Video Article

A Standardized Method for Measuring Internal Lung Surface Area via Mouse Pneumonectomy and Prosthesis Implantation

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Abstract

Pulmonary morphology, physiology, and respiratory functions change in both physiological and pathological conditions. Internal lung surface area (ISA), representing the gas-exchange capacity of the lung, is a critical criterion to assess respiratory function. However, observer bias can significantly influence measured values for lung morphological parameters. The protocol that we describe here minimizes variations during measurements of two morphological parameters used for ISA calculation: internal lung volume (ILV) and mean linear intercept (MLI). Using ISA as a morphometric and functional parameter to determine the outcome of alveolar regeneration in both pneumonectomy (PNX) and prosthesis implantation mouse models, we found that the increased ISA following PNX treatment was significantly blocked by implantation of a prosthesis into the thoracic cavity[1]. The ability to accurately quantify ISA is not only expected to improve the reliability and reproducibility of lung function studies in injured-induced alveolar regeneration models, but also to promote mechanistic discoveries of multiple pulmonary diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/56114/

Introduction

The fundamental function of the lung is the exchange of oxygen and carbon dioxide between blood vessels and the atmosphere. Lung diseases such as bronchopulmonary dysplasia (BPD), chronic obstructive pulmonary disease (COPD), and acute respiratory infections, result in decreased ISA[2]. Researchers studying lung disease have developed several quantitative methods to evaluate morphological changes in lungs, including MLI, ILV, number of gas exchange units, ISA, and lung tissue compliance[2,3]. Pioneering studies by Weibel *et al.*[4] and Duguid *et al.*[5] together established that ISA can be used as a direct measure of lung gas-exchange capacity in human lungs and can be used as a criterion to determine emphysema severity. A number of studies published in the last five years have used lung morphological parameters (*e.g.,* ISA and MLI) to assess morphological and functional changes in the lungs of mice during development[6] and during recovery from injury PNX[1,7]. ISA is calculated using **Equation 1**[8,9]:

$$\text{Internal lung surface area (ISA)} = \frac{4 \text{ x (ILV)}}{\text{(MLI)}}$$

, where ILV is the internal lung volume and MLI is an intermediary parameter that represents the pulmonary peripheral airspace size[10].

PNX, the surgical removal of one or more lung lobes, has been widely reported to induce alveolar regeneration in many species, including humans[11], mice[1], dogs[12], rats[13], and rabbits[14,15]. A study of mice lungs at fourteen days post-PNX showed that both the expansion of pre-existing alveoli and the *de novo* formation of alveoli contribute to the restoration of ISA, ILV, and the number of alveoli in the remaining lung tissues[1]. We and others have shown that the insertion of materials such as sponge, wax, or a custom-shaped prosthesis into the empty thoracic cavity following PNX (*i.e.*, prosthesis implantation) impairs alveolar regeneration. It is now firmly established that mechanical force functions as one of the most important factors for initiating alveolar regeneration[1,16,17]. Such studies have highlighted the effectiveness of using ISA values from PNX-treated and Prosthesis-implanted lungs as a criterion to quantitatively evaluate alveolar regeneration.

Observer bias is known to significantly influence measured values for lung morphological parameters (*e.g.*, MIL and ILV). Standardized protocols can be used to obviate this bias in determining both ILV and MLI, which are the two parameters used in the calculation of ISA. Here, we provide highly-detailed, standardized protocols for measuring these lung parameters. Importantly, the ability to accurately quantify ISA promises

to improve the reliability and reproducibility of studies of lung function in injury-induced alveolar regeneration models and should facilitate mechanistic discoveries in multiple pulmonary diseases.

Protocol

All procedures used in this protocol were carried out in accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the National Institute of Biological Sciences, Beijing. 8 week-old CD-1 male mice were housed in a specific pathogen free (SPF) facility until the experiments were conducted. Surgeries were performed using completely anesthetized mice (*i.e.*, without any toe pinch responses). After surgery, mice were kept in a warm, humid room with sufficient food and fresh water. Mice were sacrificed using an overdose of anesthetic delivered by intraperitoneal injection.

1. Mouse PNX Surgery

1. Fully anesthetize the mice with sodium phenobarbital (120 mg / kg body weight) and buprenorphine (0.1 mg / kg body weight) via intraperitoneal (I.P.) injection. Perform surgery when mice no longer react to toe pinching.
2. Remove hair on the left thorax of the mice with chemical depilatory treatment (~3 x 3 cm^2 area).
3. Secure each mouse on an intubation platform with its ventral side facing the operator (**Figure 1A**).
4. Pull out the mouse tongue and illuminate the vocal cords with a small animal laryngoscope containing a notch for guiding catheters[18] (**Figure 1A**).
5. Distinguish the vocal cords by observing the movements of the vocal cords during breathing. Gently insert a 20 G intravenous intubation cannula into the trachea at an anterior angle[19].
6. Place mice in a right lateral recumbent position and connect the cannula to a mechanical ventilator (*e.g.,* pressure controlled; see the **Table of Materials**). Check the insertion of the cannula into the trachea by observing the breathing movements of the mouse chest (**Figure 1B**).
7. Set the inspiratory pressure of the ventilator to 12 cm H_2O and set the respiratory rate to 120 breaths per min (**Figure 1B**).
8. Decontaminate the skin in the surgical area with betadine and 70% ethanol.
9. Make a 2 - 3 cm posterolateral thoracotomy incision in the space at the 5th intercostal space, cut through skin and muscles with Noyes Spring Scissors (cutting edge: 14 mm; tip diameter: 0.275 mm) (**Figure 2B, C**). Surgical instruments used for thoracotomy procedure are sterilized prior to use.
10. Make a 1.5 cm incision at the 5th intercostal space to expose the left lung (**Figure 2D, E**). During the operation, use a high temperature cauterizer to stop bleeding.
11. Lift one-third of the left lung lobe from the chest with blunt tip forceps (**Figure 2F**), and then use a cotton swab to pull out the entire left lung (**Figure 2G**).
12. Identify the pulmonary artery and bronchi of the left lung lobe (**Figure 2G**).
13. Tightly ligate the bronchi and vessels at the hilum with a silk surgical suture and cut out the left lung lobe at 3 - 4 mm from the ligation (**Figure 2H, I**).
    NOTE: Be careful not to cut off the suture knots on the left hilum, which can cause pneumothorax (*i.e.*, air or gas in the cavity of the thorax).
14. Close the chest wall with 1 suture, and then stitch the muscle layer and the skin layer sequentially, using 5 - 6 interrupted sutures. Leave a 3 - 4 mm gap between each suture (**Figure 2M, 2N**).
    NOTE: Keep the surgical suture needle away from the heart; inadvertent cardiac puncture will result in immediate death.
15. Disinfect the surgical area with povidone-iodine.
16. After the surgical operation, place the mouse on a 38 °C thermal pad and connect the mouse to the ventilator until spontaneous breathing movements commence (**Figure 2O**).

2. Prosthesis Implantation

1. Perform steps 1.1 - 1.13 of the PNX procedure (that is, up to the point when the left lung lobe of the mouse is removed).
2. Clamp the center of the silicone prosthesis (customer made, 12 mm in length, 3 mm in thickness, 7 mm in width, 0.2 g, ellipsoid-shape) using blunt forceps (**Figure 2J**). Sterilize silicone prosthesis prior to insertion.
3. Hold the rib with forceps with one hand to expose the thoracic cavity, and then insert the prosthesis into the left empty thoracic cavity with another hand.
   NOTE: The insertion angle is approximately 45 degrees between the frontal plane of the prosthesis and the thoracic surface (**Figure 2K, L**). Be very gentle when inserting the prosthesis. Excessive force will result in pleural rupture.
4. Adjust the orientation of the prosthesis with blunt forceps to ensure that the prosthesis occupies the left empty thoracic cavity.
5. Perform steps 1.14 - 1.16 of the mouse PNX procedure.

3. Measurement of ILV

1. Prepare a custom device ("inflation tube") that consists of a plunger removed from a disposable serological pipette (10 mL), a 40 cm long flexible tube with a needle adapter, a flow rate control valve, and an 18 G needle. After assembly, secure the pipette on a board with tape (**Figure 3A**). The distance between the top of the pipette and the experimental bench must be at least 30 cm.
2. Prepare fresh 4% paraformaldehyde (PFA) fixation solution by dissolving 20 g PFA in 500 mL pre-heated 1x phosphate buffered saline (PBS) in a 55 °C water bath, shaking manually once every 10 min until the solution is clear. After cooling to room temperature, filter the solution with a 0.45 µm filter.
   CAUTION: Wear appropriate personal protective equipment (PPE) when handling PFA.
3. Sacrifice mice with an overdose injection of anesthetic (0.8% phenobarbital sodium, 1,000 U/mL heparin).
4. Secure each mouse on a polystyrene dissection plate and spray it with 70% alcohol.
5. Carefully open the mouse chest and cut out the sternum using scissors to thoroughly expose the lung lobes.

6. Remove excessive tissue using scissors to expose the trachea. Make sure to separate the trachea from the esophagus.
7. Cut the abdominal aorta and insert a 25-gauge needle into the right ventricle of heart; connect the needle to a 20 mL syringe prior to this insertion. Slowly push 1x PBS into the heart to remove blood cells until lungs turn white. Typically, 5 - 10 mL PBS is required to clear the pulmonary blood vessels.
8. Fill the custom-constructed inflation tube with 4% fresh PFA and remove all the bubbles from the inflation tube.
9. Insert the 18-gauge needle of the inflation tube into the trachea and clip the trachea with vessel clips to avoid fluid leakage.
10. **Inflate lungs with 4% PFA at a constant transpulmonary pressure of 25 cm/H_2O[2,20]. Incubate the lungs at room temperature for 2 h to achieve fully expanded lungs. This "pre-fix" step is critical for preserving lung morphology.**
    1. By monitoring the inflation tube, record the value of the initial 4% PFA volume and record the final volume. The internal lung volume equals the initial 4% PFA volume minus the final 4% PFA volume.
11. Ligate the trachea and using scissors, gently dissect out the lungs (keeping the lungs intact) from surrounding connective tissues. Be very gentle to avoid damaging the lungs.
12. Incubate the lungs in a 50-mL conical tube filled with 4% PFA for 12 h at 4 °C with gentle shaking on an shaker (50 rpm). Proceed to tissue processing and staining (see section 4).

4. Tissue Embedding, Sectioning, and Hematoxylin & Eosin (H&E) Staining

1. After fixation, use Noyes Spring Scissors to trim the heart and excessive connective tissues off the lungs. Gently separate the individual lung lobes by cutting off the bronchus that connects the lung lobes to the trachea.
2. Extensively wash the lung lobes 3 - 4 times in 50 mL 1x PBS (30 min/wash) on an orbital shaker (50 rpm).
3. Following the final wash, cryoprotect the lung lobes by immersing them in a 30% sucrose solution (in 1x PBS) at 4 °C until the tissue sinks to the bottom of the 50-mL conical tubes (approximately 12 h).
4. Prior to embedding and cryosectioning the tissues, remove the lung lobe samples from the tubes with forceps, retain the accessory lobes for the histological analysis, dab the remaining sucrose solution from the surface of the accessory lobe samples, and then thoroughly immerse the sample into a Petri dish containing optimal cutting temperature (O.C.T) compound for approximately 30 min.
5. Freeze the O.C.T-embedded accessory lobe samples in liquid nitrogen using cryomolds. Position the largest surface area of the lobe parallel to the bottom of the mold.
6. Prepare a total of three 10-µm-thick sections for each sample during cryosectioning for histological analysis. Discard the first 1 mm of tissue, collect one 10-µm-thick section, discard 0.5 mm of tissue, collect another section, discard 0.5 mm of tissue, and collect the third (final) section.
7. Air dry the sections for 1 h before performing H&E staining.
8. **Perform H&E staining**
    1. Wash the sections in 3 - 4 changes of tap water and then stain the sections in fresh hematoxylin for 2 min; rinse the section under running tap water; immerse the section two times in a 1% HCl-70% ethanol solution to remove excess hematoxylin.
    2. Stain the section in fresh eosin for 3 min; dehydrate the sections with two successive 30 s washes in 95% ethanol and two 30 s washes with 100% ethanol; clear the sections in xylene for 30 s, repeat the clearing step once in fresh xylene; mount the slides with mounting medium using glass coverslips.

5. Quantification of MLI

1. Acquire digital images of the H&E stained accessory lobe sections (20X magnification) using a bright-field microscope.
2. To quantify the MLI, select a total of 15 non-overlapping views (1,000 µm x 1,000 µm) randomly from the suitable areas (without arteries and veins, major airways, and alveolar ducts) of 3 sections.
3. Place a grid with 10 evenly-distributed vertical lines and 10 equally-distributed horizontal lines of defined length (1,000 µm) on the chosen areas of view using a ruler tool; each line is thus spaced 100 µm apart (**Figure 4B**).
4. Define the value of one intercept as the linear length between two adjacent alveolar epithelia. Measure the values of all intercepts along each 1,000 µm length line.
5. For each grid, quantify the values of all intercepts among the 10 horizontal 1,000 µm length lines and the 10 vertical 1,000 µm length lines. NOTE: MLI is the average value of the intercept lengths from a total of 15 grids analyzed from among the 3 sections prepared for each of the accessory lobes.

6. Calculation of ISA

1. Calculate the ISA using **Equation 1** (see the **Introduction**). Refer to section 3 for the measurement of ILV and refer to section 5 for the quantification of MLI.

Representative Results

We performed here an experiment with a PNX-treated group and a prosthesis implantation (Prosthesis-implanted) group. These groupings are the same as the groupings used in a previously-published study from our research group[14].

The mouse PNX and prosthesis implantation procedures are shown in **Figure 2**. 8 week-old CD-1 male mice are used for the surgeries and for the quantification. In the PNX-treated group and the Prosthesis-implanted group, the left lung lobes both were resected (**Figure 2A-2I**). In the Prosthesis-implanted group, a prosthesis that mimics the size and shape of the left lung lobe was inserted into the chest after the left lung lobe was removed (**Figure 2J-2L**).

Fourteen days after surgery, a custom-made inflation tube was used to determine the ILV of the remaining right lungs (**Figure 3A**). The average ILV of the remaining right lungs of the 5 PNX-treated mice was approximately 1.4 mL, significantly higher than the 1.05 mL ILV values of the right lungs of the 5 Prosthesis-implanted mice (**Figure 3B, Table 1**).

For the MLI measurement, a total of 15 views were analyzed from among the 3 sections prepared from each mouse. **Figure 4A** shows a merged image from an accessory lobe section and the morphological standard for a chosen area (*e.g.,* view 1 - 3) or a non-chosen area (*e.g.,* view 4 - 5) used for the quantification of MLI. Here, view 3 from an accessory lung lobe section of the PNX-treated group was taken as an example for the measurement of MLI (**Figure 4B**). An enlarged picture of view 3 is also displayed for illustration (**Figure 5A**). Line 3 is presented as an example: the length of an intercepted alveolar air space indicated by the double-headed arrow lines. Our analysis showed that the MLI values in the remaining right lungs of the PNX-treated mice were significantly greater than those of the remaining right lungs of the Prosthesis-implanted mice (**Figure 5B, Table 1**). All data are presented as the mean ± S.E.M (**Figure 5B**).

ISA was calculated using **Equation 1**. **Table 1** shows the ILV values, MLI values, and ISA of all lungs. The ISA of the Prosthesis-implanted mice was significantly smaller than that of the PNX-treated mice, demonstrating that the insertion of a prosthesis impaired PNX-induced regeneration.

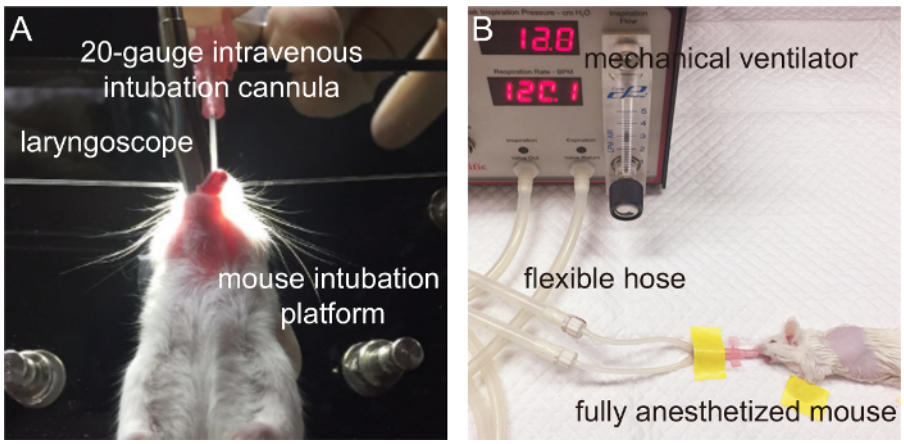

Figure 1: Mouse Endotracheal Intubation and Mechanical Ventilation. (**A**) Endotracheal intubation with a 20 G intravenous intubation cannula via laryngoscopy. (**B**) Connect the fully-anesthetized mouse to a pressure-controlled mechanical ventilator before the surgery. Please click here to view a larger version of this figure.

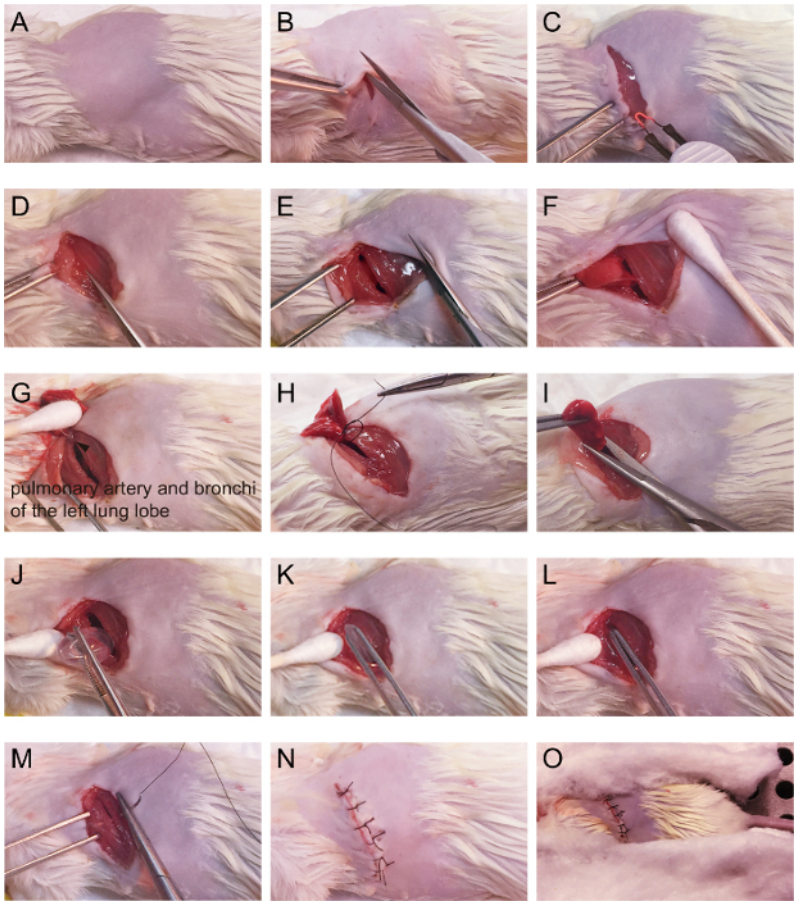

Figure 2: Mouse Pneumonectomy (PNX) and Prosthesis Implantation. (**A-C**) Cut the skin and the muscle layer; stop bleeding with a high temperature cauterizer during the surgical operation. (**D-E**) Make a 1.5 cm incision at the 5th intercostal space. (**F-H**) Pull out the left lung lobe with blunt forceps and identify the pulmonary artery and bronchi, ligate at the hilum. Arrowheads represent the pulmonary artery and bronchi of the left lung lobe. (**I**) The left lung lobe was resected at 3 - 4 mm from the ligation. (**J-L**) Insert a prosthesis into the left thoracic cavity. (**M, N**) Suture the chest, the muscle layer, and the skin layer. (**O**) Monitor the mice until spontaneous breathing movements commence. Please click here to view a larger version of this figure.

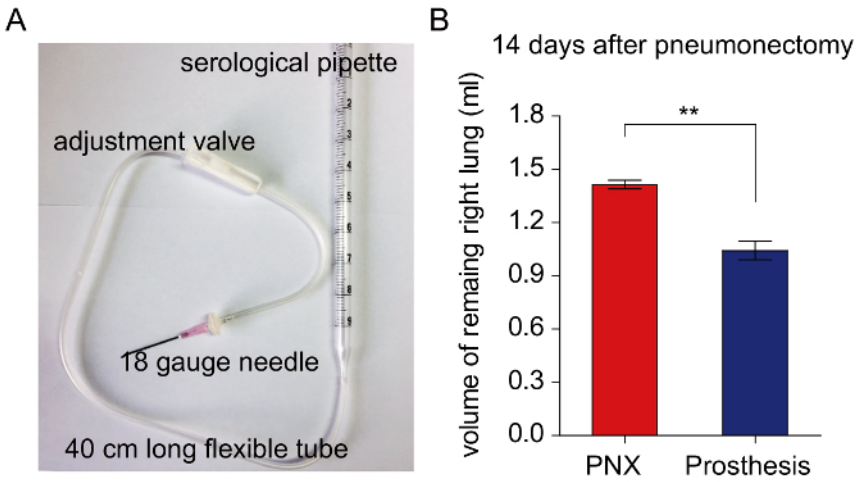

Figure 3: Measurement of the Internal Lung Volumes (ILVs) of the Remaining Right Lungs. (**A**) A custom device ("inflation tube") for measuring the internal lung volumes. (**B**) The ILVs (mean ± S.E.M.) of the remaining right lungs of the PNX-treated group and Prosthesis-implanted group were measured at 14 days post-PNX. **, p <0.01, Student's t-test. Please click here to view a larger version of this figure.

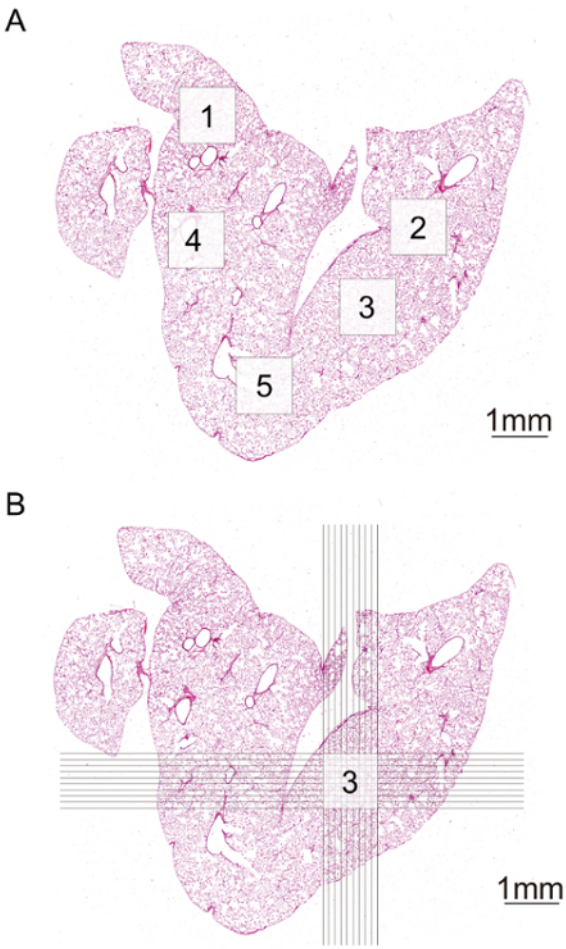

Figure 4: Quantification of the Mean Linear Intercept (MLI) of Accessory Lobes in the Remaining Right Lungs. (**A**) A merged image of an accessory lobe section is shown. Examples of chosen areas (*e.g.,* view 1 - 3) and non-chosen areas (*e.g.,* view 4 - 5) used for MLI quantification. (**B**) 10 evenly-spaced vertical lines and 10 evenly-spaced horizontal lines of defined length (1,000 µm) were placed on the chosen area. Scale bar = 1 mm. Please click here to view a larger version of this figure.

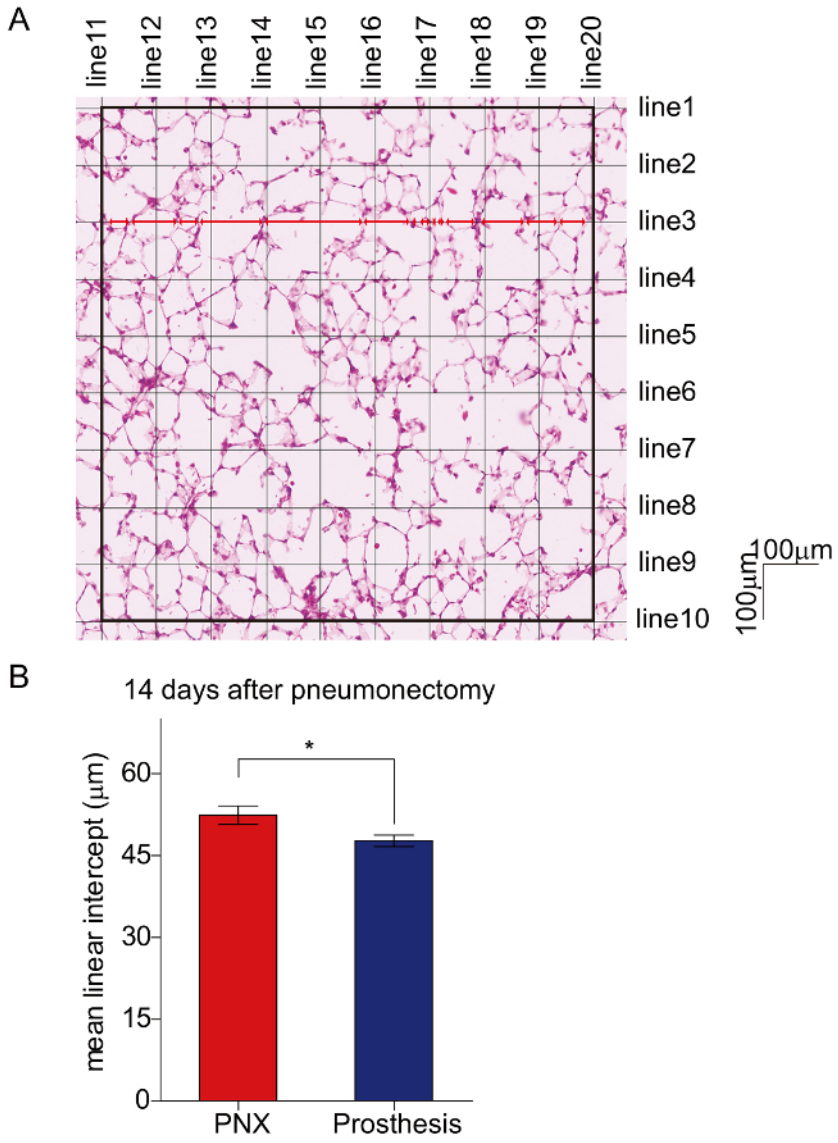

Figure 5: Quantification of the MLI in PNX-treated Lungs and Prosthesis-implanted Lungs. (**A**) An enlarged picture of view 3 in **Figure 4B** is shown. Red-colored lines with double arrowheads represent the length of one linear intercept. (**B**) The MLI values (mean ± S.E.M.) of accessory lobes of PNX-treated mice and Prosthesis-implanted mice were measured at 14 days post-PNX. *, p <0.05, Student's t-test. Scale bar: 100 μm. Please click here to view a larger version of this figure.

14 days after surgery	internal lung volume (ml)	mean linear intercept (mm)	internal lung surface area=4ILV/ MLI (cm^2)
PNX-treated	1.5	57.8	1038.06
	1.35	49.6	1088.71
	1.42	48.5	1171.13
	1.4	51.5	1087.38
	1.4	54.6	1025.64
Prosthesis-implanted	1.1	49.6	887.10
	1	50.5	792.08
	1.1	47.3	930.23
	1.15	44.8	1026.79
	0.86	46.3	742.98

Table 1: Calculation of the ISA Values of PNX-treated and Prosthesis-implanted Mice. The values of ILV, MLI, and ISA of the accessory lobes at 14 days post-PNX.

Discussion

In this protocol, we provide detailed descriptions about the measurement of pulmonary parameters after mouse left lung PNX and prosthesis implantation. ISA is now considered to be a key metric for the assessment of respiratory function in many pulmonary diseases and in injury-induced alveolar regeneration. However, although the pulmonary research community is in agreement about the utility of ISA as a useful metric, to date, there has been little consideration of the standardization of the measurement of ILV and MLI, the two parameters used to calculate ISA. Obviously, as with any measurement, it is important to attempt to obtain unbiased data. The core goal of the present research effort is to establish a standardized protocol for use by the murine pulmonary research community.

We attempted to reduce sources of measurement bias in a number of ways as we developed this protocol. We found that variation in ILV measurements could be reduced by preventing fluid leakage by insuring that the size of the needle inserted into the trachea is dimensionally matched (we found that 18-gauge needles closely matched mice tracheae). We also found that the mouse chest wall needs to be thoroughly removed prior to the PFA inflation, as this minimizes the potential influence of the chest wall on the ILV measurements. MLI values depend to a large extent on alveolar morphology. Accordingly, in addition to the importance of using age-matched and sex-matched mice, the proper maintenance of alveolar morphology during the lung fixation procedures is critically important. We here used a widely-adopted fixation method: we inflated lungs at a transpulmonary pressure of 25 cm H_2O with freshly prepared 4% PFA to fully dilate the lung tissue. In our experience, lower distending pressures can lead to tissue contraction, abnormal alveolar morphology, and ultimately result in lower MLI values.

Observations in our previous studies have indicated that the accessory lobe of the remaining lung exhibits maximal volumetric expansion, as compared to the other three lobes of the right lung, after PNX regeneration; the accessory lobe of the remaining lung also exhibits maximal increases in the value of morphological parameters (*e.g.*, MLI)[21,22,23,24]. We therefore only analyzed sections from accessory lobes to avoid variation from different lung lobes. To help reduce variation between the various sections used in the quantification of MLI, we controlled the orientation of the lobe during embedding: we placed the largest surface of the lobe parallel to the bottom of the cryomold. We also carefully controlled the thickness of sample slices, the sequence of section sampling, and the cutting position during sectioning. In addition to sample processing, another important aspect of standardization is that all arteries and veins, pleura, major airways, and alveolar ducts need to be excluded from the tissue areas that are assessed during MLI quantification. Arteries, veins, and alveolar ducts are all much larger than alveoli (by 4 - 10 times), so excluding these large structures is important for obtaining reliable intercept measurements. For each group, 5 mice are adequate for the quantification. For each mouse, a total of 15 non-overlapping views (1,000 µm x 1,000 µm) were selected randomly from the suitable areas (without arteries and veins, major airways, and alveolar ducts) of the 3 sections of the accessory lobe. The sample embedding method and MLI measurement can be also applied to paraffin treated lung tissues.

Whereas others have defined MLI as the total line length divided by the number of intercepts with crossed alveolar walls[25], we here used a linear intercept as the linear length between two adjacent alveolar epithelial walls in MLI calculations, disregarding the thickness of mesenchyme from the MLI data. Accordingly, we calculated ISA using the ILV but not the total lung volume.

For the PNX and prosthesis implantation procedures, the survival rate of mice undergoing PNX ranged from 85% to 90%. The survival rate of mice undergoing prosthesis implantation was about ~80%. During all surgeries, several steps should be taken to improve mice survival. 1) The proper insertion of the catheter in the trachea is a prerequisite for a successful PNX operation. With the guidance of a laryngoscope, the trachea of the mouse can be easily observed to facilitate safe and effective endotracheal intubation. 2) Do not puncture the lungs or hearts during the procedure. Ensure that the thorax of the fifth left intercostal space is widely opened and that the hilum of the left lobe is clearly identified prior to lobectomy. When performing left lung lobe resection, ensure that the left lobe is intact via the use of blunt forceps to avoid pulmonary hemorrhage and/or lobar rupture. During wound closure, keep the tip of the surgical needle away from the heart and lungs. 3) Be gentle when inserting the prosthesis into the empty thorax cavity, as excessive force can cause rupture of the pleura. 4) Mice should be placed on a 38 °C thermal pad and monitored until their senses recover, as postoperative hypothermia is known to increase mouse morbidity.

After the removal of the left lung lobe, both the pulmonary respiratory units and internal lung gas exchange area were significantly reduced. 14 days after the surgery, the ILV, MLI, and ISA in the remaining lung tissue were significantly larger in the PNX-treated group of mice than in the Prosthesis-implanted group, strongly suggesting that the insertion of a prosthesis blocked PNX-induced alveolar regeneration. Thus, both PNX

and prosthesis-implantation mouse models can be used as powerful tools for investigation of the cellular and molecular events that occur during mechanical force induced re-alveolarization. Additionally, the ISA values of *Yap* AT2 null lungs were significant smaller than those of control lungs at post-PNX day 14[1], indicating that our protocol is also suitable for detecting impaired regeneration of genetic mutant mice. The rigorous and standardized quantitative methods presented in this study can be applied to measure the lung parameters and ISA in developmental studies and with genetically modified animal models of multiple diseases, including emphysema in chronic obstructive lung diseases, alveolar regeneration following lung injuries, and lung development defects.

Disclosures

The authors have nothing to disclose.

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