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Perfusion and inflation of the mouse lung for tumor histology

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LONG ABSTRACT:

The ability to evaluate lung histology is critical for the fields of lung cancer research and cancer metastasis. It is equally important to rapidly and efficiently perform necropsies from studies without sacrificing the quality of tissues procured. The goal of this protocol is thus to present a method to rapidly perfuse, inflate, and fix mouse lungs for downstream histological analysis. This method does not standardize lung inflation thus not requiring any special procedures or equipment and instead simply instilling fixative directly through the trachea following perfusion through the heart. This allows for sufficient estimation of tumor size, histology, and scoring. This method 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.

SHORT ABSTRACT:

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.

Keywords

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

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, tail vein, or other methods to establish tumors within the lung. All of these models share the common need for the 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

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excess blood, and inflating and fixing the lungs to clearly visualize lung architecture. Speed is a critical component of this procedure as at times it is 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 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. Sacrifice mouse using an approved IACUC method. Shown here to be cervical dislocation of an anesthetized mouse.
2. Using surgical scissors, make a horizontal incision in middle of lower abdomen. Next, insert scissors into the small hole created from incision and cut vertically up the center midline to just below the neck of the mouse.
3. Pull the skin back and inspect the axillary lymph nodes.
4. Using the scissors, make a small 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.
5. With the flat of the scissors, or using forceps, move the liver to expose the diaphragm. Inspect the diaphragm for tumor growth or metastases. Then, gently snip diaphragm on your right side, allowing it to expand. Gently cut diaphragm

from right to left to expose the thoracic cavity and lungs. Be careful not to cut the lungs.

6. Cut up through the lateral extreme of the left rib cage (on your right) to inspect the left lobe of the lungs.

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.

Note: 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.

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.

9. Make an incision in the renal artery.

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

11.1 For lung inflation, use a 3mL syringe with a 22g 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/second 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.

11.2 (Optional) Prior to withdrawing needle and lung inflation, suture thread can be used 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.

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

12. Cut the heart to remove it from lungs.

13. Place the lungs a cassette labeled with the mouse ID or study ID. Place cassette in 10% buffered formalin and fixed for 24–48hrs. Lungs can be left in fixative for over a year if desired.

14. 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 which 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.

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]. It is recommended if this is a concern to 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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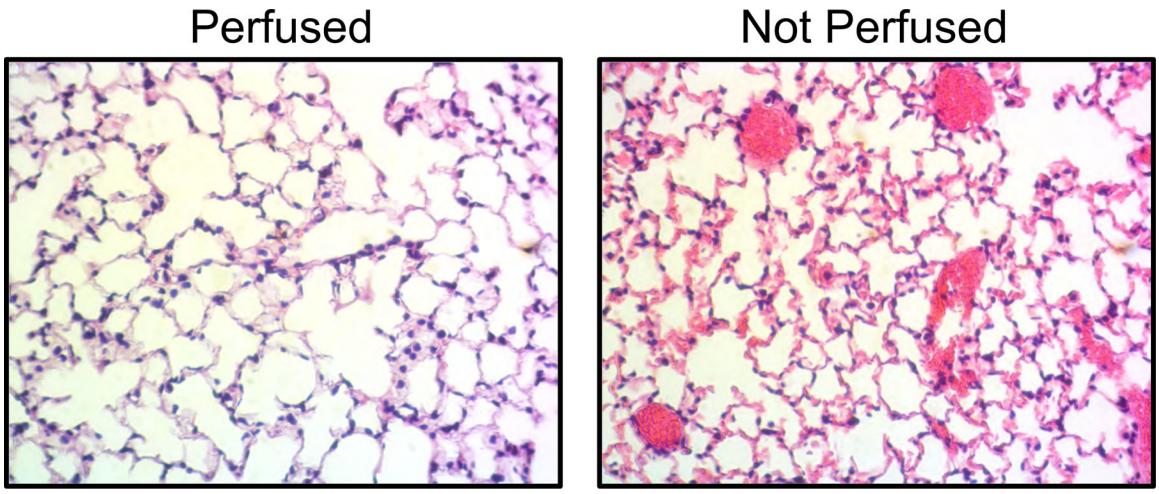

Figure 1.
Representative H&E staining of perfused and non-perfused lungs. 1A) Lungs perfused with PBS, 2B) lungs not perfused.

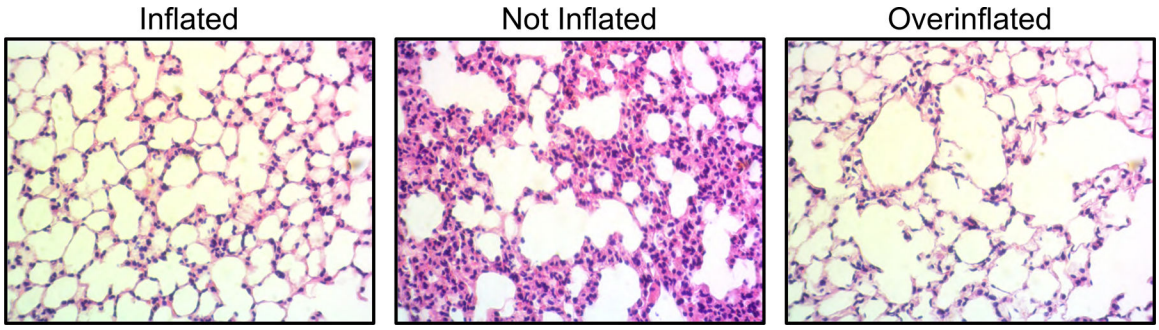

Figure 2.

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

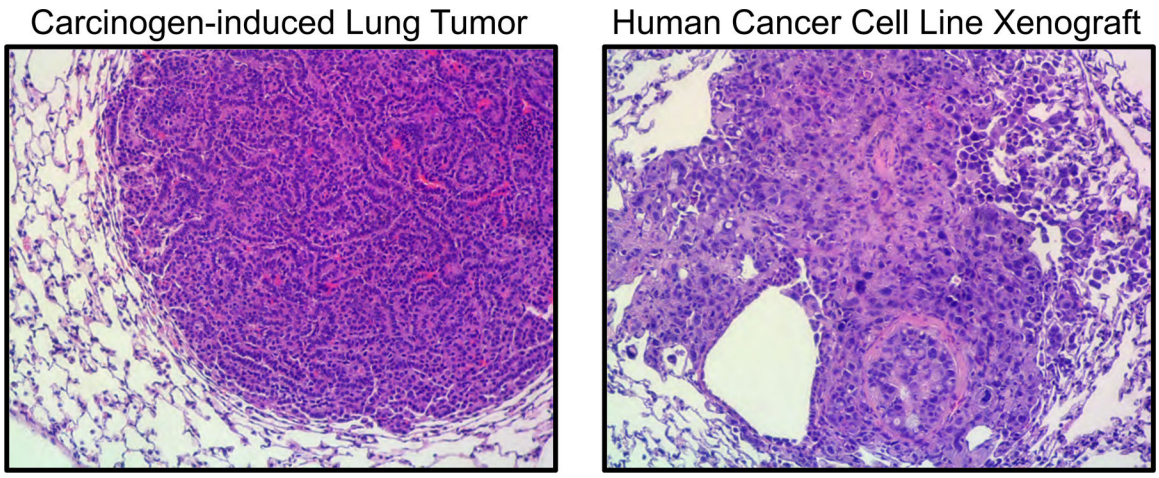

Figure 3.

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