SUPPLEMENTAL

Methods

Alternative technique I

Briefly, a horizontal incision was made across the abdomen of a ketamine+xylazine anesthetized, systemically heparinized rat. The abdominal aorta and vena cava were identified, exposed and then severed. As the rat bled out, the chest cavity was opened, and the left atrial appendage was dissected. The rat was perfused with 20 mL of room temperature heparinized-saline via direct puncture of the right ventricle with a 20G needle. The flushing solution syringe was changed to a syringe filled with 46˚C barium/gelatin perfusate and the perfusate was injected until it exited the dissected left atrial appendage. Lungs were inflated with 10% phosphate buffered formalin by gravity instillation via the trachea. Lungs were removed *en bloc* and stored as described in the optimized technique.

Alternative technique II

This protocol was adapted from previously published protocols used for PAH rat lung micro-CT.[14,15] The protocol was modified to accommodate our barium/gelatin perfusate, as opposed to microfil, and our lack of a constant-rate syringe pump. The procedure began as described in alternative technique I. Once the chest was open, the main pulmonary artery (mPA) was cannulated through the right ventricle and secured with sutures. Blood was flushed by hand with 10 mL of room temperature heparinized-saline at a rate of 2 mL per minute, which was maintained by eye using a timer. The syringe attached to the cannula in the mPA was changed to one filled with 46˚C barium/gelatin perfusate. Barium/gelatin perfusate was injected at a constant rate of 2 mL per minute until the technician injecting reached an undeniable point of resistance.

Lungs were inflated with 10% phosphate buffered formalin by gravity instillation via the trachea. Lungs were removed *en bloc* and stored as described in the optimized technique.

Alternative technique III

This was the initial protocol adopted by the lab from which we developed our optimized technique. Briefly, a horizontal incision was made across the abdomen of a ketamine+xylazine anesthetized, systemically heparinized rat. The abdominal aorta and vena cava were identified, exposed and then severed. As the rat bled out, the chest cavity was opened, and the left atrial appendage was dissected. The mPA was cannulated through the right ventricle and secured with sutures. The blood was flushed quickly (flow rate ~20 mL/min) by hand with 20 mL of room temperature heparinized-saline. The syringe attached to the cannula in the mPA was changed to one filled with 46˚C barium/gelatin perfusate and the lungs were filled quickly (flow rate ~20 mL/min) until an obvious point of resistance was reached. While the lungs were being filled with perfusate, they were slowly inflated with air by a syringe via the trachea. The perfusion was completed as previously described.

Table S1. Troubleshooting issues during perfusion

Trouble	Possible causes	Solution
Incomplete flushing of lungs	Too little flushing solution used	Use 200+mL for flush
	Rat body temp low	Maintain rat on 37°C heating pad throughout procedure
	Flushing solution temp too low	Use 37°C heparinized saline for flush
	No ventilator	Maintain intubated rat on small animal ventilator throughout
	Open chest immediately for perfusion - heart stops beating	Keep chest closed while jugular vein is used for flush and abdominal aorta is used to drain
	46°C flush used too early - blood left in the lungs will clot	Need to wait until lungs appear white and efflux runs clear before 46°C flush
	Catheter wrongly positioned in jugular vein - falsely high pressure reading	a) Re-position catheter in jugular vein b) Maintain flow rate of 20 ml/min despite pressure reading
Poor contrast perfusion - suboptimal micro-CT image	Perfusion pressure too low	Maintain *in vivo* RVSP measured for each rat throughout perfusion
	Perfusion pressure too high	
	Lung temperature too low - premature perfusate solidification	Use heparinized saline maintained in 46°C water bath for flush before barium perfusion
	Perfusate temperature too low - premature perfusate solidification	Pre-prepare syringes with barium perfusate and store in 46°C water bath until use
	Barium contamination on outside of lung	Wash lungs in 46°C water before placing in formalin

* RVSP= right ventricular systolic pressure

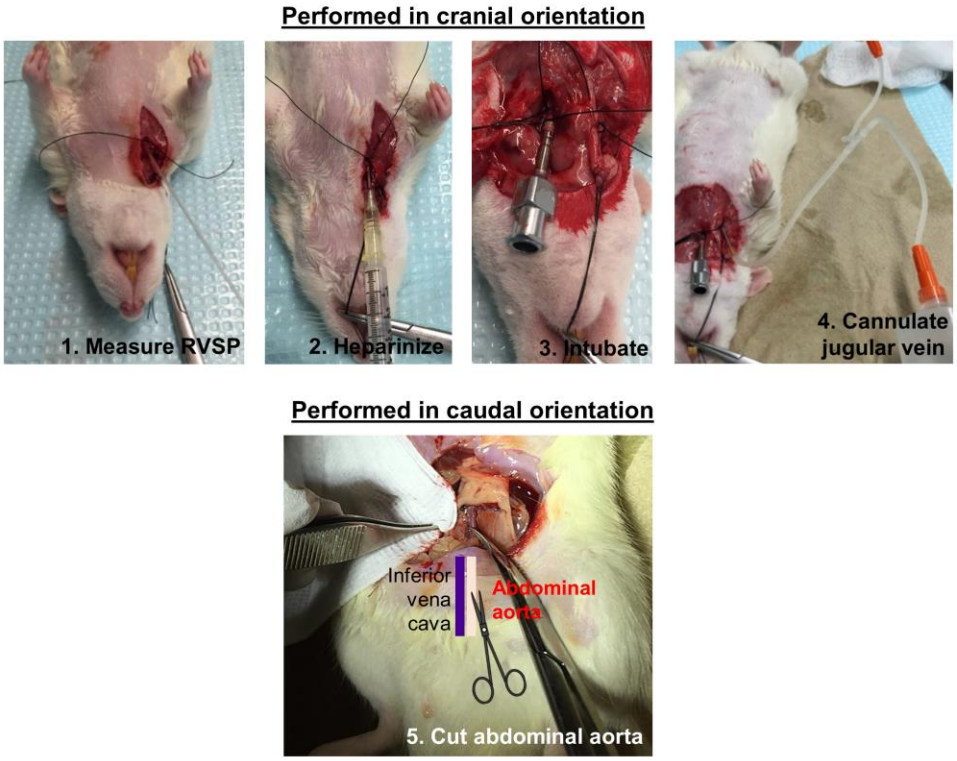

Figure S1

Figure S1. Step by step images of procedures completed before perfusion begins. First, *in vivo* RVSP is measured by right heart catheterization via the right jugular vein in a ketamine+xylazine anesthetized animal. The rat is then systemically heparinized and intubated.

Following this step, the jugular vein is catheterized with T-tubing attached to a 60cc syringe filled with 37°C heparinized saline and the pressure transducer. The rat is rotated to a caudal position before carefully cutting the abdominal aorta to start blood drainage.

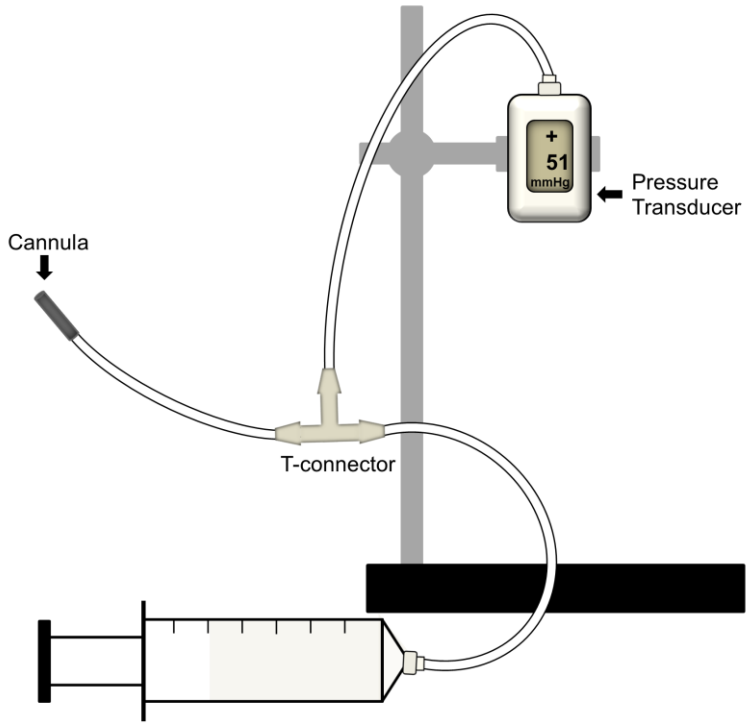

Figure S2

Figure S2. Setup diagram for T-connector tubing, syringe, catheter and pressure transducer. Poly-ethylene tubing was attached to a T-connector. One horizontal end of the

tubing ends in a catheter, while the other horizontal end has a luer lock to attach to the syringe used for perfusion. The vertical portion of the tubing is connected to the pressure transducer, which is mounted on a stand.

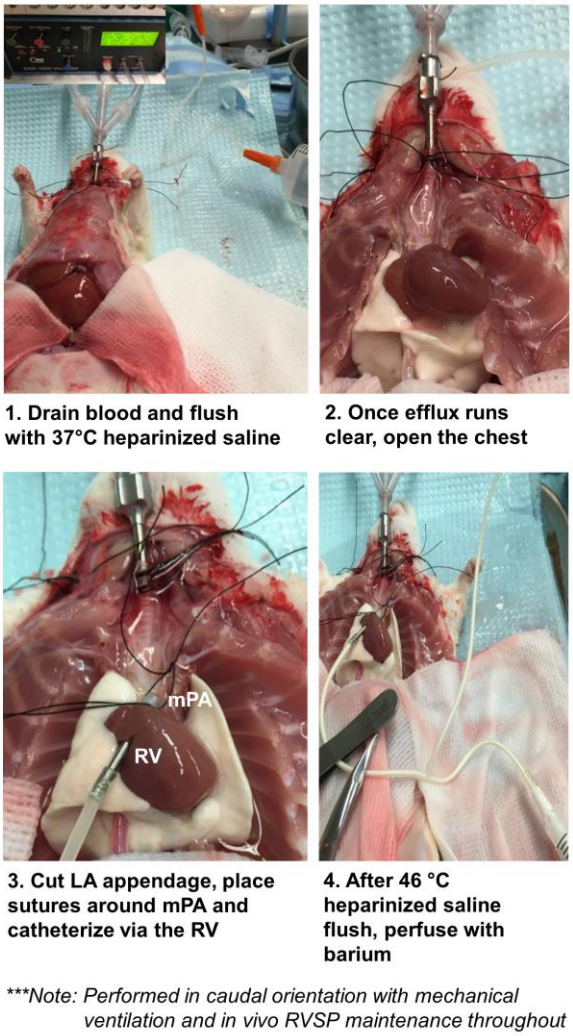

Figure S3

Figure S3. Step by step images of procedures completed during flush and contrast-perfusion. After cutting the abdominal aorta, the intubation tube is connected to the small animal ventilator. Meanwhile, flushing from the jugular vein catheter begins. Flushing continues until

efflux from the abdominal aorta runs clear. The lungs are checked for whiteness by dissecting the diaphragm under the xiphoid process. Flushing continues while the chest cavity is opened. Following this step, the left atrial appendage is cut, and the heart is prepared for main pulmonary artery (mPA) catheterization. Once the mPA is catheterized, flushing continues until the chest cavity is clear of blood. The lungs are then flushed with 46°C heparinized saline until they reach body temperature. Finally, the barium is perfused via the mPA catheterization.

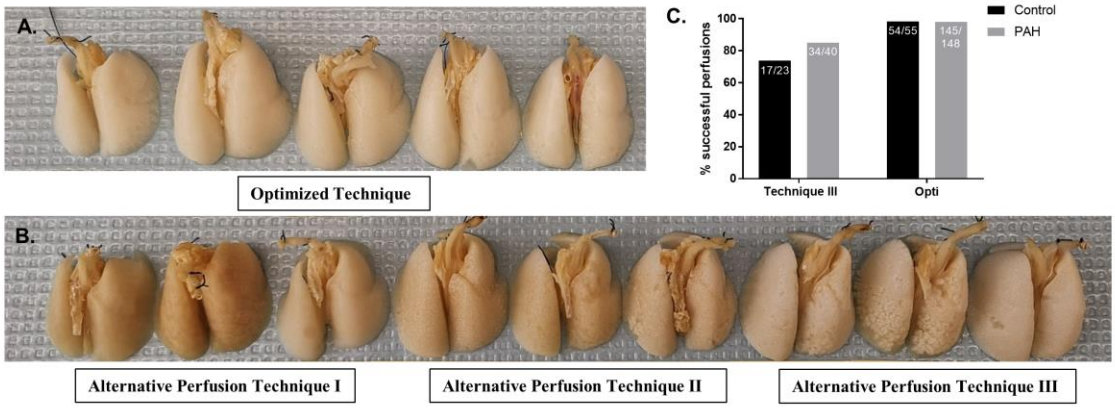

Figure S4

Figure S4. Formalin fixed, barium perfused lungs from alternative perfusion techniques I, II and III are obviously flawed compared to samples perfused with the optimized technique. A) Brilliant white lungs demonstrate efficient flushing of blood and instillation of

barium-perfusate that results from our optimized perfusion technique (lungs produced micro-CT scans from Fig.3G). B) Dark coloration from oxidized blood left in lungs and inconsistent barium distribution is apparent in samples from alternative perfusion techniques I, II, and III. Micro-CT images resulting from these samples are shown in Fig.3. C) Technique III had a success rate of 74% and 85% for control and PH animals, respectively; whereas the optimized protocol had a success rate of ~98% for both control and PH animals. “Success” was defined as brilliant white lungs post-perfusion that went on to produce a high quality micro-CT image.

Figure S5. 360° video of high resolution micro-CT scan from whole lung of normal control SD rat. An in-house engineered specimen container, large enough to fit whole rat lung, was used to image normal control SD rat lungs perfused with the technique described here. Link: https://players.brightcove.net/4988507115001/BJ5hvqqbQ_default/index.html?videoId=ref:sj-vid-1-pul-10.1177_2045894019883613

Figure S6. 360° video of high resolution micro-CT scan from whole lung of D21 post-MCT SD rat. An in-house engineered specimen container, large enough to fit whole rat lung, was used to image D21 post-MCT PAH SD rat lungs perfused with the technique described here. Link: https://players.brightcove.net/4988507115001/BJ5hvqqbQ_default/index.html?videoId=ref:sj-vid-2-pul-10.1177_2045894019883613