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Vascular Casting of Adult and Early Postnatal Mouse Lungs for micro-CT Imaging

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Corresponding Author:	Russell Knutsen National Heart Lung and Blood Institute Bethesda, MD UNITED STATES
Corresponding Author's Institution:	National Heart Lung and Blood Institute
Corresponding Author E-Mail:	russell.knutsen@nih.gov
Order of Authors:	Russell Knutsen
	Leah M. Gober
	Joseph R. Sukinik
	Danielle R. Donahue
	Elise K. Kronquist
	Mark D. Levin
	Sean E. McLean
	Beth A. Kozel
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DEPARTMENT OF HEALTH & HUMAN SERVICES

National Institutes of Health
National Heart, Lung, and Blood Institute
Bethesda, Maryland 20892

January 17, 2020

Dear Dr. Kyle Jewhurst and JoVE Editors

We submit to you our manuscript entitled, “Vascular Casting of Adult and Early Postnatal Mouse Lungs for micro-CT Imaging”, for your review. In it, we outline a method for visualizing the pulmonary arterial vasculature. We refine existing procedures to establish a fluid and reproducible technique. Our method pushes tissue size and age boundaries to early postnatal mice which is helpful for developmental studies. Using the knowledge gained from the pulmonary procedure, we also briefly discuss casting alternate systemic vascular beds and some helpful tips to ensure success!

Thank you for considering our manuscript,

Beth A. Kozel, M.D. Ph.D.
Investigator and Lasker Clinical Research Scholar
Director, Laboratory of Vascular and Matrix Genetics
National Heart, Lung, and Blood Institute, National Institutes of Health
Beth.Kozel@nih.gov; Phone: 301-451-2888

TITLE:
Vascular Casting of Adult and Early Postnatal Mouse Lungs for Micro-CT Imaging

AUTHORS AND AFFILIATIONS:
Russell H. Knutsen[1], Leah M. Gober[1], Joseph R. Sukinik[1], Danielle R. Donahue[2], Elise K. Kronquist[1], Mark D. Levin[1], Sean E. McLean[3], Beth A. Kozel[1]

[1]Translational Vascular Medicine Branch, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA
[2]Mouse Imaging Facility, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA
[3]Division of Pediatric Surgery, Department of Surgery, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Corresponding Author:
Beth A. Kozel (Beth.kozel@nih.gov)

Email Addresses of Co-authors:
Russell H. Knutsen (russell.knutsen@nih.gov)
Leah M. Gober (leah.m.gober@gmail.com)
Joseph R. Sukinik (jrsukinik@gmail.com)
Danielle R. Donahue (donahued@mail.nih.gov)
Elise K. Kronquist (elise.kronquist@nih.gov)
Mark D. Levin (mark.levin@nih.gov)
Sean E. McLean (sean_mclean@unc.med.edu)

KEYWORDS:
lung, micro computed tomography, perfusion, vascular, arterial, imaging, cast

SUMMARY:
The aim of this technique is ex vivo visualization of pulmonary arterial networks of early postnatal and adult mice through lung inflation and injection of a radio-opaque polymer-based compound via the pulmonary artery. Potential applications for casted tissues are also discussed.

ABSTRACT:
Blood vessels form intricate networks in 3-dimensional space. Consequently, it is difficult to visually appreciate how vascular networks interact and behave by observing the surface of a tissue. This method provides a means to visualize the complex 3-dimensional vascular architecture of the lung.

To accomplish this, a catheter is inserted into the pulmonary artery and the vasculature is simultaneously flushed of blood and chemically dilated to limit resistance. Lungs are then inflated through the trachea at a standard pressure and the polymer compound is infused into the vascular bed at a standard flow rate. Once the entire arterial network is filled and allowed to

cure, the lung vasculature may be visualized directly or imaged on a micro-CT (µCT) scanner.

When performed successfully, one can appreciate the pulmonary arterial network in mice ranging from early postnatal ages to adults. Additionally, while demonstrated in the pulmonary arterial bed, this method can be applied to any vascular bed with optimized catheter placement and endpoints.

INTRODUCTION:

The focus of this technique is the visualization of pulmonary arterial architecture using a polymer-based compound in mice. While extensive work has been performed on systemic vascular beds such as brain, heart, and kidney[1-5], less information is available regarding the preparation and filing of the pulmonary arterial network. The aim of this study, therefore, is to expand upon previous work[6-8] and provide a detailed written and visual reference that investigators can easily follow to produce high-resolution images of the pulmonary arterial tree.

While numerous methods exist for labeling and imaging lung vasculature, such as magnetic resonance imaging, echocardiography, or CT angiography[9,10], many of these modalities fail to adequately fill and/or capture the small vessels, limiting the scope of what can be studied. Methods such as serial sectioning and reconstruction provide high resolution but are time/labor-intensive[11-13]. Surrounding soft tissue integrity is compromised in traditional corrosion casting[10,13-16]. Even animal age and size become factors when attempting to introduce a catheter or, the resolution is lacking. The polymer injection technique, on the other hand, fills arteries to the capillary level and when combined with µCT, allows for unparalleled resolution[5]. Samples from mouse lungs as young as postnatal day 14 have been successfully casted[8] and processed in a matter of hours. These can be rescanned indefinitely, or even sent for histological preparation/electron microscopy (EM) without compromising the existing soft tissue[17]. The main limitations to this method are the upfront cost of CT equipment/software, challenges with accurately monitoring intravascular pressure, and the inability to acquire data longitudinally in the same animal.

This paper builds on existing work to further optimize the pulmonary artery injection technique and push age/size related boundaries down to postnatal day 1 (P1) to yield striking results. It is most useful for teams that want to study arterial vascular networks. Accordingly, we provide new guidance for catheter placement/stabilization, increased control over fill rate/volume, and highlight notable pitfalls for increased casting success. Resulting casts can then be used for future characterization and morphologic analysis. Perhaps more importantly, this is the first visual demonstration, to our knowledge, that walks the user through this intricate procedure.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (ACUC) of the National Heart Lung and Blood Institute.

1. Preparation

1.1. Inject the mouse intraperitoneally with heparin (1 unit/g mouse body weight) and allow it to ambulate for 2 min.

1.2. Euthanize the animal in a CO_2 chamber.

1.3. Arrange the mouse in a supine position on a surgical board and secure all four limbs to the board with tape. Use magnification for fine dissection.

2. Exposing lungs and trachea

2.1. Spray the ventral side of the mouse with 70% ethanol to minimize hair interference.

2.2. Grasp the abdominal skin with forceps and make a small incision with scissors in the umbilical region. Slide the tips of scissors into the fascial layer between the abdominal musculature and skin and begin separating the two layers. Work rostrally, removing the skin from the abdomen, ribcage, and neck.

2.3. Open the abdominal musculature with scissors and cut laterally on both sides until the diaphragm is exposed.

2.4. Gently grasp the xiphoid process and slightly lift the ribcage maximizing the view of the caudal lungs through the thin, semitransparent diaphragm. Carefully make a small incision in the diaphragm just beneath the xiphoid process. The lungs will collapse and retract away from the diaphragm. Dissect the diaphragm away from the ribcage, taking care not to nick the lung parenchyma.

2.5. Locate and sever the inferior vena cava (IVC) and esophagus where they pass through the diaphragm. Use gauze to clean up any pooling blood in the thoracic cavity, avoiding contact with the lungs.

2.6. Grasp the xiphoid once again and gently lift. Cut the ribcage bilaterally (roughly at the midaxillary line) avoiding contact with the lungs. Remove the anterior ribcage entirely, making the final cut along the sternal angle just before the manubrium.

2.7. Using a prefilled syringe, liberally wet the lungs with phosphate-buffered saline (PBS, pH 7.4) to prevent drying out. Continue this routine throughout the procedure.

2.8. Using forceps, grasp the manubrium and gently elevate away from the body. Using scissors, cut 1-2 mm lateral to the manubrium, severing clavicles, and remove. This will expose the thymus underneath.

2.9. Grasp each lobe of the thymus, pull apart, and remove. Repeat this procedure with the submandibular gland. Finally, remove the muscular tissue overlaying the trachea.

NOTE: Following the dissection, the heart, ascending aorta (AA), pulmonary arterial trunk (PAT), and trachea should be visible. Ensure the primary arterial branches off the trunk are not divided or injured.

3. PA catheterization and blood perfusion

3.1. To assemble Unit 1, thread 15 cm of PE-10 tubing onto the hub of a 30 G needle and attach to a 1 mL syringe prefilled with 10^{-4} M sodium nitroprusside (SNP) in PBS. Prime the tubing by advancing the plunger until all the air is purged from this unit (**Figure 1**).

CAUTION: SNP is toxic if swallowed. Avoid contact with the skin and eyes. Wash skin thoroughly after handling. Wear appropriate personal protective equipment.

3.1.1. Alternatively, assemble Unit 2. For mice postnatal day 7 (P7) and younger, use a hemostat to detach an additional 30 G needle from its hub and thread the needle onto the open end of the tubing of Unit 1 (**Figure 1**).

3.2. Instead of a needle, use curved sharp forceps to grasp one end of a 10 cm length of 7-0 silk. Penetrate the apex of the heart entering from one side and passing the tips of the forceps through the muscle and out of the other side. Grasp the silk with another set of forceps and pull approximately a 2 cm length through and tie off. Take the remaining 8 cm end of the suture, tugging the heart caudally, and tape the end to the surgical board.

NOTE: This will create tension, further exposing the great vessels and tethering the heart in place, allowing for easier placement of the catheter in the pulmonary artery.

3.3. Hook the tips of curved forceps under both the AA and PAT. Pull a 3 cm length of 7-0 silk back through the opening and create a single-throw loose suture.

3.4. Using scissors make a 1-2 mm incision toward the apex of the heart, penetrating the thin-walled right ventricle (RV), to allow for the insertion of the catheter (Unit 1). Prior to the insertion, confirm there is no air in the system. Introduce the primed tubing into the right ventricle and gently advance into the semitransparent thin-walled PAT.

3.4.1. Visually verify that the catheter has not advanced into either the left or right pulmonary branches and does not abut the pulmonary artery branchpoint. Using tape, secure the distal portion of the tubing to the surgical board.

NOTE: To identify the RV, use forceps to pinch the right side of the heart. Unlike the left ventricle, the relatively thin free wall of the RV should be easily grasped.

3.4.2. For mice younger than P7, attach Unit 2 to a micromanipulator and introduce the needle end of the unit into the PAT as described above using the manipulator.

3.5. Gently tighten the loose suture around both great vessels and cut the 8 cm length of suture created in step 3.2 to return the heart to a natural resting position. The catheter is now firmly secured within the PAT.

3.6. Clip the left auricle of the heart to allow perfusate to exit the system.

3.7. Secure SNP-containing syringe (Unit 1 or Unit 2, size dependent) in the syringe pump and perfuse the solution at a rate of 0.05 mL/min to flush the blood and maximally dilate the vasculature. Blood/perfusate will exit via the clipped auricle. Continue perfusion until perfusate runs clear (~200 µL in an adult mouse, less for younger animals).

NOTE: When perfusing the low viscosity PBS/SNP, a relatively higher infusion rate was used in the interest of saving time. The more viscous polymer compound is infused at a slower rate to prevent overfilling, rupture, and maximize control over distal endpoints.

4. Tracheostomy and lung inflation

4.1. Construct the lung inflation unit (**Figure 2**).

4.1.1. Connect a flexible plastic 24 G intravenous (IV) catheter (needle removed)/butterfly infusion set to a stopcock, attached to an open 50 mL syringe (no plunger). Hang the syringe from a ring stand.

4.1.2. Add 10% buffered formalin to the syringe. Open the stopcock, allowing formalin to enter the tubing and purge all air from the system. Close the stopcock and raise the syringe until the meniscus is 20 cm above the trachea[8].

CAUTION: Formalin is flammable, carcinogenic, acutely toxic when ingested, and causes skin irritation, serious eye damage, skin sensitization, and germ cell mutagenicity. Avoid ingestion and contact with skin and eyes. Avoid inhalation of the vapor or mist. Keep away from sources of ignition. Wear appropriate personal protective equipment.

4.2. Place two loose sutures inferior to the cricoid cartilage 2-4 mm apart.

4.3. Using scissors, make a small incision in the cricothyroid ligament superior to the sutures.

4.4. Insert the IV catheter into the opening and advance the tip beyond the two loose sutures.

4.5. Tighten the sutures around the trachea and open the stopcock. Allow the formalin to enter the lungs by gravity and wait for 5 min for the lungs to fully inflate. If the lungs adhere to the ribcage during inflation, grasp the outside of the ribcage with blunt tipped forceps and move in all directions to assist in freeing the lobes. Do not make direct contact with lungs.

4.6. After 5 min, back the IV catheter beyond the first suture and ligate. Repeat for the second suture. The lungs are now inflated in a closed, pressurized state.

5. Casting the vasculature

5.1. In a 1.5 mL tube, prepare 1 mL of an 8:1:1 solution[8] of polymer:diluent:curing agent and gently invert several times to ensure good mixing.

5.2. Remove the plunger from a 1 cc syringe, cover the opposite end with a gloved finger, and pour the polymer compound into the syringe. Carefully reinsert the plunger, invert, and advance the plunger to remove all air and form a meniscus at the tip of the syringe.

5.3. Remove the SNP/PBS syringe from the hub of the needle and drip additional PBS into the hub to create a meniscus. Carefully check the hub for trapped air, dislodge if necessary, and reform the meniscus. Join the hub to the syringe filled with the polymer compound.

NOTE: Creating a meniscus on both ends significantly reduces the chance for air to enter the system.

5.4. Attach the polymer compound filled syringe to the syringe pump and infuse at 0.02 mL/min.

NOTE: For smaller lungs, a slower rate can be helpful to prevent overfilling but, is not essential.

5.5. Monitor the compound as it freely moves down the PE tubing and note the syringe volume as it enters the PAT. Continue filling until all lobes are filled completely down to the capillary level and stop the syringe pump. Check the syringe volume again.

NOTE: After several runs an estimated volume can be used to gauge an approximate endpoint (~35 µL for an adult mouse and ~5 µL for a P1 pup). After the pump is halted, the residual pressure in the system will continue to push the polymer compound into the pulmonary arteries. All lung lobes should fill at a similar rate.

5.6. Cover the lungs with a fiber optic cleaning wipe, liberally apply PBS, and allow the carcass to sit undisturbed for 30-40 min at room temperature. During this period, the polymer compound will cure and harden.

5.7. Remove the catheter, sever the arms/lower half of the mouse, and place the head/thorax into a 50 mL conical filled with 10% buffered formalin overnight.

5.8. After fixation, grasp the trachea and gently separate the heart/lung unit from the remaining rib cage and thorax. Place the heart/lung block in a formalin filled scintillation vial. Discard the rest.

6. Casting alternative vascular beds for casting (Table 1)

NOTE: Each target vascular bed may require different catheter placements, infusion rates, and optimal filling times. Thus, multiple animals will be necessary to cast multiple organs.

6.1. For systemic vascular beds superior or inferior to the diaphragm follow steps 1.1-2.5 as above. See additional notes on the portal system and diaphragm (**Table 1**).

6.2. Grasp the xiphoid process with a hemostat and cut the ribcage bilaterally (roughly in the midaxillary line) just prior to the internal thoracic arteries.

6.3. Fold the still connected ribcage over such that it is resting on the animal's neck/head, fully exposing the chest cavity.

6.4. Follow step 3.1 above, then remove the lungs. Once the thoracic aorta (TA) is visible, hook the tips of curved forceps underneath it, ~10 mm superior to the diaphragm. Grasp a 3 cm length of 7-0 silk, pull back through the opening under the TA, and create a single-throw loose suture. Repeat this procedure ~8 mm above the diaphragm.

6.5. For structures superior to the diaphragm, use spring scissors to create a small hole (~30% of the total circumference) on the ventral portion of the TA, ~2 mm inferior to the loose sutures placed in step 6.4.

6.5.1. For structures inferior to the diaphragm, instead, create a small hole ~2 mm superior to the loose sutures.

6.6. Depending on the animal size, introduce Unit 1 or 2 into the vessel, advance beyond loose sutures, and gently ligate the vessel.

6.7. Follow step 3.7, setting the syringe pump at a rate of 1.0 mL/min and perfusing a minimum of 5 mL. Perfusate will exit via the IVC.

6.8. Follow steps 5.1 - 5.4 adjusting the infusion rate to 0.05 mL/min, visually monitoring the target tissue in real-time.

NOTE: Infusion volume will be organ and animal age specific. The volume can be further limited by ligating arterial branches leading to non-target vascular beds (i.e., brain, liver, kidney, intestine).

6.9. Follow 5.6 then remove target tissue and place in formalin.

7. Sample mount, scan, and reconstruction for micro-CT

7.1. Using paraffin film, create a flat surface on the scanning bed and center the wet sample on this surface (**Figure 3A**).

NOTE: If motion artifact is detected, the sample may require further stabilization.

7.2. Lightly tent/cover sample with additional paraffin film to prevent dehydration. Take special care not to rest the paraffin film on the sample causing deformation to the tissue (**Figure 3B**).

7.3. Scan the sample using settings outlined in **Table 2** and standardize these parameters within a given experiment.

NOTE: This is experiment/endpoint dependent. Standardize the chosen parameters for the ease of comparison between samples.

7.4. Transfer the reconstructed scans for post-processing and analysis.

REPRESENTATIVE RESULTS:

A successful cast will exhibit uniform filling of the entire pulmonary arterial network. We demonstrate this in C57Bl/6J mice ranging in age: Postnatal day P90 (**Figure 4A**), P30 (**Figure 4B**), P7 (**Figure 4C**), and P1 (**Figure 4D**). By controlling the rate of flow and visually monitoring the fill in real-time, reliable endpoints of the most distal vasculature were achieved (**Figure 5A**).

Common challenges include damage to the lungs, incomplete filling, underfilling, or overfilling, wedging the catheter, and animal size.

If there is damage to the lung/airway, small leaks will prevent the lungs from holding pressure (**Figure 5B,C**). In the absence of complete inflation, it becomes difficult to make accurate quantitative and spatial comparisons across samples. To minimize the risk to the lung parenchyma, avoid cutting too closely to the lungs when removing the ribcage and keep the lungs moist with PBS throughout the procedure to avoid dehydration and adherence to surrounding structures. If a lobe adheres to the rib cage during inflation, gently grasp the outside of the ribcage (away from the lung) with forceps and move it in a direction to free the lobes. Alternatively, a blunt instrument, such as a spatula, with a smooth edge can be used to lift or push the inflated lung away from the ribcage. When inflating the lungs, adhere to suggested pressure parameters and avoid over-inflation as this can lead to rupture of the airway. Finally, do not remove lungs from thoracic cavity until post-fixation is complete. The trachea, lungs, and heart should be removed en bloc from the remaining portions of the thoracic cavity.

Patchy (**Figure 5D**) or incomplete (**Figure 5E**) filling can arise from an “airlock”, in which air was introduced into the vascular system via the catheter, blocking downstream flow of the compound. To minimize the chance of an airlock, vigilantly purge air from the tip of the catheter prior to insertion (Step 3.4) and during the syringe transition from SNP/PBS to polymer compound. If the fill remains patchy or incomplete, it could be an indication of increased vascular resistance as a result of focal/long segment stenosis or tortuosity. Blood clots can also lead to incomplete filling and are easily avoided by using heparin prior to the procedure.

Improper injection volume will lead to underfilling or overfilling. Underfilling occurs when too little compound was introduced into the vasculature (**Figure 5F**). Alternatively, overfilling, or introducing too much polymer compound too rapidly can cause either arterial rupture (**Figure 5G**) or, more commonly, venous transit (**Figure 5H**). Both problems can be alleviated by using a syringe pump. Investigators should carefully adhere to the proposed rate and volume restrictions or establish their own rates based upon their specific model and optimization. Monitoring polymer compound perfusion in real time under magnification is critical, and filling of small arterioles/capillaries should be used as an endpoint.

Advancing the catheter too far down the pulmonary trunk can cause the tip to wedge into one pulmonary artery branch and create an imbalance in the flow. As a result, one side fills faster than the other (**Figure 5I**), which frequently leads to overfilling in one lung and underfilling in the other. While catheter wedging is the most likely reason in this scenario, "airlock" and lack of heparin can also be contributing factors.

Finally, smaller animals present their own set of additional obstacles. Younger animals demand steady hands and small mistakes are less forgiving. High quality instruments, specifically designed for microsurgery, become more important at early postnatal timepoints. Use of a micromanipulator assists greatly in not only placement but preventing catheter dislocation. It is also essential to utilize the syringe pump on small animals to accurately control and manage endpoints.

While specifically shown for the pulmonary vasculature, this procedure can easily be applied to systemic target vascular beds as well (**Table 1**). In addition to the challenges listed above, choosing the right entry point is crucial. Casting via the thoracic aorta produces excellent results for most vascular beds. It should be noted, however, that inserting the catheter as proximal to the target site as possible and ligating non-target vasculature assists in flow and volume control. These refinements combined with appropriate direct monitoring of distal vascular endpoints (**Figure 6A-F**) and standard infusion rates optimize the filling. Many examples of such casting methods exist in the literature and are too numerous for complete referencing. However, additional details may be found in organ specific text such as these[4,5,7,18-21].

After casting, samples can be processed for µCT scanning (**Figure 7A,B**). For post-processing, a commercial software package (see **Table of Materials**) produced a 3D volume rendering of the pulmonary vascular tree presented as still images (**Figure 7C**), or movies. Further statistical analyses exploring vascular characteristics such as segment length and number, tortuosity, order (generation or rank), volume, and arcade length can also be performed. In addition to µCT scanning, the casted samples can also be cleared to obtain gross images or processed and cut for histological analysis[8].

FIGURE AND TABLE LEGENDS:

Figure 1: Catheter and needle setup. Syringes are shown with attached tubing and needles (Unit1 and Unit2). Inset: closeup of needle and tubing.

Figure 2: Lung inflation setup. Ring stand, clamp, a syringe filled with formalin, and tubing with a catheter attached.

Figure 3: Micro-CT sample preparation pre-scan. (**A**) Here the specimen was centered on a paraffin film base, (**B**) Here the sample was centered and covered on the parafilm base.

Figure 4: Vascular-casted lungs at varying developmental stages from 3 months to 1 day old. Dorsal view of lungs, (**A**) P90, (**B**) P30, (**C**) P7, and (**D**) P1

Figure 5: Examples of ideal filling and common errors during polymer compound infusion. (A) When filling endpoint was reached, a robust and fine vascular network was observed. (**B**) Fully inflated formalin perfused lungs are represented by a white dashed line, (**C**) Underinflated/deflated lungs are shown. This was observed due to a compromised pulmonary airway. The original inflated position is represented by a white dashed line and the deflated position is represented by a black dotted line, (**D**) Patchy filling: the vasculature of portions of the lobe remains unfilled while other areas were entirely filled, (**E**) Incomplete filling: the polymer compound failed to penetrate entire sections of lung, (**F**) Underfilling: the polymer compound failed to fill distal vasculature, (**G**) Rupture: the arrow is pointing to the polymer compound extruded from vasculature, (**H**) Venous filling: note the arrow pointing to the arterial segments entirely filled and extending into the venous system. Veins and venules were of significantly larger caliber, (**I**) Catheter wedge: Here the catheter was shunted into one artery preventing the vasculature of the right lobes from filling completely while the left lobe was overfilled.

Figure 6. Vascular casting and endpoints in additional organs. (**A**) Kidney: the punctate appearance of polymer compound in the glomerulus provided the endpoint. (B) Liver: note the small vessels visible at the edges of the organ. (**C**) Stomach: small vessels were visible and fully filled. D. Large intestine: Small vessels are easily identifiable and filled. (**E**) Diaphragm: the muscle here is thin and translucent with small filled vessels apparent. (**F**). Brain: small vessels were visible in the cortex.

Figure 7. CT images and 3D volume rendering of polymer compound filled lungs. (**A**) A single grey-scaled reconstructed lung slice, (**B**) This was a maximum intensity projection of a CT scan produced from polymer filled lungs, (**C**) A 3D volume rendering of the vascular arcade was generated using commercially available software (see **Table of Materials**).

Table 1. Casting alternative vascular beds.

Table 2. µCT Scanning Parameters.

DISCUSSION:

Executed properly, this method yields striking images of pulmonary arterial networks, allowing for comparison and experimentation in rodent models. Several critical steps along the way

ensure success. First, investigators must heparinize the animal in the preparatory stage to prevent blood clots from forming in the pulmonary vasculature and chambers of the heart. This allows for the complete arterial transit of polymer compound. Second, when puncturing the diaphragm and removing the ribcage, take care to protect the lungs from inadvertent damage, cuts or injury. Any leak in the airway will prevent complete inflation and render comparisons between samples inaccurate. Third, tethering the heart at the apex aids catheter placement. Fourth, the use of a strong vasodilator such as SNP will assist in both the removal of blood and complete filling of arterioles and capillaries[5,8]. Fifth, when placing the catheter into the PAT, take care not to bury the tip into the bifurcation. This will cause an imbalance in flow, shunting polymer compound to either the left or right side, yielding an unequal pressure gradient. Sixth, the use of a syringe pump will allow the user to control the rate and titer the volume to both mouse strain and age. Lastly, leave the heart/lungs attached to the remainder of the thoracic cavity, fix overnight, and remove the following day. The lungs will be well fixed and the potential for deflation due to accidental nicks during separation will be minimized.

While this methodology achieves the desired results, alternative techniques may be helpful to some users. To aid in the placement of the catheter, a micromanipulator may be employed. We chose a version with a small profile and magnetic base to minimize encroachment in an already limited working area while providing a stable base (if using a magnetic base make sure to place a steel plate under the working space to allow the magnet to engage). This allows the user to precisely place the tip of the catheter in the PAT at an angle that follows the natural trajectory of the artery. Additionally, the catheter is secure and at less risk of being dislodged. Another option is the use of a trumpeted catheter tip[8]. While not trivial to create, a trumpeted catheter is far more secure and less inclined to accidentally slide out of the PAT. Changing the ratio of polymer:diluent alters the viscosity and the ease with which small vessels are filled. Depending on the target vasculature and experimental endpoints this can be a valuable consideration. Euthanasia via CO_2 may cause pulmonary hemorrhage in a small percentage of animals and is strain dependent[22]. Consider an alternative euthanasia protocol should this impact experimental endpoints. When inflating the lungs, the use of formalin aids fixation of the organ in place at the given pressure. A physiologically neutral buffer can be substituted should peripheral vessels need to be filled in an unfixed state. If infusion rate and control are of less importance to a given experiment, perfusion by hand is also possible. Hand injection requires practice and real-time monitoring under magnification to avoid overfilling or vessel rupture[8]. Finally, the tissue mount/conditions, scanning parameters, and minimal post-processing we employed for this paper should serve merely as a starting point. Different scanners, tissues, experimental endpoints/user needs may demand alternative parameters.

While the vascular images generated from this technique are impressive, there are limitations. Primarily, the above method is suboptimal for measuring vascular caliber due to the inability to monitor and control intravascular pressure during the infusion. Other groups have managed to somewhat address these pressure concerns in systemic vasculature by monitoring driving pressure[4,23], however, such concerns are further amplified on the pulmonary side due to the relatively thin pulmonary artery walls that are easily distensible with small changes in pressures[24] and the inability to precisely measure and statically control pulmonary intravascular pressure.

A second limitation to this method is that it remains a postmortem, single timepoint experiment, limiting its utility in studies that require truly physiologic conditions or a time course. Other, live animal measures, such as CT pulmonary angiography (CTPA) or contrast-enhanced μCT (CE-CT) offer the possibility of functional and morphologic measures. Repeated scans/longitudinal studies as well as measurements at different points in the cardiac/pulmonary cycle, can be explored[10,25-28]. These methods can be reliably used, in addition to echocardiography, to measure the arterial caliber. However, both CTPA and echocardiography measures are currently limited to the assessment of the proximal vasculature. For echocardiogram, the assessment is limited to the pulmonary trunk while CTPA allows adequate calculation of the branch pulmonary artery caliber potentially 1-2 orders further, but resolution is limited, obscuring distal portions of the vasculature[7]. Radiation dosage is also a concern that should be carefully monitored when using CT especially in multi-scan longitudinal studies[29,30]. For either of these applications, μCT equipment, scan time, and analysis software may be expensive and require specialized staff training. Animal imaging core facilities at some institutions may ease this burden.

As an alternative to this compound, some groups utilize traditional corrosion casting techniques accompanied by soft tissue removal[31,32]. These methods yield results similar to this polymer compound, but the end product is brittle, leading to potential artifact[15]. In addition, the removal of soft tissue eliminates the potential for future histology[33]. Another option is to leave the soft tissue intact and perform a follow-up step wherein the soft tissue is "cleared" rendering the sample virtually transparent[34,35]. Tissue clearing gives the user some ability to see deeper within a sample but, on the whole, remains inferior to μCT as it cannot provide the same 3D visualization. Serial histologic sectioning and array tomography are methods that offer exceptionally high resolution. While this technique opens the door to exciting new possibilities, the workload is exponentially higher and not particularly conducive to large cohorts[11,12]. 3D x-ray histology is a non-destructive approach that couples both μCT and traditional histology or even EM[36-38]. It takes a more high level view of pathology by utilizing μCT to globally identify and accurately scout regions of interest that are then followed up with routine histology[39]. Substituting lower resolution contrast agents (or in some cases no contrast) with polymer compound into the vasculature might serve to elevate both techniques when possible. Another non-destructive approach that is computationally intensive yet, potentially enhances the contrast, is phase-retrieval μCT imaging[40,41]. This method can be valuable when employed on noisy data where contrast is weak or not possible[42]. The polymer compound employed in this technique, however, does not suffer from this limitation. That said, phase-retrieval may be useful where the polymer compound is possibly diluted, for example in distal vasculature[43]. Finally, stereology has been a standard in lung quantitative structural analysis for years[44]. It uses random, systematic sampling on cross sections of tissue to make 3-D inferences assuming that the chosen samples are sufficiently representative. While a powerful tool, it has the potential to lead to error and bias. Combining CT imaging with stereology, however, holds great promise[45].

The outlined method is relatively straightforward and with training a success rate of >90% is achievable. Once mastered, it allows for the complete and reliable casting of lung vasculature. In fixative, tissue and polymer remain stable indefinitely for future scans, potential histology, or

EM[46,47]. We've shown that this technique can be used in animals as young as P1 through adulthood and believe embryonic casting, via the pulmonary artery, is within reach. It should be noted that this technique can be applied to virtually any other vascular bed by simply altering the catheter entry point and determining appropriate endpoints.

ACKNOWLEDGMENTS:

This research was supported in part by the NHLBI Intramural Research Program (DIR HL-006247). We would like to thank the NIH Mouse Imaging Facility for guidance in image acquisition and analysis.

DISCLOSURES:

The authors have nothing to disclose

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663

Figure 1

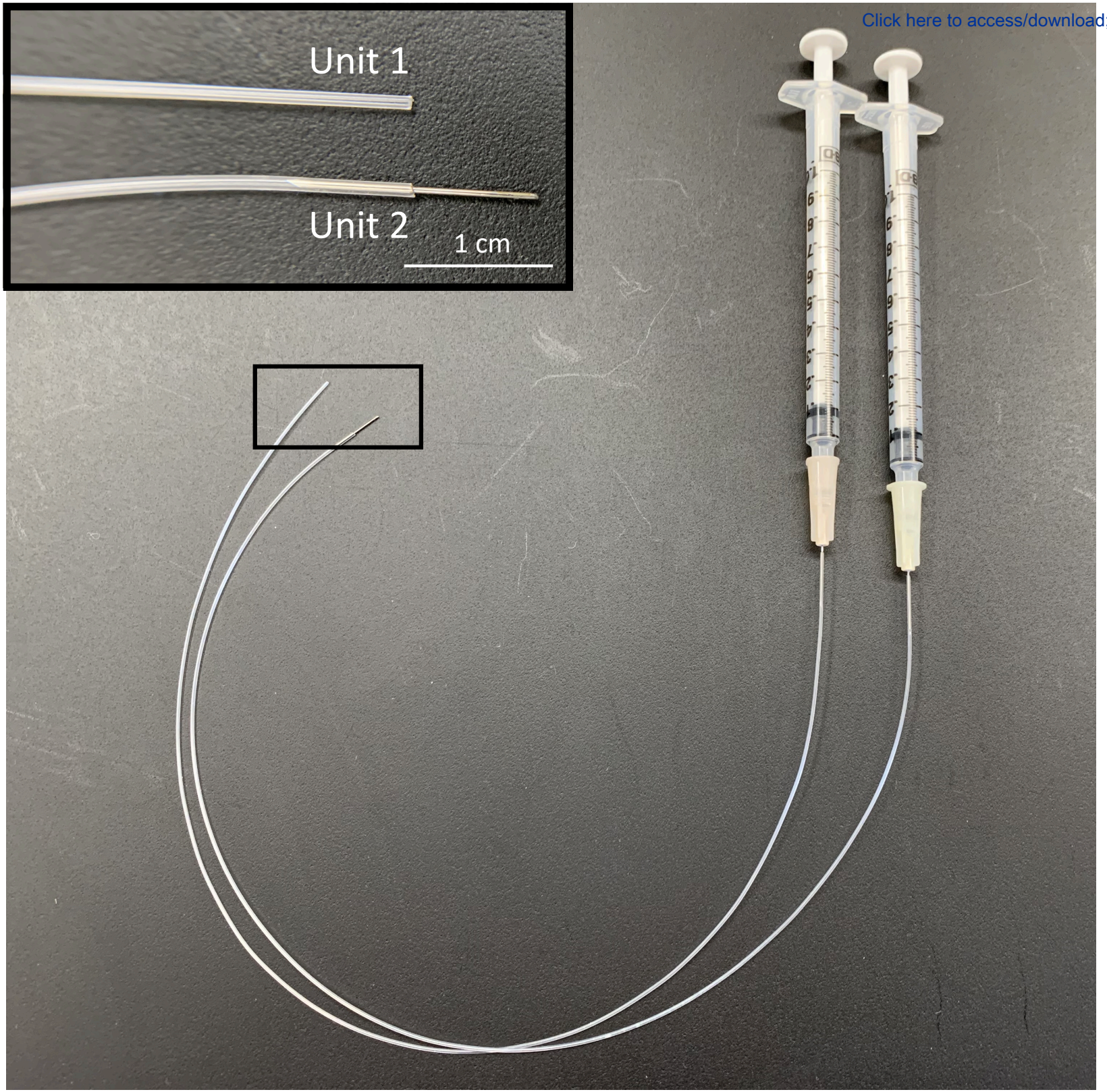

Figure 2

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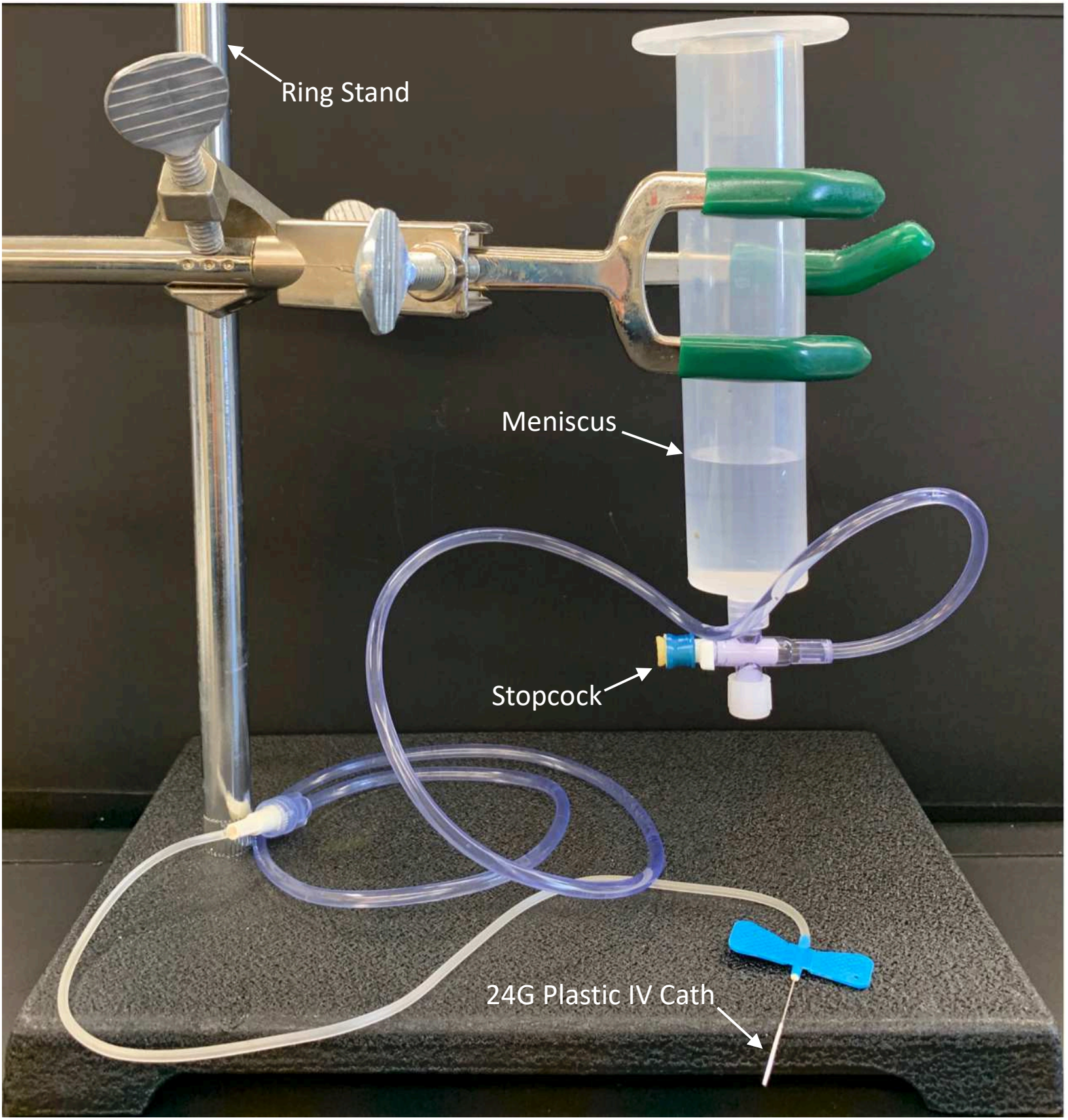

Figure 3

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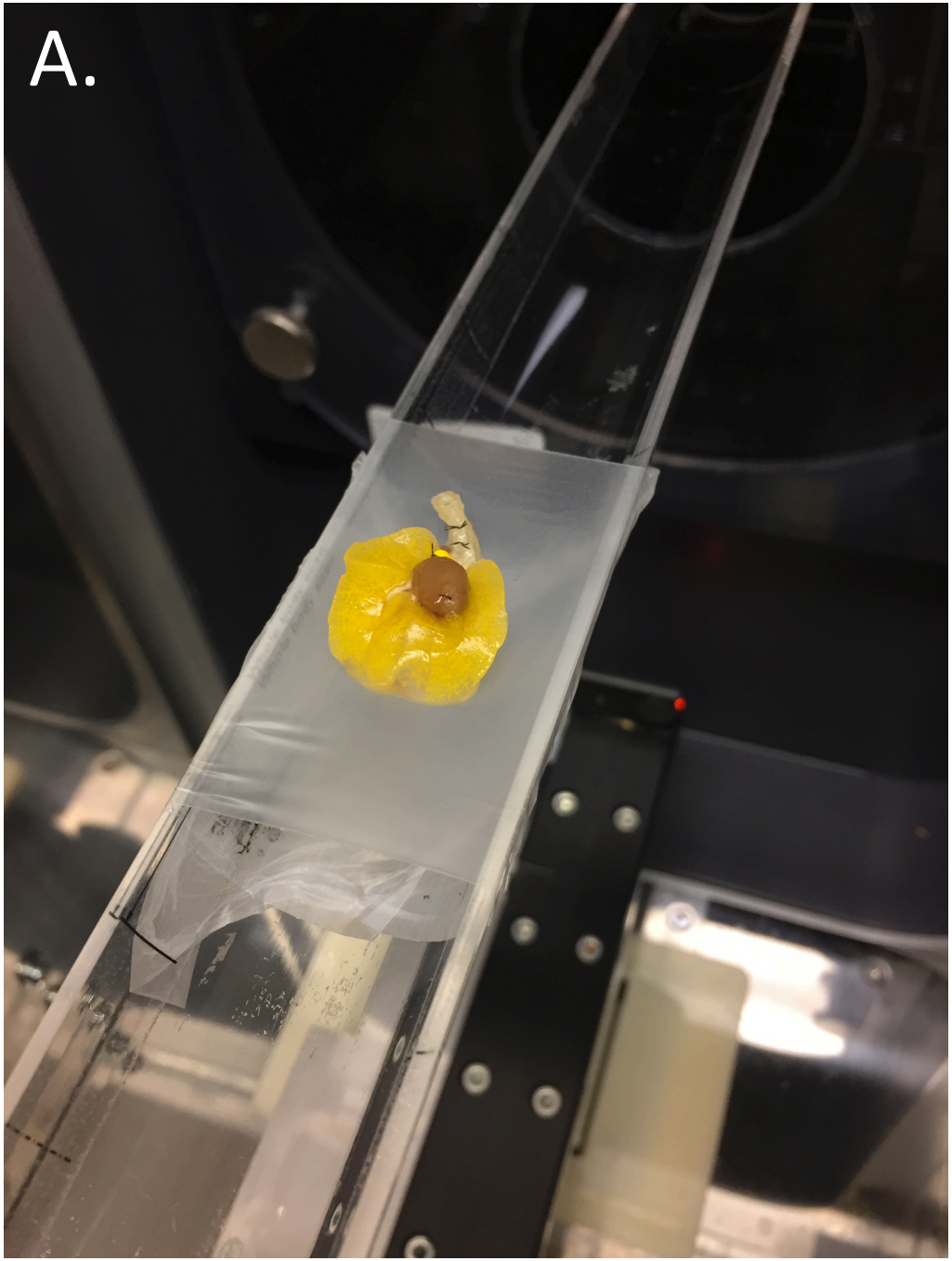

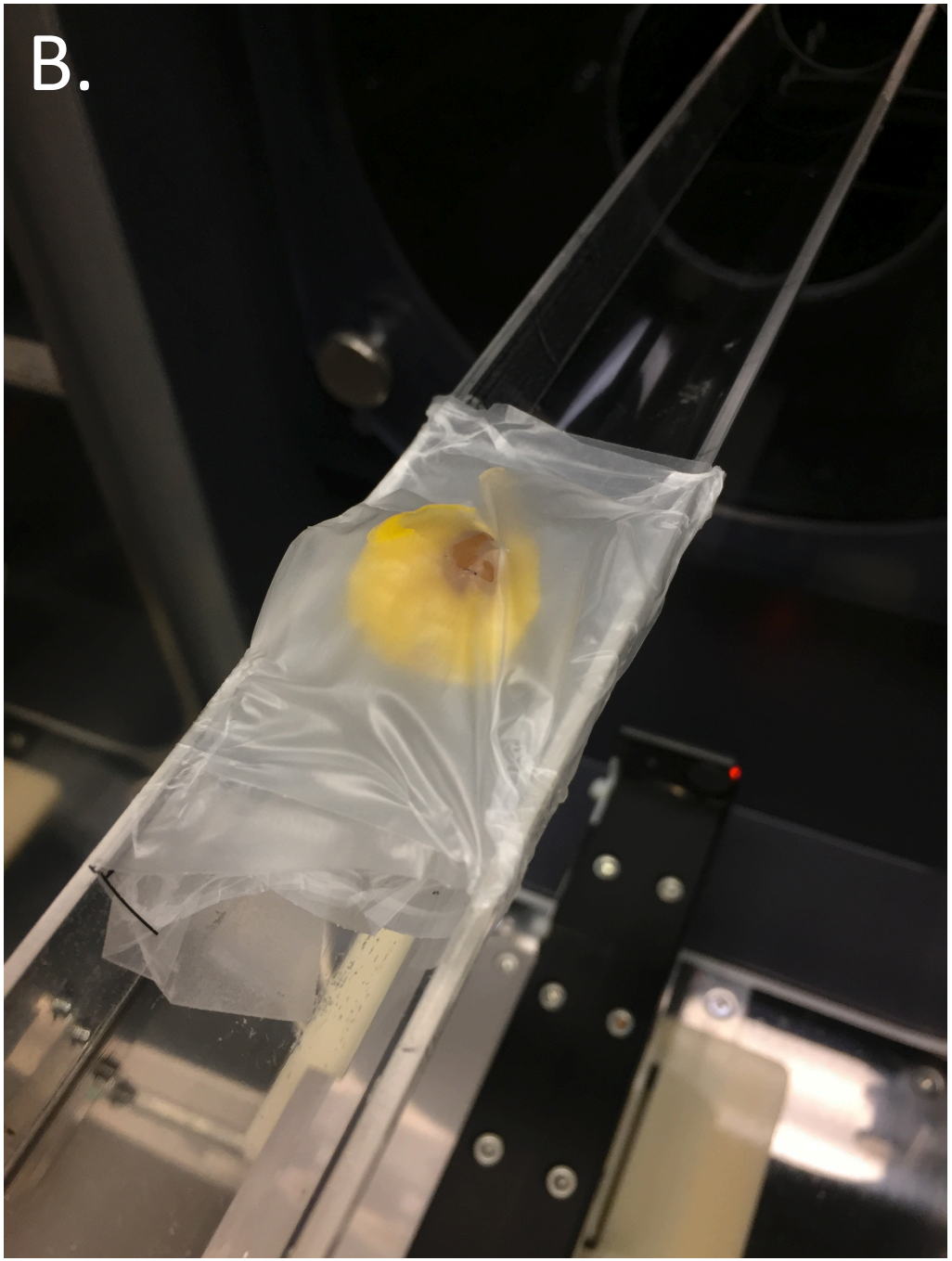

Figure 4

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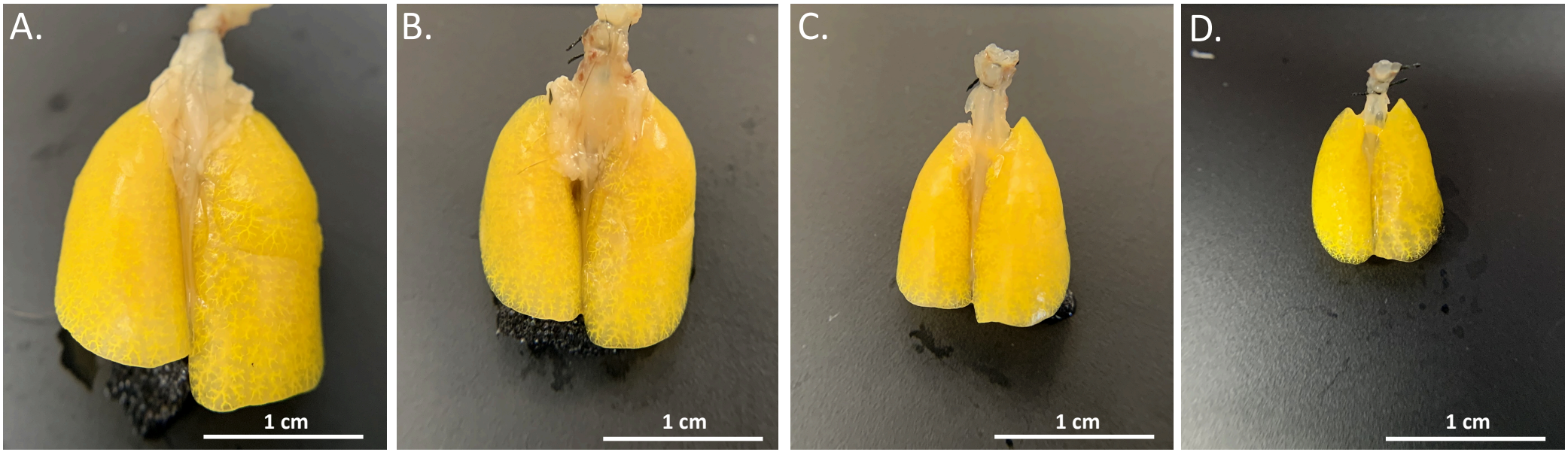

Figure 5

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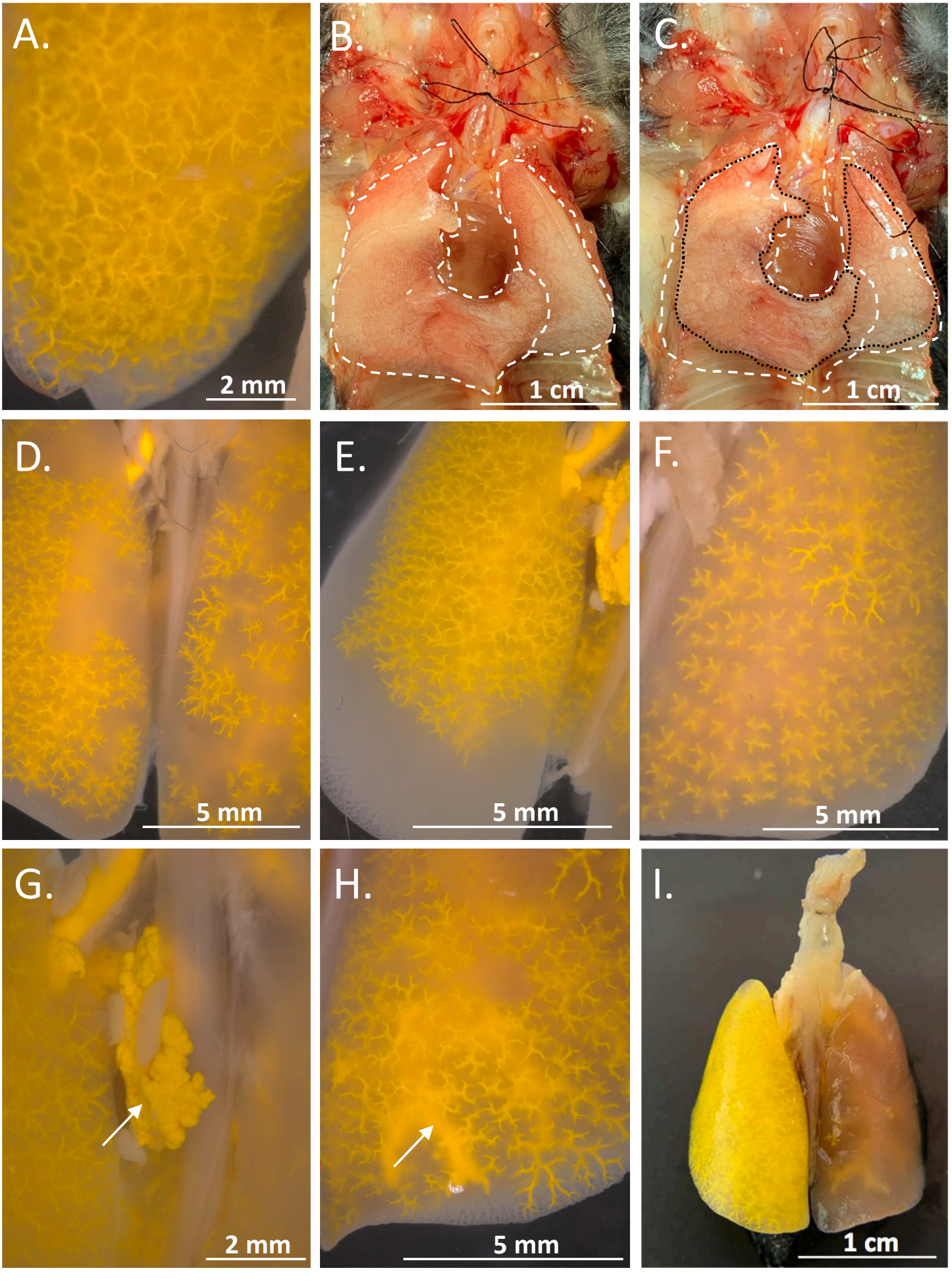

Figure 6

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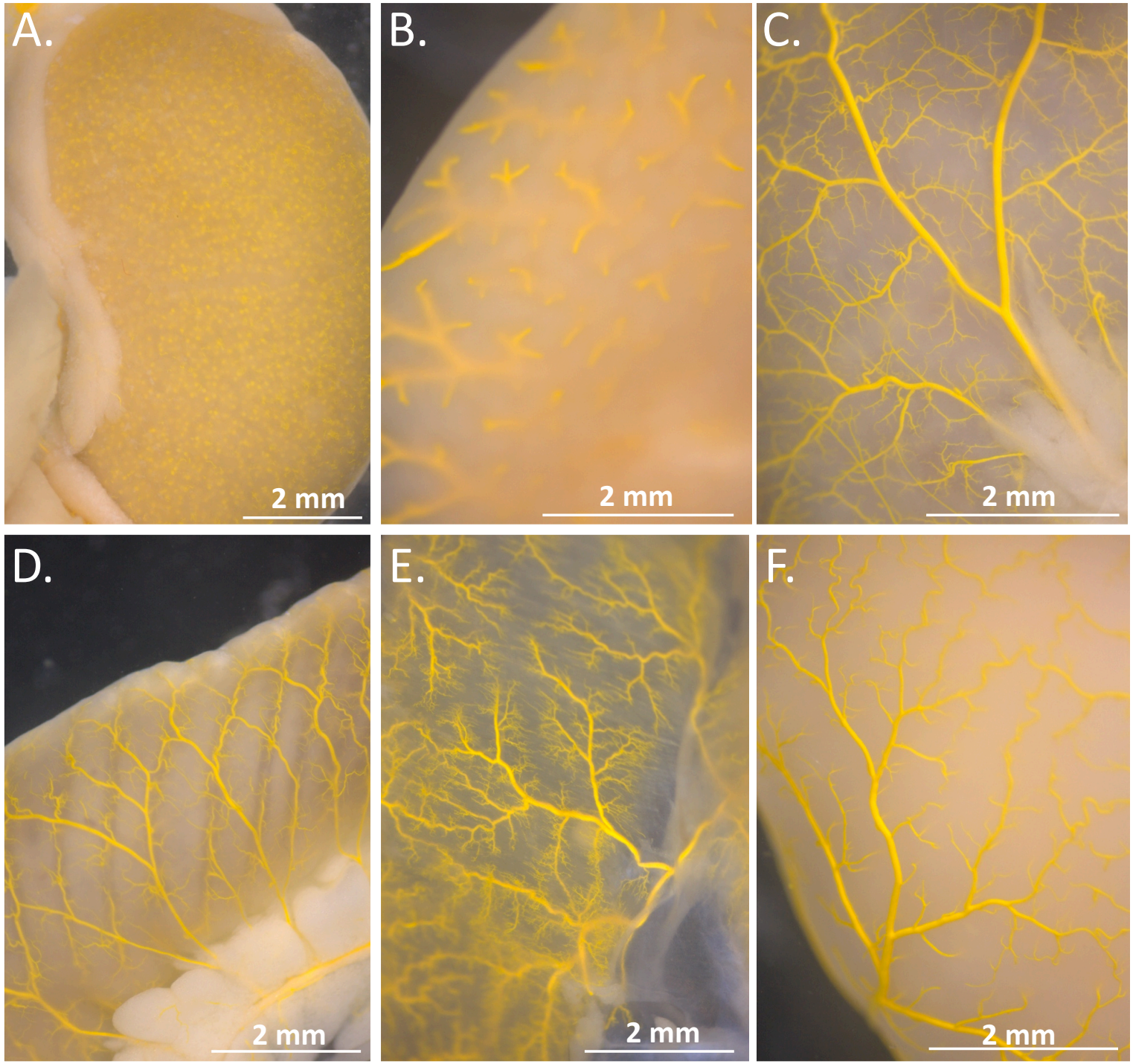

Figure 7

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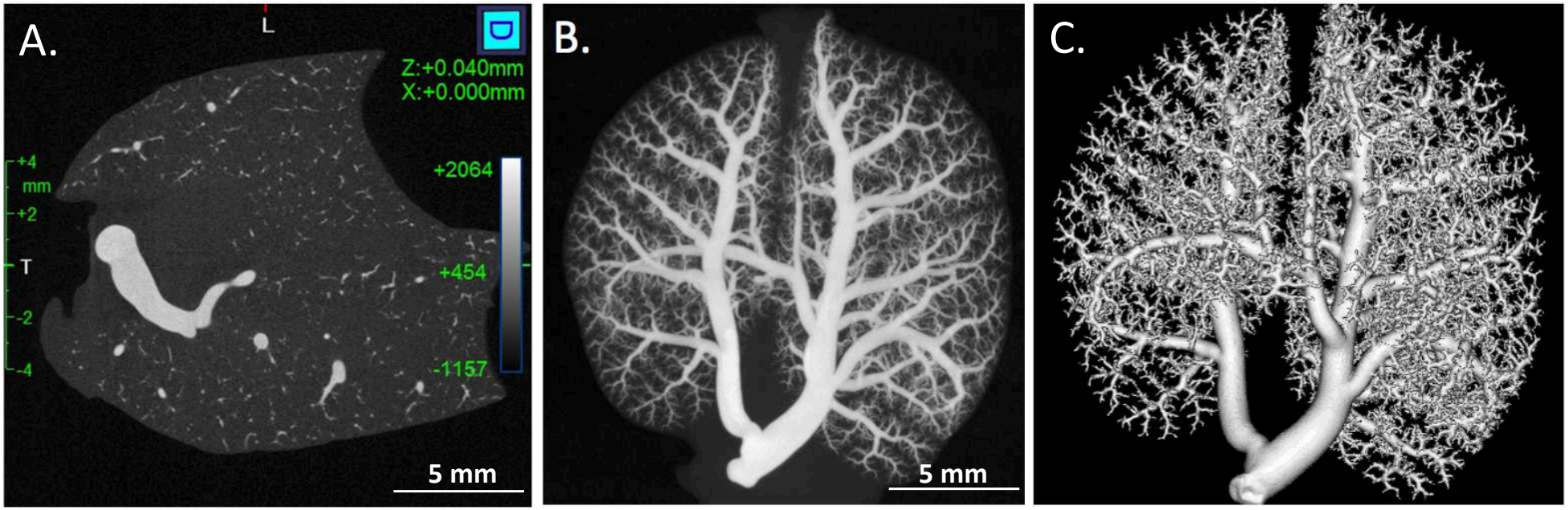

Table 1

Target Arterial Vascular Bed	Catheter placement	Infusion direction	Infusion rate	Notes
Brain	Thoracic aorta pointing cranially	Retrograde into the carotids	.05ml/min	Cannulate thoracic aorta, flip mouse to the prone position, open scalp, and visually monitor progress of polymer through skull.
Diaphragm	Left Ventrical	Anterograde into internal thoracic, phrenic, and intercostal	.05ml/min	Open a window in the side of the ribcage, leaving the majority of the ribcage and the diaphragm intact. Cannulate left ventrical, clip right atrium, and monitor progress from the caudal side of the diaphragm.
Upper limb musculature	Thoracic aorta pointing cranially	Retrograde into the brachiocephalic and left subclavian	.02ml/min	To optimize limb flow, tie off the carotid arteries and remove limb skin to allow visual monitoring of polymer transit into the limb musculature.
Kidney	Thoracic aorta pointing caudally	Anterograde into renal arteries	.05ml/min	The internal vasculature is filled blindly. To avoid venous transit, stop injecting when polymer is visible in a uniform punctate pattern across kidney.
Portal System	Portal vein	Anterograde into portal system	.02ml/min	Gently fold liver up to expose the portal vein.
Hepatic	Thoracic aorta pointing caudally	Anterograde into the hepatic artery	.05ml/min	Tie off portal vein prior to infusion to avoid venous transit from gut flowing into liver.
Stomach/ Intestine	Thoracic aorta pointing caudally	Anterograde into the celiac, superior mesenteric and/or inferior mesenteric	.05ml/min	Some regions of the gut are supplied by multiple arteries and may fill at different times. To avoid venous transit, tie off arteries not required for areas of interest and visually monitor the progress of the polymer.

Intra-abdominal fat pads	Thoracic aorta pointing caudally	Anterograde but vessel depends on fat pad being studied	.05ml/min	Fat pads are supplied by multiple arteries and may fill at different times. To avoid venous transit, tie off arteries not required for precise area of interest and visually monitor the progress of the polymer.
Lower limb musculature	Infrarenal aorta pointing caudally	Anterograde into the femoral arteries	.02ml/min	Remove limb skin to allow visual monitoring of polymer transit into the limb musculature.

Table 2

CT settings	
kVp	90
Target Material	Tungsten
Power	8W
Filtration	Cu 0.06 mm + Al 0.5 mm
Projection Number	6424
Detector Size	Flat panel CMOS - 2944 x 2352 pixels
Field of View (FOV)	36 mm
Voxel Size	72 µm
Spatial Resolution	voxel size x 1.5
Acquisition Time	14 min
Reconstruction	FBP and commercial algorithm
Binning	1x1

Table of Materials

Click here to access/download;Table of Materials;Materials_JoVE_Knutsen Revised.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1cc syringe	Becton Dickinson	309659	
20ml Glass Scintillation Vials	Fisher	03-340-25P	
30G Needle	Becton Dickinson	305106	
50mL conical tubes	Cornin	352098	For sample Storage and scanning
60cc syringe	Becton Dickinson	309653	
7-0 silk suture	Teleflex	103-S	
Analyze 12.0 Software	AnalyzeDirect Inc.	N/A	Primary Software
Amira 6.7 Software	Thermo Scientific	N/A	Alternative Sofware
CeramaCut Scissors 9cm	Fine Science tools	14958-09	
Ceramic Coated Curved Forceps	Fine Science tools	11272-50	
CO2 Tank	Robert's Oxygen Co.	n/a	
Dual syringe pump	Cole Parmer	EW-74900-10	
Dumont Mini-Forceps	Fine Science tools	11200-14	
Ethanol	Pharmco	111000200	
Formalin	Sigma - Life Sciences	HT501128	
Gauze	Covidien	441215	
Hemostat	Fine Science tools	13013-14	
Heparin (1000USP Units/ml)	Hospira	NDC 0409-2720-	
Horos Software	Horos Project	N/A	Alternative Sofware
induction chamber	n/a	n/a	
Kimwipe	Fisher	06-666	fiber optic cleaning wipe
Labelling Tape	Fisher	15966	
Magnetic Base	Kanetec	N/A	
Micro-CT system	SkyScan	1172	
Microfil (Polymer Compound)	Flowech Inc.	Kit B - MV-122	8 oz. of MV compound; 8 oz. of diluent; MV-Curing Age
Micromanipulator	Stoelting	56131	
Monoject 1/2 ml Insulin Syringe	Covidien	1188528012	
Octagon Forceps Straight Teeth	Fine Science tools	11042-08	

Parafilm	Bemis company, Inc.	#PM999	
PE-10 tubing	Instech	BTPE-10	
Phospahte buffered Saline	BioRad	#161-0780	
Ring Stand	Fisher	S13747	Height 24in.
Sodium Nitroprusside	sigma	71778-25G	
Steel Plate	N/A	N/A	16 x 16 in. area, 1/16 in thick
Straight Spring Scissors	Fine Science tools	15000-08	
SURFLO 24G Teflon I.V. Catheter	Santa Cruz Biotechnology	360103	
Surgical Board	Fisher	12-587-20	This is a converted slide holder
Universal 3-prong clamp	Fisher	S24280	
Winged Inf. Set 25X3/4, 12" Tubi	Nipro	PR25G19	
Zeiss Stemi-508 Dissection Scope	Zeiss	n/a	

nt

Dear Editor and Reviewers,

Thank you for your comments. Please see a detailed response to the concerns below.

Russ Knutsen and Beth Kozel

Editorial comments:

Changes to be made by the Author(s):

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. Addressed as requested
2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points Addressed as requested
3. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol. Word count: 163
4. Please describe all abbreviations during the first time use. Addressed as requested
5. Please expand the Introduction to include all of the following: This was done as requested with the relevant responses being found in the following lines.

a) A clear statement of the overall goal of this method line 62-63 and 65-68

b) The rationale behind the development and/or use of this technique line 63-65 and 70-77

c) The advantages over alternative techniques with applicable references to previous studies line 77-85

d) A description of the context of the technique in the wider body of literature lines 70-77

e) Information to help readers to determine whether the method is appropriate for their application lines 87-93

6. JoVE cannot publish manuscripts containing commercial language. 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 and Reagents. E.g., Instech, Plymouth Meeting, PA, Sigma, St. Louis, MO, Beckton Dickinson, Franklin Lakes, NJ, Teleflex, Coventry, CT, Microfil, Kimwipe, etc. Addressed as requested
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” Addressed as requested
8. The Protocol should contain only action items that direct the reader to do something. Addressed as requested
9. Please ensure you answer the “how” question, i.e., how is the step performed? Please include all the button clicks in the software, knob turns etc. Please use complete sentences to

describe the action. Addressed as requested (See changes to steps 2.6, 2.8, 3.1, 3.2, 3.4, and 6.4)
10. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. 121 non-continuous lines (2.75 pages) have been highlighted accounting for headings and spaces in the clean version of the manuscript.
11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." N/A
12. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. Please ensure all figures are referenced in the text. For the figures describing the protocol, please reference them in the protocol section. Addressed as requested
13. Please do not abbreviate the journal titles in the reference section. Addressed as requested
14. Please sort the table in alphabetical order. Addressed as requested

Reviewers' comments:
Reviewer #1:
In their manuscript entitled "Vascular Casting of Adult and Early Postnatal Mouse Lungs for micro-CT Imaging", the authors detail the casting process of mouse lungs as means of visualising the vascular tree in 3D using µCT.

The casting method is described in great detail and discussed adequately in the discussion section.

However, as it is stated in the title the casting is done specifically for volume imaging by means of µCT, and that second-leg of the study is almost completely absent from both the "protocol" and "discussion" sections. This needs to be addressed so that the manuscript can meet readers'/viewers' expectations. Specifically:

In "protocol" A new section in the protocol (Section 7 - lines 327-343) and discussion (lines 518-521) has been added to briefly address these concerns. Additionally, a new table identifying the parameters we chose (Table 2) and a Figure (Fig 3; Legend – lines 434-436) clarifying the sample mount have been included. Different users' experiences may vary based on their available equipment. Consequently, we've focused on the most generalizable approach. We've also limited discussion of post-processing specifics as there are many different analysis options that far exceeds the scope of this paper. We hope this addresses your concerns.
*Please give details about the imaging protocol used for imaging the casted tissue; That is: kVp, target material, power, filtration, number of projections, size of the detector, voxel size, spatial resolution and acquisition time. Please see Table 2 for these parameters.

*Please give details about the sample mounting approach and scanning environment. Was the samples scanned wet or dry? How it was stabilised? This has been addressed in the new protocol section (step 7.1 and 7.2). The CT scanner we used uses a gantry that rotates around the sample vs. the sample actually rotating. Stabilization, therefore, is less critical as the sample itself is stationary during the scan.
*How were the data reconstructed? FBP or Iterative reconstruction? Using commercial or custom developed algorithms? Please see table 2 for details.
*Was any post-processing done on the reconstructed data? We performed minimal post-processing, which is described in the figure legend of Figure 7C.

*Please give details of the software used for visualisation. In this case, the software we used was Analyze. JoVE does not allow commercial language in the text. Please see Line 418 in manuscript for general statement and materials table for specific software packages.

In "results"
*Please provide representative single grey-scaled reconstructed slice from the µCT volume (not binarized) so that readers can evaluate the quality of the imaging. This has been added in Figure 7 as panel A. The legend has also been updated.

In "discussion"
*In lines 379 & 397 authors state that this methodology achieves "robust and highly reproducible results" but no data are provided to support these statements nor any relevant literature is referenced. Please rectify or remove the statement. The statement has been removed in three positions (lines 45, 409, and 482).
*Can this method be used to visualise the bronchial tree too, and if so what kind of modifications would be needed (if any)? While this may be useful for proximal structures, once the fill enters the more distal airways, there is not (in our limited experience) enough space between bronchioles/alveoli to allow optimal visualization, grossly or by CT. The other challenge would be filling the airway at a set pressure. At 8:1:1 the microfil is too viscous for gravity to overcome the wall tension of the PE tubing. Using larger tubing and a larger gauge catheter might help overcome this challenge but, accurately measuring inflation pressure would need to be addressed. Given the range of technical challenges that would need to be addressed, we found this to be outside the scope of the current manuscript.

*Can the casting approach described here be used to co-visualise the bronchial and the vascular trees in the same specimen? When studied by CT, the casting reagent is identified by its radiodensity. Loading both the vasculature and proximal bronchial tree with Microfil would likely create challenges when trying to differentiate and visualize the two circuits simultaneously. Using two different casting agents that differ in density might alleviate this but differentiating between both circuits distally would be challenging. This would certainly be interesting and worth investigating but, beyond the scope of this paper.
*Please discuss pros- and cons- of this approach compared to non-destructive µCT approaches such as phase-retrieval µCT imaging and 3D x-ray histology Please see lines 561-570

Reviewer #2:
In this manuscript, authors have described the method of mouse pulmonary artery casting. The manuscript is well written and well organized. However, there are several points that need to be clarified.

Minor points
1. Line 92: Authors euthanize the mice with carbon dioxide. It is known that euthanasia with carbon dioxide damage the alveolar structures and lead to pathological pictures of the alveoli (e.g. pulmonary edema). This point needs to be clarified. Thank you for this comment. Short exposure to CO2 is unlikely to damage alveoli to the extent that it would change lung structure. Damage to pulmonary structures via C02 is also strain dependent. We used pure C57Bl/6 mice and they are less susceptible to pulmonary hemorrhage (BALB/c is more susceptible for example) by this method. Our focus is also vascular. Evidence of damage may manifest itself in the form of deflated lungs, possible leakage of Microfil in terminal vessels, or noted structural differences in vascular architecture post-filling and we have not observed this. A line has been added in the discussion under modifications noting this (line 511-513)
2. Line 123-125: Description of the procedure is hard to imagine, please improve it. Step 2.8 - The language in steps 2.6 and 2.8 has been altered to address this concern (Now lines 133-135 and 140-143). Additionally, these steps have been specifically highlighted for filmable content. The video should further clarify this step.
3. Line 146-149: Description of the procedure is also hard to imagine, please improve it. Step 3.2 – Language has been added to clarify (line 167-172) and this step has been specifically highlighted for filmable content. The video will further clarify this step.
4. Line 156: please describe 1. How to locate the right ventricle (RV) 2. Which part of the RV is excised and 3. Which instrument is used to make incision, 4. How big and how deep is the incision? Step 3.4. - 1. The RV is thin walled and easily distinguishable from the LV. Added "thin walled" to text (line181) and made a note addressing this (lines 188-189). 2. The RV is not excised. An incision, however, is made and location not important. It is merely an access point to the target PAT. Added "toward the apex of the heart" (line180) 3. Added "scissors" to text (line180). 4. Added "penetrating" to text – the depth needs to actually penetrate the RV completely to gain access to PAT (line181). The size should be large enough to accommodate the cath/PE10 tubing. "1-2mm" has been added to text (line180).
5. Line 177: Purpose of fixing the airway is not clear. Does this improve quality of casting procedure and/or quality of the image? The procedure may affect physiological structures of the pulmonary vasculature, especially at the peripheral level. Section 4 – Fixing the airway assists in fixing the lungs internally at the given pressure which is crucial when making comparisons across age/genotype. We used a physiologic pressure to guard against overinflation and mitigate against damage to peripheral structures. Substituting a physiologically neutral buffer (PBS, PSS, etc.) would be an alternative for researchers that require vessels to be cast in an unfixed state. We added language under Discussion/modifications (lines 513-515) to address this.
6. Line 218: Microfill is very viscous and does it go through PE10 and needle smoothly? Step 5.4 - We have had no problems advancing the Microfil through the PE10 tubing. It advances very

smoothly with absolutely no complications. Added “freely” to step 5.5 line 264.
7. Line 221: Please clarify how to prevent the dislocation of the tip of PE-10 during the infusion of Microfill. Please refer to step 3.4 and 3.5. The tightened suture around the PAT does most of the work but taping the distal portion of the PE10 tubing to the surgical board also prevents dislocation. Additionally, in the discussion under modifications, using a micromanipulator and/or creating a “trumpeted” tip can assist in preventing dislocation (line 501-509).
8. Line 230: At room temperature? Step 5.6 – added language.
9. Line 232: Please describe which type of vessels (e.g. 50 ml tube) can be used to fix the bulk tissue (thorax). Step 5.7 – added language (line 278)
10. Line 238: With this procedure (systemic perfusion), all organs (e.g. brain, muscle) in the table can be nicely casted? Please clarify. Section 6 – Yes, complete and comprehensive casts can easily be achieved in each vascular bed. Added language referring to Table1 (line 284) and included a note (lines 286-288)
11. Line 355: Latex means Microfill? Please clarify. Thank you, latex is incorrect and has been removed. Changed to “polymer compound”
12. Please describe detailed settings of micro CT for the imaging. See Table 2.
13. Fig 3 A and B: why right lobes appears to be smaller than left lobe? Now Fig 4A and B - This is a dorsal view and the right side is larger than the left

Reviewer #3:
Manuscript Summary:
In this review paper, the authors set out to describe in detail their state-of-art method for 3-dimensional visualisation of the pulmonary vascular architectures using micro-CT imaging with Microfil contrast agent and vascular casting technique. The manuscript is instructive, well-written and scientifically sound, and would be of interest to the readers of the Journal of Visualized Experiments (JoVE). The quality of the research work presented in the paper is high, and the authors explained the details involved in each step clearly and provided detailed information on tools and materials utilized. Moreover, the authors provided an insightful and interesting discussion with pertinent points relevant to the limitations and potential future directions. However, the manuscript could be further improved by addressing some specific areas. The following are my comments:

Major Concerns:
1. (Line 206) under the subheading of 'Casting the vasculature', the authors described that they prepared a solution mixture of three ingredients: Microfil, Diluent and Curing agent - by the ratio of 8:1:1 respectively.

- However, according to the Flowtech website of the Microfil company (https://www.flowtech-inc.com/about/), the ratio appears to be different - 'occasionally it may be necessary to decrease viscosity by changing the mix ratio to either 2 or 3 parts MV-Diluent for each part MV compound. If your study requires such action, the correct level of MV Curing Agent is 10% (by weight) of the amount of MV compound used.' My reading is that the ratio should be (1): (2-3): (0.3-0.4) of the mixture of the three ingredients: Microfil, Diluent and Curing agent respectfully.

The ratio is very different from the company's recommendation. The authors did not provide reasons for their departure from the recommendation by the company. Is this a typo? If their ratio is uniquely derived from trial and error attempts. It seems pertinent to provide more information. My concern is this mixture ratio is markedly different from the Flowtech company, therefore how effective is this mixture in term of its polymerisation after injection. We increased the viscosity to prevent venous transit, following the guidelines from reference 8 (Phillips, M. R. *et al.* A method for evaluating the murine pulmonary vasculature using micro-computed tomography. *J Surg Res.* **207** 115-122, (2017)). We've inserted the reference. We've had no problems with polymerization time post injection or polymer integrity acutely or longitudinally (months later). Additionally, altering the viscosity does not affect these parameters in any meaningful way in our experience.

Minor Concerns:
1. The Abstract:
- The Abstract is concise and includes the method used. However, the authors should revise the last paragraph (Line 54-57: 'When performed successfully, one can appreciate the complete pulmonary arterial network, enabling analysis of vessel number, density, length, and branching pattern. While demonstrated in the pulmonary arterial bed, this method can be applied to any vascular bed with optimized catheter placement and endpoints.').
This manuscript is confined to describing the vascular casting technique, and did not include methods on vascular characterisation or vascular network analysis. It seems appropriate to put the text within the context of the ultimate goals rather than as statements within the Abstract. For instance, 'When performed successfully, the casts could be utilized for vascular characterisation or vascular network analysis.' Line 54-57 appears more appropriate for the Discussion rather than the Abstract. We have made the change as suggested (line56-57)

2. Protocols: Few technical details should be expanded and clarified to ensure that readers understand exactly what the authors meant:

2.1 (Line 172 & 218) 'The authors infused perfusion solution at 0.05 ml/min, whereas perfuse Microfil at 0.02 ml/min.'
- Could the authors please explain why they decided to use different infusion rate between those two solutions? We chose different rates because the PBS/NaNP solution has a low viscosity and the goal is a complete perfusion. Consequently, we utilized a higher rate to shorten the duration of the protocol, allowing additional animals to be processed on the same day. For the Microfil, we reduced the speed to 0.02ml/min at this critical step to prevent venous transit and better control the endpoint (See lines 206-208).

2.2 (Line 223) The authors described that 'After Microfil injection completion, stop the syringe pump and then allow the carcass to sit undisturbed for 30-40 minutes to let the Microfil cure and harden'.
- My view is that the pulmonary circulation should be in a close system in order to prevent Microfil leakage out via the clipped left auricle. If it is correct, the authors should add one step about how to tighten loose suture. The goal of this paper is to fill the arterial side and, thus,

leakage of polymer from the auricle not an issue as Microfil does not enter the venous system.

2.3 (Line 233) The authors stated 'Place the head/thorax into in 10% buffered formalin overnight'
- The authors should provide specific temperature (cool room or room temperature) by which they kept the specimens. Added language (line 274)

2.4 (Line 329) The authors mentioned 'a variety of software packages.'
- The authors should be more specific with the software that they used or give some examples. Now line 539 - JoVE does not allow commercial language to be added to text. Please see Materials table for added software options (Software: Analyze, Amira, and Horos – Lines 8, 9, and 20)

2.5 (Line 355 & 370) The authors mentioned 'Latex'.
- What does Latex mean? It seems the authors would like to refer to Microfil, however, the Microfil is a radiopaque silicone rubber, and is not Latex. Please clarify. "Latex" has been changed to "polymer compound" throughout text.

3. Challenges:
- The issues of air bubble: How did the authors clear any air from the system properly? Especially, (line 213) in between changing solution from blood clearing perfusion to Microfil casting (during change syringe), how best to get rid of the air bubble? Step 5.2 and 5.3 addresses this and we agree it may not be easy to visualize. To that end, this step has been specifically highlighted for filmable content. The video will further clarify this step. Additionally, some language has been changed in each step (lines 248-249 and 253)
- How to overcome the imbalance in flow and unequal pressure gradient? Please provide more details. This is specifically addressed in the protocol (please see step 3.4 lines 184-186) We've also restructured the results to better highlight this (lines 389-398).
- What is success rate of the vascular cast generation? Please provide more details. With training, success rate is >90%. This is acknowledged in line 577.
- The authors showed many examples of common errors during Microfil perfusion (Figure 4 & 5). It would be more instructive to the readers if the authors explain cause(s) of each errors and how to solve those problems. These figures have now been combined into one (now Figure 5). Additionally, we reorganized this section to better explain each of the common errors (lines 354-398).

4. Figures and tables:
- The figure 4B, 6A, 6B are unclear: the resolution is poor and the images appear blurred and out of focus. Please replace. We appreciate the reviewers concern. The challenge lies in the 3D structure of the vasculature leading to different parts of the image being in and out of focus. In addition, some of the leakage produces haziness in the tissue which is actually part of what the reviewer noticed. We worked to create new images but some of the "imperfections" result from the "error" in the technique. 4B – We merged fig 4 and 5. and 4B has been removed from

the manuscript entirely. 6A – The punctate appearance on the surface of the kidney are vessels nearing the surface but somewhat obscured by surrounding soft tissue. We replaced the original image with an improved one. 6B – these vessels are deeper within the tissue and, as a result, resolution suffers due to soft tissue obscuring the target. We retook several pictures and saw no improvement. This is true to what the user would be able to visually observe in determining the endpoint.
- Figure 4 and 5 provide similar information and could be combined. We agree and have combined the 2 figures into the current figure 5.
- Figure 7 should show the scalebar or magnification. We've placed a scale bar on the 2D images, Fig8A and 8B. Displaying a 2D scale on a 3D object (8C) however, is not generally recommended as the aspect ratio and viewing angle can influence the scale between various parts of the volume rendering.

5. Abbreviations:
- The authors should provide the full name for every abbreviation when first mentioned, for example, 'postnatal day 1 (P1)'. Thank you, we've scoured the text and made every effort to address this.

Reviewer #4:
Manuscript Summary:
Knutsen et al present a thorough protocol on the vascular casting of murine lung arterial network. This method show a reproducible and highly applicable approach to visualize vascular system in 3D. The manuscript is well written and contains a detailed protocol of different steps and discusses challenges connected with this method. Please see our comments and suggestions below.

Major Concerns:
Protocol comments:
Point 2.5 please explain why oesophagus needs to be severed The esophagus, which runs parallel to the vena cava in the chest cavity, obscures the surgical field. Language has been added to further clarify the anatomy (line 129-130). This step has been specifically highlighted for filmable content. The video will further clarify this step.
Section 3 is a very crucial part of the protocol. In the video, please explain this part carefully step by step. Perhaps detailed pictures would also help for researchers performing the procedure without the video in front of the, ie at the bench. We agree and are glad that you picked up on that. Given the shutdown of lab space at the NIH, we were not able to go back and take the pictures you requested but do think that this will be well covered in the video. This step has been specifically highlighted for filmable content.

Point 3.2 Please clarify how do you penetrate the heart with the silk (there is no mention of a needle) There is no need for a needle. Using sharp curved forceps allows for this maneuver in

lieu of a needle. We added language in the text to hopefully clarify. Additionally, this step has been specifically highlighted for filmable content.
Point 3.4 How and when do you prime the tubing. The priming step is 3.1 when the units are first assembled. This is accomplished when the PBS/SNP is pushed through the syringe and PE10 tubing effectively displacing the air. In step 3.4 you are just confirming no air has crept back in the tip of the PE10 tubing. We've added language to step 3.1 (line 157) and 3.4 (line 182) to make this a bit clearer. This step has been specifically highlighted for filmable content. The video will further clarify this step.
Point 3.7 Please specify how do you perfuse and whether you connect the syringe to the tubing The connection of syringe and tubing are made in step 3.1. We've added "Unit 1 and Unit 2" here to help reference that (line 201). In terms of how to perfuse – at this stage the catheter (PE10 tubing) is firmly secured in the PAT and the syringe portion of the "unit" placed in the syringe pump. Once you turn the pump on it will begin pushing the PBS/SNP through the vasculature thereby perfusing the circuit.
Point 4.1 Please rephrase "Close stopcock and raise the syringe until the meniscus is 20 cm above the trachea". It is unclear how meniscus or of which tube/liquid should be 20 cm above trachea. This is the lung inflation unit in fig2. The tube, liquid meniscus, and stopcock can all be visualized here. We added labels on the figure to clear up any confusion.
Points 4.2 to 4.6 should be very carefully explained during a video or images should be provided to show these steps. These steps have been specifically highlighted for filmable content. We intend to carefully detail each of these steps in the video
Point 5.7 when the catheter is removed is the PAT ligated or is there no risk of microfil leaking out? If the PAT is ligated please mention it in the protocol The Microfil is polymerized at this point (see step 5.6 stating this) so there is no need to ligate.
Point 6.4 Please specify what do you mean by "opening" in this sentence: "Pull a 3 cm length of 7-0 silk back through opening and create a single-throw loose suture." This is the same concept as in step 3.3. I've altered the language to make it more clear (line 299-302).

Figures:
Figure 4A and 4B is not mentioned in the manuscript text Figures 4 and 5 have been condensed into just Fig 5 now. 4A is now 5F, 4B has been removed entirely, and 4C is now 5A. All of them are now referenced in the text.
Figure 5 a and b point out to the line showing collapsed lungs in a text Now 5B and 5C. We've added a white dashed line to both 5B and 5C to show the original inflated position and added a black dotted line on 5C to highlight the deflated position. Also added text to the fig legend stating this.
Figure 5F please provide a close up image of the rupture Now 5G – We have replaced this image with a close up.
Figure 6D the description says large intestine, looks like a rib cage? Please double check Now 6D -We checked and it is, in fact, large intestine. Please note the stool in the colon. The lower right portion of figure is the marginal artery of Drummond that runs parallel to the intestine and supplies the smaller feed branches.
Figure 7 is not mentioned anywhere in the manuscript text -> line 328 refers to figure 7A not 6A please correct, line 330 refers to figure 7B not 6B please correct Thanks for catching this. This is

now 7A, 7B, and 7C and it has been addressed (line 417 and 420).

Material:
The material list contains:
-hydrochloric acid - This has been deleted from the materials list.
-parafilm – This has been added to the protocol (line 329).
-steel plate – Added under modification section in discussion (line 504).
-slide holder – This is actually the surgical board we use. Reworded the Materials list to reflect this.
-20ml Glass Scintillation Vials – Added to step 5.8.

These items are not mentioned anywhere in the protocol. Please add the information.

Comments and questions:
Can you please discuss challenges of early postnatal vs adult mouse lung casting The main obstacle is size. The smaller the animal the less forgiving small mistakes are. Please see the new paragraph in the results section (lines 399-404).
Are the different tissue filled with Microfil cleared in figure images? If yes please describe your clearing protocol. No tissue in this manuscript has been cleared. Each organ/tissue has been removed from the animal, placed in fixative, and imaged individually. Depending on the tissue some may appear more translucent than others but, no clearing steps have been performed.
Page 23 continuation of the table: either fit table on one page or add additional column stating the organ it concerns This was a formatting error. It has been changed to fit on a single page
Page 26 and 27 comments/description - the comments seem incomplete - maybe missing images or information? This is another formatting error. We have consolidated the information on a single page now.

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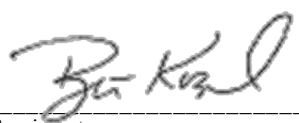

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