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Title: Air-Inflation of Murine Lungs with Vascular Perfusion-Fixation

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Author Questionnaire

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

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**.

☒ Statements are read by JoVE VO talent

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **No**

Protocol Length
Number of Shots: **47**

Videographer: When filming the protocol, please avoid filming the faces of any of the demonstrators

Introduction

1. Introductory Interview Statements

NOTE to JoVE VO Talent: Please record all introduction and conclusion statements

REQUIRED:

1.1. This is an inexpensive method that combines the advantages of air inflation and vascular perfusion-fixation for preserving lungs for structural and functional analyses. **[1]**.

1.1.1. *4.7.2 for 'vascular perfusion-fixation'*

1.2. The main advantage of this technique is the preservation of both cell morphology and location within the airspaces of the lung. **[1]**.

1.2.1. *4.2.2.*

OPTIONAL:

1.3. Care should be taken while placing the Luer stub adapter into the trachea and the perfusion needle into the right ventricle to ensure adequate inflation and fixation.

1.3.1. *Suggested B-roll: 3.7.1 for 'luer stub adapter'*

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at National Jewish Health.

Protocol

2. Air-Inflation Apparatus Preparation

Videographer: When filming the protocol, please avoid filming the faces of any of the demonstrators

2.1. To set up the air-inflation apparatus, place a syringe for the water column into a ring holder **[1]** and measure a vertical height of 25 centimeters from the animal platform to 25 centimeters on the water column **[2]**.

2.1.1. WIDE: Talent placing syringe into ring holder

2.1.2. Talent measuring/marking height

2.2. Attach the end of the water column tube to the stopcock on the air chamber **[1]** and attach a tube from the female Luer of the air chamber to the stopcock on the animal processing container **[2]**.

2.2.1. Talent attaching water column tube to stopcock

2.2.2. Talent attaching female Luer tube to stopcock

2.3. Confirm that the cap to the air chamber **[1]** and the stopcock on the outside of the animal processing container are closed **[2]** and that the stopcock on the tubing leading from the water column to the air-inflation chamber is open **[3]**.

2.3.1. Cap closure being checked

2.3.2. Stopcock closure being checked

2.3.3. Stopcock being opened/shot of open stopcock

2.4. Then fill the syringe with water to the 25-centimeter mark **[1]**. Water will flow through the syringe and tubing into the air chamber **[2]**. Once the pressure has equalized, the water will stop flowing **[3-TXT]**.

2.4.1. Talent filling syringe

2.4.2. Water leaving syringe through tubing

2.4.3. Water stopping flowing **TEXT: See text for water leakage correction details**

3. Animal Preparation

3.1. To prepare the lungs for inflation, make one lateral incision in the abdominal wall below the rib cage of a euthanized mouse **[1]** and a second, lateral, abdominal wall incision above the hips **[2]**.

3.1.1. WIDE: Talent making incision *Videographer: More Talent than mouse in shot*

3.1.2. Shot of first incision, then second incision being made

3.2. Cut along the midline from the inferior incision toward the superior incision **[1]** and use blunt scissors to carefully make an incision into the lateral side of the diaphragm. The lungs should collapse as soon as the diaphragm is punctured **[2-TXT]**.

3.2.1. Incision being made between incisions

3.2.2. Incision being made in diaphragm/lungs collapsing **TEXT: Caution: Do not puncture lungs**

3.3. Cut transversely along the diaphragm to open the thoracic cavity **[1]** and cut superiorly along the sternum from the xiphoid process to the jugular notch and laterally above the rib cage to fully expose the heart and lungs **[2]**. *Videographer: This step is important!*

3.3.1. Diaphragm being cut

3.3.2. Incision(s) being made/heart and lungs being exposed

3.4. Pin down the sides of the ribcage **[1]** and make a midline incision in the neck above the trachea **[2]**.

3.4.1. Pin(s) being placed

3.4.2. Incision being made

3.5. Remove the skin, muscle, thyroid gland, and connective tissue surrounding the trachea **[1]** and use curved forceps to slide two pieces of suture under the posterior trachea **[2]**.

3.5.1. Tissue being removed

3.5.2. Suture/thread being placed

3.6. Use one piece of suture to hold the inflation Luer-stub adapter in place **[1]** and use an 18-gauge x 1-inch needle to make a small hole in the trachea **[2]**. *Videographer: This step is important!*

3.6.1. Suture being placed

3.6.2. Hole being made

3.7. Insert a 20-gauge Luer-stub adapter into the hole **[1]** and tie the suture around the trachea immediately distal to where the Luer-stub adapter enters the hole **[2]**. *Videographer: This step is important!*

3.7.1. Adapter being inserted

3.7.2. Suture being tied

3.8. Then transfer the animal to the animal processing container **[1]** and attach the Luer-stub adapter to the female Luer on the inside of the animal processing container **[2]**.

3.8.1. Talent placing animal into container *Videographer: More Talent than mouse in shot*

3.8.2. Adapter being attached to Luer

4. Lung Air-Inflation, Perfusion, and Fixation

4.1. To inflate the lungs, place the 25-gauge x 5/8-inch needle attached to the perfusion apparatus tubing into the right ventricle of the heart **[1]** and cut the abdominal aorta to allow blood to drain from the heart and to promote the flow of perfusate through the lungs **[2]**. *Videographer: This step is important!*

4.1.1. WIDE: Talent placing needle *Videographer: More Talent than mouse in shot*

4.1.2. Shot of needle in place, then aorta being cut

4.2. Open the stopcock on the outside of the animal processing container **[1]** and inflate the lungs at 25 centimeters of water for 5 minutes **[2]**, while that the water level in the syringe does not decrease too quickly **[3]**. *Videographer: This step is important!*

4.2.1. Stopcock being opened

4.2.2. Lungs being inflated

4.2.3. Water level decreasing

4.3. During the last minute of the lung inflation, turn on the peristaltic pump to a flow rate of 10 milliliters/minute **[1]**. Heparin solution should flow from the bottle through the tubing into the animal **[2]**.

4.3.1. Talent turning on pump *Videographer: This step is important!*

4.3.2. Solution flowing into animal

4.4. After inflating the lungs for 5 minutes, turn off the pump **[1]** and switch the perfusate from heparin to fixative **[2]**.

4.4.1. Talent turning off pump

4.4.2. Talent selecting fixative

4.5. Lower the water column syringe to 20 centimeters. It is normal for air bubbles to move within the water column as the pressure changes **[1]**.

4.5.1. Talent lowering water column

4.6. Check the water level in the syringe. It should be at the 25-centimeter mark **[1-TXT]**.

4.6.1. Shot of water level in syringe **TEXT: Add more H_2O to syringe as necessary**

4.7. Then wait 1 minute to allow the lungs to deflate from 25 to 20 centimeters of water pressure **[1]** before restarting the pump at a 6.5 milliliter/minute flow rate for 10-15 minutes of vascular fixative perfusion **[2]**.

4.7.1. Lungs deflating

4.7.2. Fixative being perfused into lungs

5. Lung Extraction

5.1. For extraction of the inflated and fixed lung tissue, tightly tie the second piece of thread around the trachea distal to the Luer-stub adapter **[1]** and remove the Luer-stub adapter from the trachea **[2]**.

5.1.1. WIDE: Talent tying suture *Videographer: More Talent than mouse in shot*

5.1.2. Shot of tied suture, then adapter being removed

5.2. Remove the needle from the heart **[1]** and use blunt scissors to cut the connective tissue posterior to the mediastinum to free the lungs and heart from the thoracic cavity, taking care to avoid puncturing the lungs **[2]**.

5.2.1. Needle being removed

5.2.2. Tissue being dissected

5.3. Carefully remove the heart from the lungs **[1]** and place the lungs into 20-25 milliliters of fixative in a 50-milliliter conical tube **[2]**.

5.3.1. Heart being removed

5.3.2. Talent placing lungs into tube

5.4. Pull the suture holding the trachea through the opening of the conical tube and secure the suture by the threads of the cap **[1]**.

5.4.1. Suture being pulled/secured

5.5. Then invert the tube to ensure that the buoyant, air-inflated lungs remain fully submerged in fixative **[1]** and process the lungs for histologic studies according to standard protocols **[2]**.

5.5.1. Tube being inverted

5.5.2. LAB MEDIA: Figure 4

Results

6. Results: Representative Lung Inflation, Extraction, and Histological Evaluation

6.1. When the diaphragm is entered during dissection **[1]**, the integrity of the pleural space will be abolished and the lungs should collapse **[2]**.

6.1.1. LAB MEDIA: Figure 2B
6.1.2. LAB MEDIA: Figure 2B *Video Editor: please emphasize collapsed lung (white tissue)*

6.2. Upon the application of 25 centimeters of water pressure **[1]**, air will enter the lungs via the trachea and inflation should be easily observed **[2]**.

6.2.1. LAB MEDIA: Figure 2C
6.2.2. LAB MEDIA: Figure 2C *Video Editor: please emphasize inflated lung (white tissue)*

6.3. Once the lungs have fully expanded **[1]**, the inflation pressure can be decreased to 20 centimeters to keep the lungs inflated without over-distension **[2]**.

6.3.1. LAB MEDIA: Figure 2D
6.3.2. LAB MEDIA: Figure 2D *Video Editor: please emphasize white tissue*

6.4. The lungs should remain inflated after tracheal ligation **[1]** and removal from the thorax **[2]**.

6.4.1. LAB MEDIA: Figures 3A and 3B *Video Editor: please emphasize white tissue in Figure 3A*
6.4.2. LAB MEDIA: Figures 3A and 3B *Video Editor: please emphasize white tissue in Figure 3B*

6.5. Deflation can occur if the lungs are punctured during the animal preparation or lung extraction **[1]**.

6.5.1. LAB MEDIA: Figure 3C *Video Editor: please emphasize white tissue*

6.6. Histological analysis of inflated lung sections **[1]** reveals that very few immune cells are present in the airway lumens of tissues fixed using traditional liquid-based inflation **[2]**.

6.6.1. LAB MEDIA: Figure 4

6.6.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize empty space in right Figure 4A image*

6.7. In contrast, inflammatory cells are preserved within the airspaces of tissues fixed via vascular perfusion with air-inflation **[1]**.

6.7.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize cells in empty space in right Figure 4B image/arrows in right Figure 4B image*

Conclusion

7. Conclusion Interview Statements

NOTE to JoVE VO Talent: Please record all introduction and conclusion statements

7.1. Following air-inflation with perfusion fixation, lung tissue may be embedded in formalin for tissue sectioning and subsequent analysis via histology techniques including staining, immunohistochemistry and immunofluorescent imaging. **[1]**.

7.1.1. *5.5.1 for 'embedded in formalin'*