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Perfusion and inflation of the mouse lung for tumor histology

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Vineeta Bajaj, Ph.D.

July 10, 2020

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RE: resubmission of manuscript JoVE60605

Dr. Bajaj:

Thank for this opportunity to resubmit our invited manuscript titled: Perfusion and inflation of the mouse lung for tumor histology. We have extensive experience in the use of multiple mouse models of lung cancer and the histological evaluation of lung tumors in mice.

We greatly appreciate the reviewer's comments on our previous submission and have taken every effort to address them. We feel as though the incorporated changes to our protocol, and new figure, significantly improve the manuscript. I apologize for the delay in resubmission; however, personal tragedies prevented us from doing so until now.

Please feel free to contact me if there are any questions regarding our submission

Sincerely,

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TITLE:

Perfusion and Inflation of the Mouse Lung for Tumor Histology

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KEYWORDS:

Lung cancer, mouse, lung inflation, perfusion, fixation, lung tumor histology, lung metastasis

SUMMARY:

The purpose of this method is to present a simple and efficient method for the perfusion, inflation, and fixation of mouse lungs for the examination of lung tumor pathology and evaluation of metastases to the lung.

ABSTRACT:

The ability to evaluate lung histology is critical for the fields of lung cancer research and cancer metastasis. It is equally important to perform necropsies rapidly and efficiently from studies without sacrificing the quality of the tissues procured. The goal of this protocol is to present a method to rapidly perfuse, inflate, and fix mouse lungs for downstream histological analysis. This method does not standardize lung inflation; thus, it does not require any special procedures or equipment and instead simply instills fixative directly through the trachea following perfusion through the heart. This allows for sufficient estimation of tumor size, histology, and scoring. This also allows for the collection of frozen tissue prior to lung tissue fixation. This method is limited in that it does not allow for later morphometric quantification of the lung; however, it is more than sufficient for lung tumor analysis from genetically engineered mouse models (GEMMs), syngeneic models, as well as xenograft tumor and metastasis studies.

INTRODUCTION:

A variety of mouse models of lung oncogenesis and cancer metastasis to the lung exist ranging from complex GEMMs to carcinogen-induced models to syngeneic and xenograft models, where cancer cells are injected via intracardiac, intrathoracic, the tail vein, or other methods to establish tumors within the lung. All these models share the common need for histological evaluation of

lung histology and pathology. Thus, it is necessary to have a robust yet rapid method to perform necropsies of mice while perfusing the lungs to remove excess blood, and inflating and fixing the lungs to clearly visualize lung architecture. Speed is a critical component of this procedure as it may be necessary to collect the lungs from dozens of mice at a single time point. This procedure can be performed in less than 6 minutes per mouse.

While this procedure is more than sufficient for evaluating tumor histology, it is not recommended for those who wish to perform stereology or morphometric measurements of the lungs. Such measurements require lung inflation to be standardized, as does the calculation of absolute surface area of the lung, absolute volume, and alveolar size and number[1]. This method is also not optimal for some imaging approaches. For example, imaging of the lungs via µCT for ex vivo morphometric analysis requires that the lungs remain filled with air[2]. When the preservation of air spaces and dimensions are the primary concern, it is recommended to fix the lungs by perfusion dehydration techniques[3,4]. One of the biggest concerns of this model is the potential for rupturing of the alveolar walls, lessening its use in studies of emphysema; however, the recommended procedure for fixation of lungs for the study of emphysema is still quite similar, as it is recommended to fix the lungs either by intratracheal instillation of 10% formalin (similar to the protocol described here) under constant fluid pressure or by in situ fixation[5].

The advantage of the described procedure here is that it does not require constant fluid pressure, instead inflating the lungs until they have fully expanded, thus decreasing the time needed for the procedure. The procedure here described closely resembles the methods recommended by an armamentarium of the Society of Toxicologic Pathology, where a subcommittee was formed to recommend the best methods of lung fixation for toxicology studies. The majority of scientists within this subcommittee recommended fixing the lungs by intratracheal instillation with a syringe, though there were varying recommendations on the time the lung was left in the fixative[6]. Thus, while a variety of methods of lung inflation and fixation exist, the method described herein is proposed to be the optimal method to quickly inflate and fix the lungs for downstream tumor histological evaluation.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham.

1. Experimental protocol

1.1. Sacrifice the mouse using an approved IACUC method. Here, we used cervical dislocation of a mouse anesthetized with 5% isoflurane. Use an appropriate mouse for the study; here, we use an 8-week old FVB mouse

1.2. Using surgical scissors, make a 3.5-5 mm horizontal incision in the middle of lower abdomen. Next, insert surgical scissors into the small hole created from incision and cut vertically up the center midline to just below the neck of the mouse.

1.3. Pull the skin back with fingers and inspect the axillary lymph nodes.

1.4. Using surgical scissors, make a 3.5 mm lateral incision to open the abdominal cavity, and then cut in the anterior direction up to the bottom of the thorax. Inspect the organs in the abdominal cavity: liver, spleen, kidneys, etc.

1.5. With the flat of the surgical scissors, or using forceps, move the liver to expose the diaphragm. Inspect the diaphragm for tumor growth or metastases. Then, gently snip the diaphragm on the operator's right side, allowing it to expand. Gently cut the diaphragm from right to left to expose the thoracic cavity and lungs. Be careful not to cut the lungs.

1.6. Cut up through the lateral extreme of the left rib cage (on operator's right) to inspect the left lobe of the lungs.

1.7. Gently move the right lobes of the lung out of the way and cut up the lateral extreme of the right rib cage and remove rib cage.

NOTE: Removal of the rib cage is optional, though removal enables clearer view of later lung inflation.

1.7.1. If fresh or frozen lung tissue is required, use hemostat forceps to clamp the bronchus of the left lobe and resect the left lung using surgical scissors prior to perfusion.

1.8. Using the forceps to lift the tissue covering the trachea, cut away any excess tissue. Then gently cut the thin tissue lining the trachea to expose the airway.

1.9. Cut through the renal artery with surgical scissors.

1.10. To perfuse the lungs, use a 3 mL syringe with a 22 G needle to inject 1x PBS with 10 U/mL heparin into the right ventricle of the heart. Slowly perfuse the lungs at approximately 300 µL/s with PBS/heparin. The lungs will frequently turn white. 2.5 mL of PBS are generally used in this step.

1.11. For lung inflation, use a 3 mL syringe with a 22 G needle, this time held parallel to the trachea. Insert the needle into the trachea and inject 10% formalin with rate of flow no greater than ~200 µL/s until the lungs have fully inflated. Once the lungs are inflated, formalin will backflow out of the trachea. Hold the needle in place for a few more seconds and then withdraw.

1.11.1. (Optional) Prior to withdrawing needle and lung inflation, use suture thread to tie off the trachea. To achieve this, use 4 inches of suture thread holding the point of the thread with a small pair of forceps. Place the thread on the dorsal side of the trachea and pull through to make a

loop around the needle. Next make an overhand knot around the needle. Pull knot tight, remove needle from trachea, close knot.

1.12. Use forceps to lift the heart, insert surgical scissors directly behind the lungs, and cut connective tissue while the lifting the heart to resect lungs.

1.13. Cut the heart to remove it from lungs.

1.14. Place the lungs a cassette labeled with the mouse ID or study ID. Place the cassette in 10% buffered formalin and fix for 24-48 h. Lungs can be left in fixative for over a year if desired.

1.15. Transfer the cassette containing the lungs to 70% ethanol and proceed to processing for histology.

REPRESENTATIVE RESULTS:

The above protocol allows for quick perfusion, inflation, and fixation of mouse lungs. The figures shown below represent the importance of each step. **Figure 1** depicts H&E stained lungs that have been perfused with PBS and lungs in which the perfusion step has been skipped or the lungs failed to perfuse correctly. As shown, excess blood in the poorly perfused lungs creates less than ideal histology and can make it challenging to fully observe lung architecture. **Figure 2** demonstrates both the importance of inflation as well as the dangers of over-inflation. It is more difficult to identify areas of hyperplasia in un-inflated lungs due to the compression and close proximity of the alveoli; however, in the overinflated lungs, many of the alveolar walls have been broken and this could be mistaken for emphysema if not careful. **Figure 3** depicts tumor histology in lungs which have been perfused and inflated using the technique described herein.

FIGURE AND TABLE LEGENDS:

Figure 1. Representative H&E staining of perfused and non-perfused lungs. (**A**) Lungs perfused with PBS. (**B**) Lungs not perfused.

Figure 2. Representative H&E staining of inflated, uninflated, and overinflated lungs. (**A**) Lungs were inflated and fixed with 10% formalin until the lungs fully expanded. (**B**) Lungs were not inflated through the trachea and instead directly placed in 10% formalin. (**C**) Lungs were inflated and fixed with 10% formalin but 10% formalin was continuously pushed into the lungs past full expansion resulting in over-inflation.

Figure 3. Representative H&E staining of perfused and inflated mouse lungs. (**A**) Lung tumors were induced using the chemical carcinogen urethane. (**B**) Spontaneous lung metastases from a human xenografted cell line

DISCUSSION:

The procedure described above for the perfusion, inflation, and fixation of mouse lungs is ideal for quick and efficient preparation of mouse lungs for lung tumor histology and pathology

analysis. The procedure does not require any special equipment and can be performed in less than 6 minutes per mouse. The procedure does not require a fixed volume for inflation nor constant fluid pressure. Because this procedure is not standardized, it is not recommended for those wishing to perform stereological or morphometric analyses of the lung. Procedures where such standardization is required have been better described[1,7].

The most critical steps of this protocol are perfusion and inflation. It is important to perfuse through the right ventricle of the heart, whereas if perfusion is done through the left ventricle, the lungs will not perfuse. It is easy to tell if perfusion is done correctly as the lungs will turn white. Instillation of fixative through the trachea allows inflation of the lungs, which allows for easier downstream histological analysis of lung architecture. It is important to stop administration of fixative as soon as the lungs have fully expanded, as over-inflation can cause alveolar wall breakage and the appearance of emphysema. Once the lungs are inflated, some formalin will backflow out of the trachea. This is normal and does not impact downstream histological analysis; however, if this is a concern the trachea can be tied off prior excising the lungs.

While this protocol uses 10% buffered formalin to fix the lungs, which is the most commonly recommended fixative[5,6], there are reports of artifacts introduced by this fixative, namely shrinkage of the lung tissue[1,8]. If this is a concern, follow the guidelines of the American Thoracic Society and European Respiratory Society for the assessment of lung structure[1]. Another potential fixative not commonly recommended but that may prove useful is Bouin's solution, which may provide better contrast for the evaluation of lung surface nodules[9,10]. In summary, the herein described protocol provides a robust and simple method for the fixation of mouse lungs for tumor histology.

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DISCLOSURES:
The authors have nothing to disclose.

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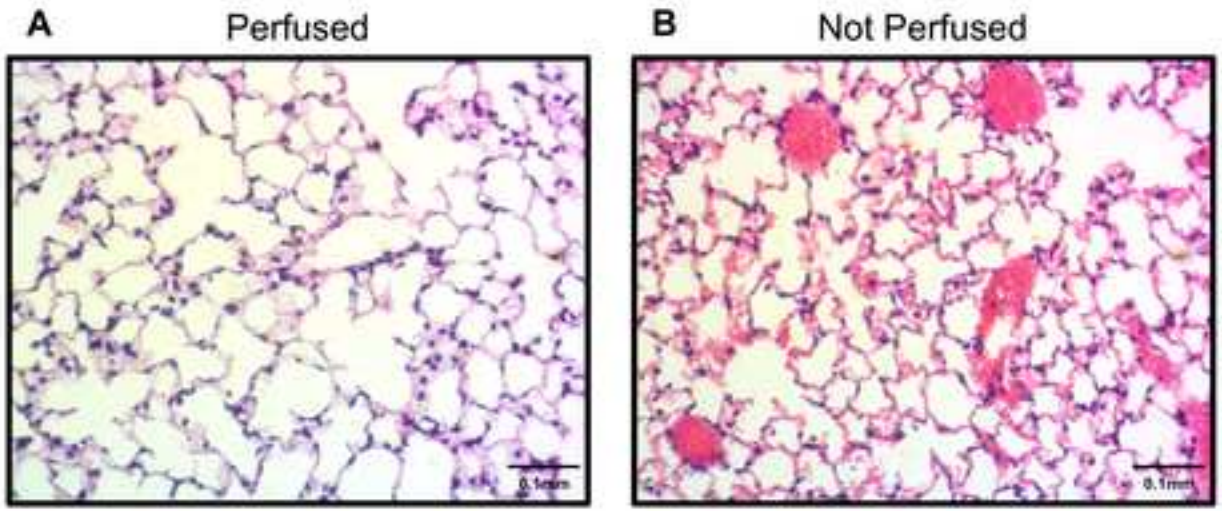

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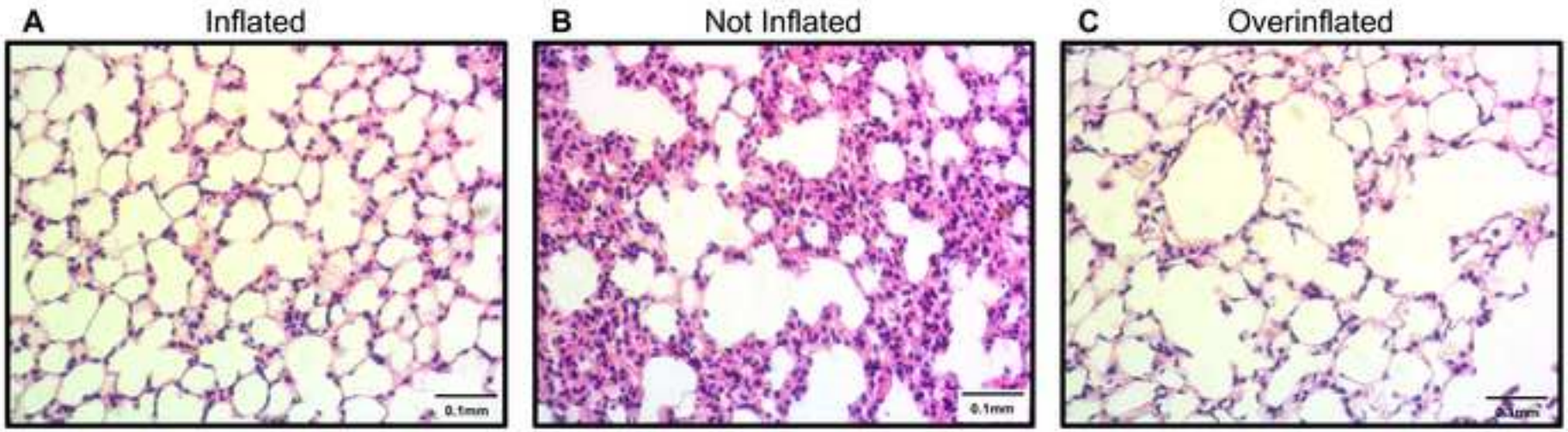

Figure 3

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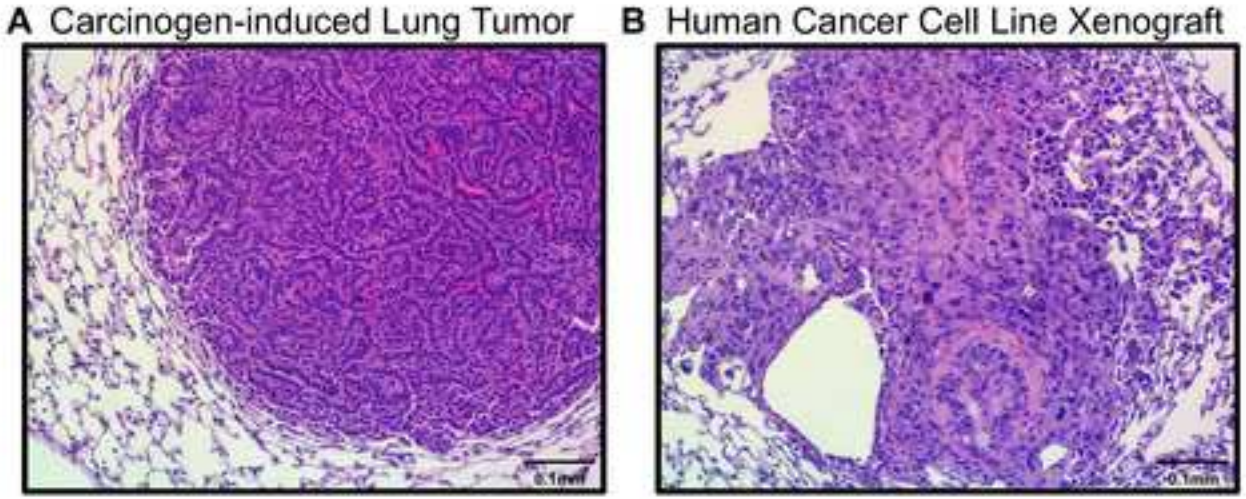

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10% buffered formalin	Fisher	23-245685	
22 G Needle	BD	305155	
3 mL syringe	BD	309656	
70% Ethanol	Decon	2405	
Forceps	Harvard Apparatus	72-8595	
Heparin	Fisher	H19	
Phosphate Buffered Saline (PBS)	Corning	21-030-CV	
Surgical scissors	Harvard Apparatus	72-8428	

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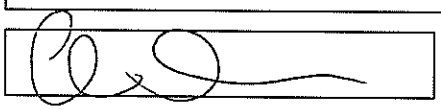
Signature:	
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Response to reviewers:

Thank you for the opportunity to resubmit our manuscript. We greatly appreciate the constructive and insightful comments provided by the reviewers. Changes were made to the manuscript that address these concerns. Our point by point response is listed below:

Reviewer #1:

"In the protocol section, step 11: After inflation of the lungs with formalin, the authors describe that they withdraw the needle out of the trachea, but they do not specify how they prevent the lungs from deflating. How do the lungs stay inflated? It would be necessary to add some details regarding that point." For this procedure on visualizing tumors within the mouse lung, we don't tie off the trachea. Inflation leaves a sufficient amount of fluid in the lung airways for histological analysis. To address this concern however, we have added an explanation on line 116 that suture thread can be used to tie off the trachea before the needle is removed.

Reviewer #2:

"It is thus curious that no images of tumors in fixed lungs are presented." We have updated the figures to include images with lung tumors from human xenograft and KRas driven lung tumor mouse models.

"And the method of simply inflating the lungs to an unknown level of inflation has been around for decades." We agree with the reviewer's statement; however, novelty is not a requirement of this publication and moreover, the straight forward method for visualizing tumor histology in the lung is not published as we present it here.

"Indeed, if the goal is simply to look at the tumor or even get a estimate of its mass, there really is no need to inflate the lung with fixative at all." To differentiate between tumors, especially when necrosis is involved, inflation greatly assists visualizing individual tumors.

"And there is even less reason to perfuse the vasculature to wash out the blood. In fact leaving the blood in place might actually help in visualization of any tumors present. It surely helps one visualizing the blood vessels in the parenchyma and likely in tumors, too" Having 15 years experience in performing these analyses, we respectfully disagree with the reviewer. Moreover, Figure 1 clearly shows how perfusion allows for better contrast in the lung.

"Line 59. "inflate lungs until they are fully expanded". The entire method is based on this approach, but full expansion is entirely subjective. How can one possibly know what this is or when one is over expanding the lungs? We agree with the reviewer's comment and have added a volume to clarify to the protocol user an approximate amount to inflate the lungs

"Indeed, if you simply had a reservoir of formalin at a certain height above the lung and tied in your needle you could uniformly inflate all your lungs. This might add 2 minutes to the procedure, but would surely make the inflations more uniform." Our method proposed here produces consistent lung inflation. Moreover, the reviewer's suggestion could add hours to the procedure time, as the extra 2 minutes offered up as an example if applied to a study of 80 mice (an moderately sized animal experiment) would add over 2.5hrs of procedure time at the least. This defeats the purpose of this method proposed.

"If you don't really care how big the lung is, then why inflate at all?" This statement was never made by the authors.

"The extra time needed to inflate from a reservoir on a ring stand would be about the same time as that need to perfuse the vasculature (which is not needed for your purposes)." These concerns are addressed above.

"Line 105. Perfusion with PBS can also damage the lungs if the pressure is too high. How much fluid is used to wash the vasculature and over what time frame? But it doesn't seem that this is even necessary to just examine a tumor." Thank you for this comment. We have added more details regarding total volume and rate of flow.

"Line 117. Why 24-48 hours? Would it be bad to leave it in fixative for a week? A month?" For a mouse lung, 24-48 hours of fixation in 10% buffered formalin is sufficient time for histological purposes. Lungs can be left in formalin for longer periods of time if desired. The protocol has been amended to indicate this.

"Line 126. Why does the blood make it challenging to observe lung architecture? It does in our experience and those by our colleagues at other institutions. There are less cells (RBCs) after perfusion of the lung and greater contrast for the evaluator.

And more importantly, your whole method is based on not worrying about standardizing inflation, since you are not concerned with quantifying changes in lung architecture" A major data point for our lung pathology enumeration is lesion number. As stated above, inflated lungs, that are well perfused, allow our research members to quantify mouse lung hyperplasia, AAH, adenoma, and carcinoma, etc, number. We are not quantifying lung architecture.

"*Page 4. In at least 3 places you emphasize the dangers of overinflation, but there's really no way to know if one is doing this until you look at the histology."* This is an important point listed by the reviewer and we thank them for their comment. We have indicated rate of flow on line 110 and have also indicated that once the lungs are inflated, formalin will back flow out of the lung and allow the user to know the lung are completely inflated. We've performed this procedure on thousands of mouse lungs and do not have complications with overinflation.

Reviewer #3:
"The fixative is instilled via the trachea and the needle is withdrawn after a very short time. After this duration, the lung can deflate due to the normal mechanics of the lung tissue, draining the fixative into the pharynx and the nasal cavity. We thank the reviewer for their comment and would like to state that we do observe that this rapid approach for inflation of the lungs does sometimes result in fixative leaving the mouse lung and a collapse small areas in the proximal lung; however, lung pathology enumeration is not affected by this.

"This deflation is of greater impact since the rib cage has been dissected, eliminating the effects of chest wall, providing the negative pressure to keep the lungs inflated. Even in cases where quantitative measurements of the alveolar airspace are not made, this reduction in lung volume, which will be heterogeneously distributed over the lung, may potentially affect regions of interest, i.e. the tumor segments. A simple fix would be to use a 22g catheter to instill the fixative, held in place by tying surgical sutures around the trachea. The catheter can be left in place for the duration of the procedure, and can also provide a tether point by which to move the lungs, minimizing damage in handling lung tissue" This is a helpful suggestion and potential alternative approach to the one we describe in this method. However, having performed thousands of these procedures we do not observe any significant changes in overall lung volume that is not normalized over the entire study cohort.

"The ribs can potentially puncture the lungs during the procedure if excised. In this case, it is recommended that the ribcage be excised to the lateral extremes, minimizing any chance of damage to the lungs" Thank you for this comment. This is how we actually perform the procedure and have updated the text of the method to be more clear.

"The authors mention that the flushed appearance of the lung is an indication of good perfusion. Since this is subjective, it is recommended that the outflow from the excised renal artery is monitored. When the outflow is clear, complete flushing of the vasculature can be confirmed." We agree with the reviewer that this is one method for determining complete flushing of the vasculature; however, given that we perfuse directly into the right ventricle, the lungs are the first organ to perfuse and thus it is not necessary to perfuse the entire mouse vasculature. We have updated the manuscript to make more clear the volume necessary for complete perfusion of the lungs which is much less than that required for the renal outflow to become clear.

"The perfusion is completed using PBS. Addition of heparin is recommended to ensure complete perfusion in the microvasculature across the whole lung, which can go unnoticed in the absence of adequate sampling." We have added the use of heparin to our materials and methods for use with PBS.