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Lung Fixation under Constant Pressure as a Method Useful for Evaluating Emphysema in Mice

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Corresponding Author:	Tadashi Sato Juntendo University Graduate School of Medicine Tokyo, Tokyo JAPAN
Corresponding Author's Institution:	Juntendo University Graduate School of Medicine
Corresponding Author E-Mail:	satotada@juntendo.ac.jp
Order of Authors:	Keiko Karasutani
	Hario Baskoro
	Tadashi Sato
	Naoko Arano
	Yohei Suzuki
	Aki Mitsui
	Naoko Shimada
	Yuzo Kodama
	Kuniaki Seyama
	Yoshinosuke Fukuchi
	Kazuhisa Takahashi
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TITLE:

Lung Fixation under Constant Pressure for Evaluation of Emphysema in Mice

AUTHORS AND AFFILIATIONS:

Keiko Karasutani, Hario Baskoro, Tadashi Sato, Naoko Arano, Yohei Suzuki, Aki Mitsui, Naoko Shimada, Yuzo Kodama, Kuniaki Seyama, Yoshinosuke Fukuchi, Kazuhisa Takahashi

Department of Respiratory Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan

Corresponding Author:

Tadashi Sato (satotada@juntendo.ac.jp)

Email Addresses of Co-authors:

Keiko Karasutani (k-karasutani@juntendo.ac.jp)
Hario Baskoro (hario@juntendo.ac.jp)
Tadashi Sato (satotada@juntendo.ac.jp)
Naoko Arano (narano@juntendo.ac.jp)
Yohei Suzuki (yohei-s@juntendo.ac.jp)
Aki Mitsui (m-akira@ga3.so-net.ne.jp)
Naoko Shimada (naokoh@juntendo.ac.jp)
Yuzo Kodama (ykodama@juntendo.ac.jp)
Kuniaki Seyama (kseyama@juntendo.ac.jp)
Yoshinosuke Fukuchi (yfukuchi@tea.ocn.ne.jp)
Kazuhisa Takahashi (kztakaha@juntendo.ac.jp)

KEYWORDS:

dhronic obstructive pulmonary disease, emphysema, lung fixation, constant pressure, emphysematous mouse model, cigarette smoke

SUMMARY:

Presented here is a useful protocol for lung fixation that creates a stable condition for histological evaluate of lung specimens from a mouse model of emphysema. The main advantage of this model is that it can fix many lungs with the same constant pressure without lung collapse or deflation.

ABSTRACT:

Emphysema is a significant feature of chronic obstructive pulmonary disease (COPD). Studies involving an emphysematous mouse model require optimal lung fixation to produce reliable histological specimens of the lung. Due to the nature of the lung's structural composition, which consists largely of air and tissue, there is a risk that it collapses or deflates during the fixation process. Various lung fixation methods exist, each of which has its own advantages and disadvantages. The lung fixation method presented here utilizes constant pressure to enable optimal tissue evaluation for studies using an emphysematous mouse lung model. The main

advantage is that it can fix many lungs with the same condition at one time. Lung specimens are obtained from chronic cigarette smoke-exposed mice. Lung fixation is performed using specialized equipment that enables the production of constant pressure. This constant pressure maintains the lung in a reasonably inflated state. Thus, this method generates a histological specimen of the lung that is suitable to evaluate cigarette smoke-induced mild emphysema.

INTRODUCTION:

COPD is one of the leading worldwide causes of death[1]. Cigarette smoke is the most important cause of COPD, but the mechanisms of pathogenesis remain incompletely defined. COPD demonstrates two main characteristics, including progressive limitation of airflow and an abnormal inflammatory response of the lung. Emphysematous disorder frequently occurs in the lungs of COPD patients[2]. The pathological findings of emphysema are characterized by alveolar wall destruction[3]. Several animal species have been used to generate COPD models in vivo (i.e., dogs, guinea pigs, monkeys, and rodents)[4]. However, the mouse has become the most commonly used in the construction of COPD models. This has many advantages, including its low cost, ability to be genetically modified, extensive genomic information availability, availability of antibodies, and ability to use a variety of mouse strains[5]. Presently, there is no mouse model that can mimic the full features of human COPD; thus, individual researchers must choose which model is most suitable for the specific COPD research[6]. The emphysematous mouse model is one of many COPD mouse models that are currently available. Additional models include the exacerbation mouse model, systemic co-morbidities model, and COPD susceptibility model[7].

The emphysematous mouse model can be generated by several types of exogenous agents, including chemical agents and cigarette smoke exposure[4]. Chemical exposure (e.g., to elastase) produces a severe type of emphysema, while cigarette smoke results in mild emphysema[8,9]. Cigarette smoke is believed to be the main cause for the pathogenesis of COPD; therefore, the choice of cigarette smoke as a means to create a COPD mouse model is reasonable[10]. Many studies have used cigarette smoke to create emphysema in the mouse. For example, Nikula et al. successfully created an emphysematous mouse model from B6C3F1 female mice by exposing them to cigarette smoke for 7 or 13 months[11]. We have also established an emphysematous mouse model via senescence marker protein/SMP-30 KO mice[12]. It is crucial to perform a lung fixation method that can properly visualize this mild emphysema model by cigarette smoke exposure.

Various methods for lung fixation have been established[13]. However, there is no gold standard method of lung tissue fixation for evaluating emphysema[14]. Several studies from this lab have shown that the fixation system presented here is useful by creating a stable condition for evaluating emphysema[12,15-18]. The main advantage of the current system is that it can fix many lungs with the same condition at one time without lung collapse or deflation. The current lung fixation system uses some special equipment that allows lung specimens to be inflated at an appropriate constant pressure for a given period. This special equipment consists of three parts, including a lower container, upper container, and pump. Lung specimens are placed in

the lower container that is connected to pressurized fixing agents, resulting in a 25 cmH_2O pressure difference in the level of agents between the upper and lower containers[19].

PROTOCOL:

The following methods have been approved by the Animal Care and Use Committees of Juntendo University School of Medicine. There are three main steps in this method: 1) mouse dissection, 2) lung exsanguination, and 3) fixation of lung tissues assisted by specialized equipment. Typically, lung specimens are processed to embedment after 48 h of fixation[12,15-18].

1. Mouse dissection

1.1. Measure the body weight of the mouse, then determine the amount of pentobarbital to administer.

1.2. Inject pentobarbital intraperitoneally at a dosage of 70 mg/kg of body weight and confirm anesthesia by the absence of reaction to toe pinch.

1.3. Inject the needle at a 45° angle until it penetrates the skin and muscle. Draw the plunger and confirm an air vacuum, then inject the pentobarbital.

1.4. Confirm anesthesia by the absence of reflex motion.

1.5. Cut the mouse skin and abdominal muscle at the medial line, aiming for the cephalic area.

1.6. Cut laterally to provide a wider working space.

2. Lung exsanguination

2.1. Expose the diaphragm layer and puncture it with forceps.

2.2. Open the thoracic space and cut the sternal area, allowing the lungs and heart to be seen clearly.

2.3. Cut the heart in four locations: right atrium, left atrium, left ventricle, and right ventricle.

2.4. Insert a cannula (22 G) into the right ventricle area and direct it to the cephalic area until it reaches the pulmonary artery, as shown in **Figure 1.**

2.5. Turn on the pump and allow the 1x phosphate-buffered saline (PBS) to circulate (approximately 200 mL/h) until all lung tissue changes to a white color.

3. Fixation of lung tissue

3.1. Remove the trachea, lungs, and heart.

3.2. Free all three organs by cutting the surrounding connective tissues.

3.3. Tie the right main bronchus with a suture thread and cut all lobes of the right lung.

3.4. (Optional): the lobes of the right lung consist of four parts. Cut these parts from the right main bronchus and divide the parts for processing as frozen tissue samples.

3.5. Insert the heart and the lobes of the left lung into fixing agents, located inside a 10 mL syringe.

CAUTION: Fixing agents are hazardous. Wear proper protective equipment (e.g., long rubber gloves) and work in a well-ventilated room.

3.6. Create a vacuum condition using a 10 mL syringe to inflate the lung, as shown in **Figure 2**.

3.7. Insert a cannula (20 G) into the trachea and tie a knot.

3.8. Inflate the lung with fixing agents to check with for leaks, using a 1 mL syringe.

3.9. Transfer to lung fixation pressure equipment, as illustrated in **Figure 3**.

3.10. After fixing periods, remove the lung sample tying the trachea off with a knot.

REPRESENTATIVE RESULTS:

As described previously, the specialized equipment, which generates extended constant pressure, can be divided into three parts (**Figure 3A**). The lower part is the point at which to insert the lung sample (**Figure 4A**). The lung is connected via a cannula (20 G) to the tip of formalin flow using a three-way stop cock (**Figure 4B**). Pressure is generated from the different surface levels of fixing agents between the lower and upper containers (**Figure 5**). The pressure difference is 25 cmH_2O; however, using the height adjustment knob, the pressure can be adjusted within the range of 25–30 cmH_2O (**Figure 5**). A pump connects the lower and upper containers via tubes (**Figure 3A**), preserving a 25 cm difference in fixing agent surface height. The direction of agent flow is described in **Figure 3B**.

Presented next is a representative result of histological findings in the lung, following 48 h of fixation. Six-month-old male SMP30-KO mice were exposed to cigarette smoke or fresh air (as control) for 8 weeks. Both tissue specimens were stained with hematoxylin and eosin. **Figure 6A** shows histological findings from the air-exposed mice, which did not exhibit marked airspace enlargement. In contrast, **Figure 6B** reveals significant airspace enlargement and alveolar wall destruction in mice that were exposed to chronic cigarette smoke.

The mean linear intercepts (MLI) was determined according to the method described by Thurlbeck et al.[20] to access the airspace size. The destructive index (DI) was determined to evaluate the destruction of the alveolar wall according to the method described by Saetta et al.[21]. These morphometric examinations of the lung specimen revealed that DI and MLI were significantly greater in the smoke-exposed SMP30-KO mice than in the air-exposed mice (**Figure 6C,D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Lung exsanguination. A cannula was inserted at the location of the right ventricle and directed to the pulmonary artery.

Figure 2: Vacuum syringe lung inflation. Vacuum condition inside the 10 mL syringe containing fixing agents to inflate the lungs.

Figure 3: Lung fixation equipment. (A) The acrylic equipment allowed a 25 cmH_2O pressure difference to inflate the lungs continuously for 48 h, utilizing a pump machine. (**B)** The direction of fixing agent flow is indicated by arrows.

Figure 4: Lower container. (A) The mouse lung specimen was positioned inside fixing agents in the lower container. (**B)** Inside the lower container, there is a sample placement box, at the top of which formalin flows through a three-way stopcock and the cannula.

Figure 5: Upper container and height adjustment knob. The upper container generated a pressure of 25 cmH_2O. There are two pairs of height adjustment knobs which can be used to adjust the height of the upper container; as a result, the pressure that is generated can be set within the range of 25–30 cmH_2O.

Figure 6: Mouse lung histologic and morphometric findings. Representative histologic images of lung sections from 8-week cigarette smoke-exposed or air-exposed SMP30-KO mice (6 month-old, male), stained with hematoxylin-eosin. Scale bar = 100 μm. (**A)** The air-exposed group did not show significant enlargement or other findings. (**B)** The cigarette smoke-exposed group showed marked airspace enlargement and alveolar wall destruction. (**C)** The mean linear intercepts (MLI). In the lungs of cigarette smoke-exposed mice, MLI was significantly greater than air-exposed mice (*$p < 0.001$). (**D)** The destructive index (DI). In the lungs of cigarette smoke-exposed mice, DI was significantly increased compared to the lungs of air-exposed mice (*$p < 0.001$). Values are presented as mean ± SD (n = 6 for each group).

DISCUSSION:

The fixation procedure for rodent lungs presented here is not novel; however, this system has several advantages. Firstly, it can fix many lungs (maximum of 20) with the same condition at one time. The Society of Toxicologic Pathology states that the pressure for gravity instillation vary from 22–25 cmH_2O[22]. Notably, several studies have performed lung fixation at a pressure

of 25 cmH_2O[13,19,23-27], which has been adopted in our laboratory using the current system[12,15-18].

Secondly, it can fix lung tissues at a constant pressure for various periods of time. In our laboratory, lung samples are usually fixed for 48 h. Many investigators use a relatively short period of time (e.g., 5–20 min)[13,28-32], then tie off the inflated lung and immerse in formalin for extended periods as desired. There is no data or research indicating a gold standard for the length of duration for lung fixation. However, the statement of the American Thoracic Society (ATS)/European Respiratory Society (ERS) describe the “silver standard”, in which airway inflation pressure must be maintained for at least 24 h[14]. The Japanese Society of Pathology also recommended fixation times of no longer than 1 week to produce consistent immunohistochemical slides; although, their recommendation is based on analysis using human specimens[33]. Relatively short fixing time periods may not be applicable to the current system, because each sample is supposed to be individually placed in the lower container. This is a limitation of the current system. In conclusion, the proper length of time for mouse lung fixation remains unknown.

Critical steps in this method are related to the risk of lung formalin leakage during the formalin fixation process. Lung formalin leakage can cause lung size shrinkage. This risk can be divided into two parts. The first part occurs during the sacrifice step. While opening the thoracic cage, it is important not to cause injury to the lung surface. The key to prevention is to approach this from the diaphragm and continue to cut the thoracic rib cage after the lung is detached from the parietal pleura. This method avoids lung injury caused by surgical equipment. Another key step occurs when tying the right main bronchus. It is important to identify which are the mouse’s right lobes. Placing the lungs in a position where they can be seen from a dorsal view enables easier identification of the location of the lungs.

The second part is during the lung fixation process using specialized equipment. A critical step occurs while inserting the lung specimen into the lower container’s formalin port. It should be confirmed that the insertion is tightly secured to prevent lung specimen detachment from the formalin port during the constant pressurization process. Another aspect to highlight is the tubing connection between the three parts of specialized equipment (lower container, upper container, and pump). All tube connections should be tightly connected. If leakage occurs, the formalin volume in the upper container will decrease, thereby reducing constant pressure.

According to recommendations from the Society of Toxicologic Pathology, intratracheal instillation of formalin has advantages for rodent lung model, which prevail over its disadvantages[22]. They have suggested the use of an intratracheal formalin fixation method when performing quantitative studies of alveolar lung morphometry. Intratracheal lung instillation has two advantages, including preservation of the airway and alveolar wall as well as visualization of lung parenchyma[22]. One study by Braber et al. revealed that the intratracheal formalin instillation method is superior in terms of preserving lung structure when compared with the vacuum inflation and whole-body perfusion methods[13]. The current method utilizes intratracheal instillation in a mouse model to optimize visualization of the alveolar area.

Regarding fixing agents, 10% formalin, which contains formaldehyde, is conventionally used. Formaldehyde is widely used as a fixing agent for immunopathological investigations because it does not completely destroy protein immunogenicity. However, the ATS/ERS statement does not recommend formalin fixation, because it does not adequately stabilize tissue structure[14]. Glutaraldehyde is recommended for airway instillation instead; however, it is subject to destroy protein immunogenicity, which results in an unsuitable fixing agent for immunohistochemistry. Several pieces of evidence have reported that the fixed lungs may be provided for morphometric evaluation (e.g., mean linear intercepts, internal surface area, and destructive index) following formalin fixation using the current fixation system[12,15-18]. Certainly, glutaraldehyde can be adopted for the current system; thus, researchers can choose both agents in the current system according to experimental needs.

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DISCLOSURES:

The authors have no competing interests to declare.

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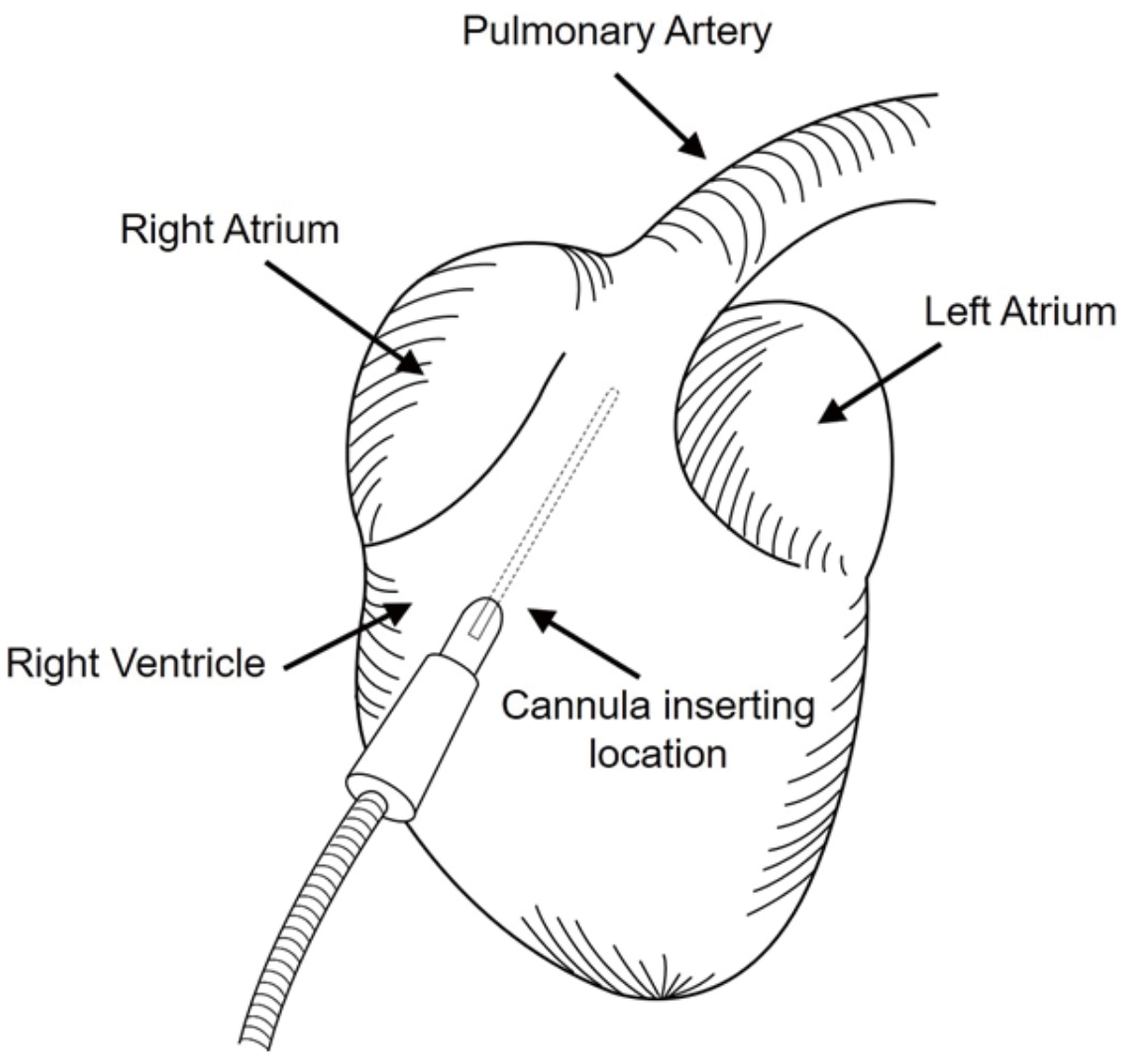
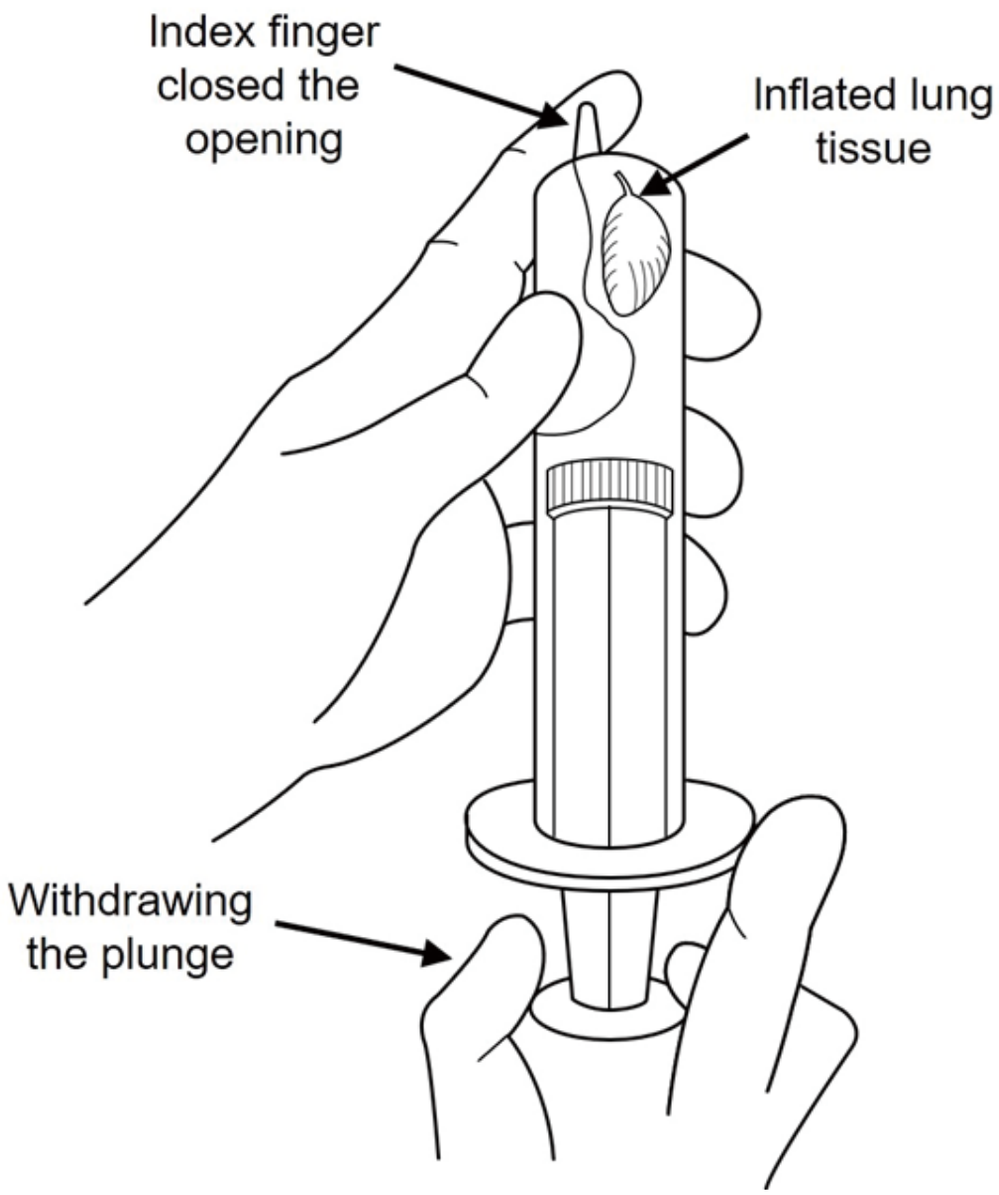
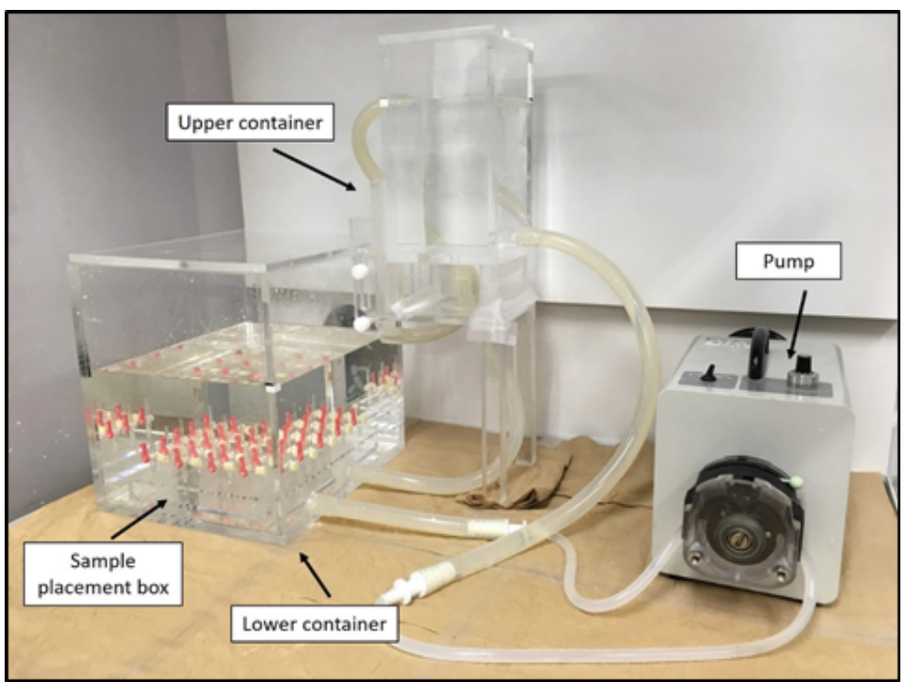
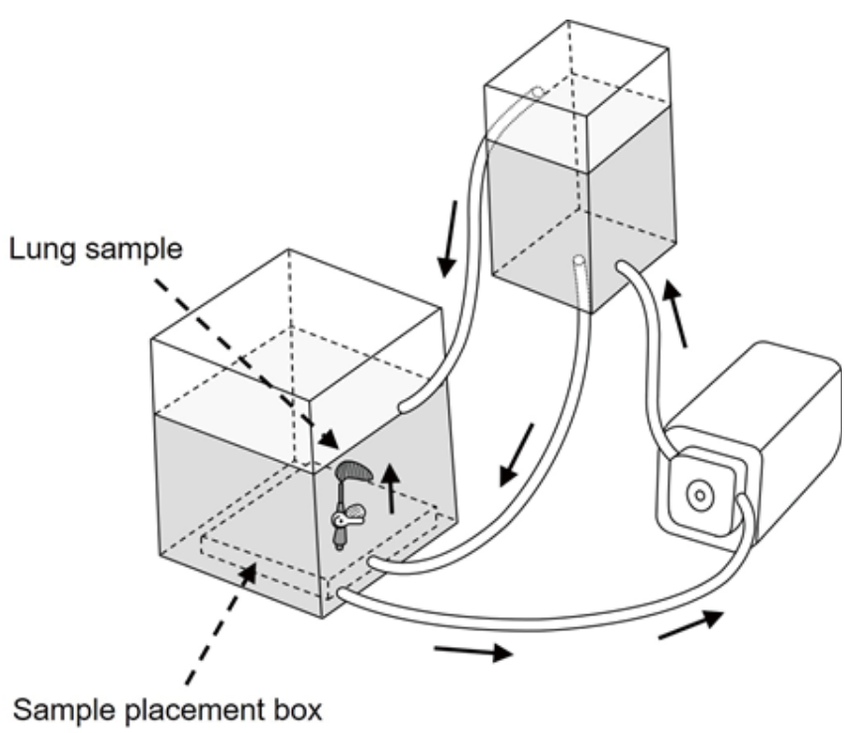




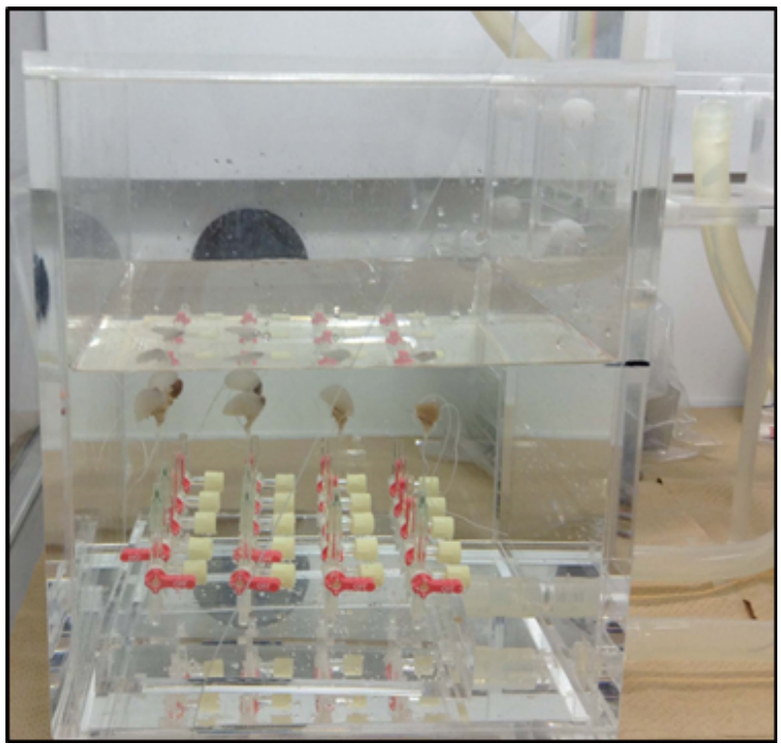
A. Lung fixation equipment



B. The direction of fixing agent flow



A. Lower container



B. Sample placement box and three-way stopcocks

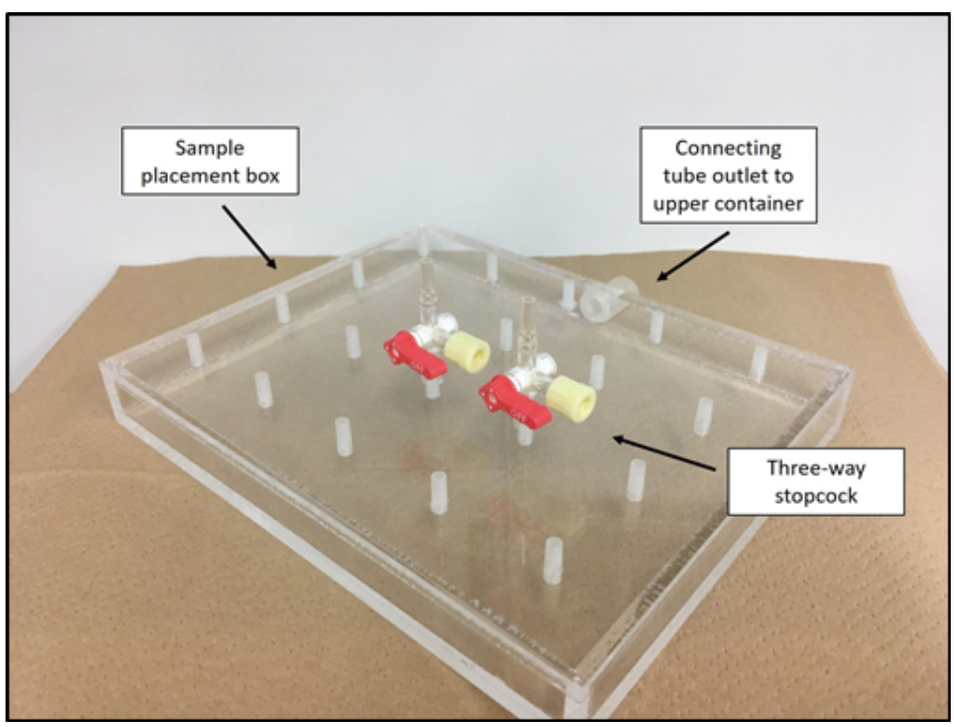
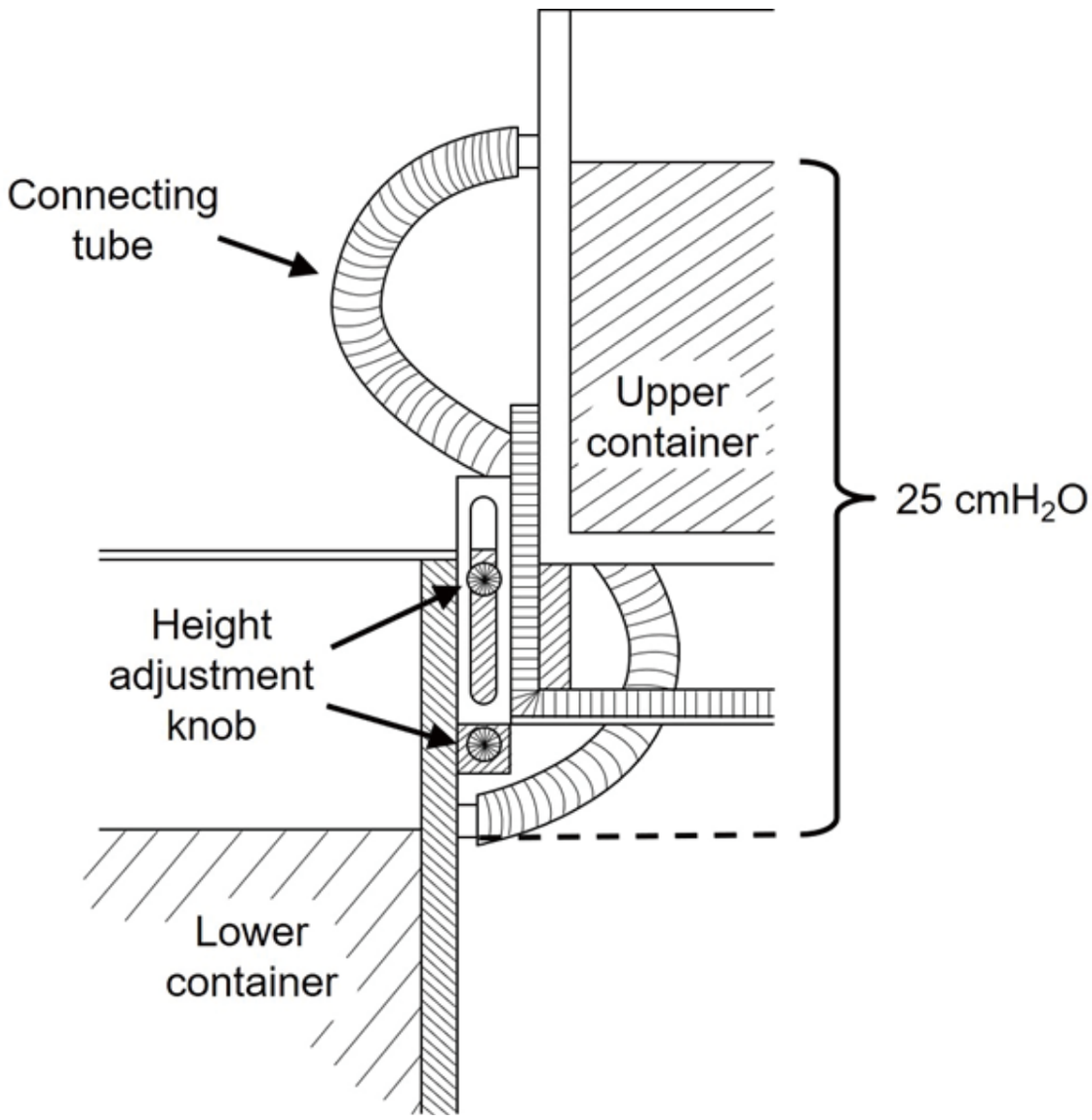
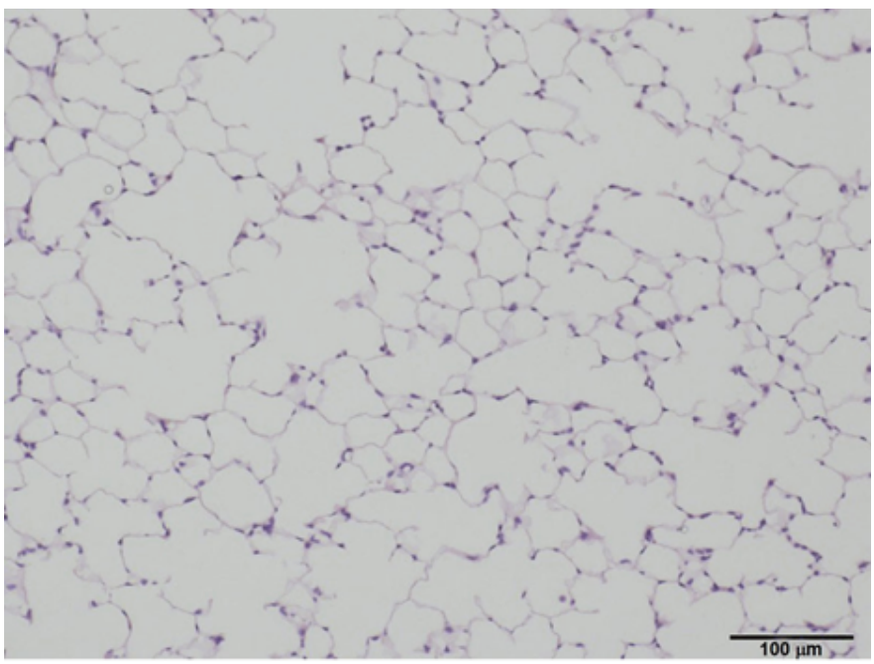


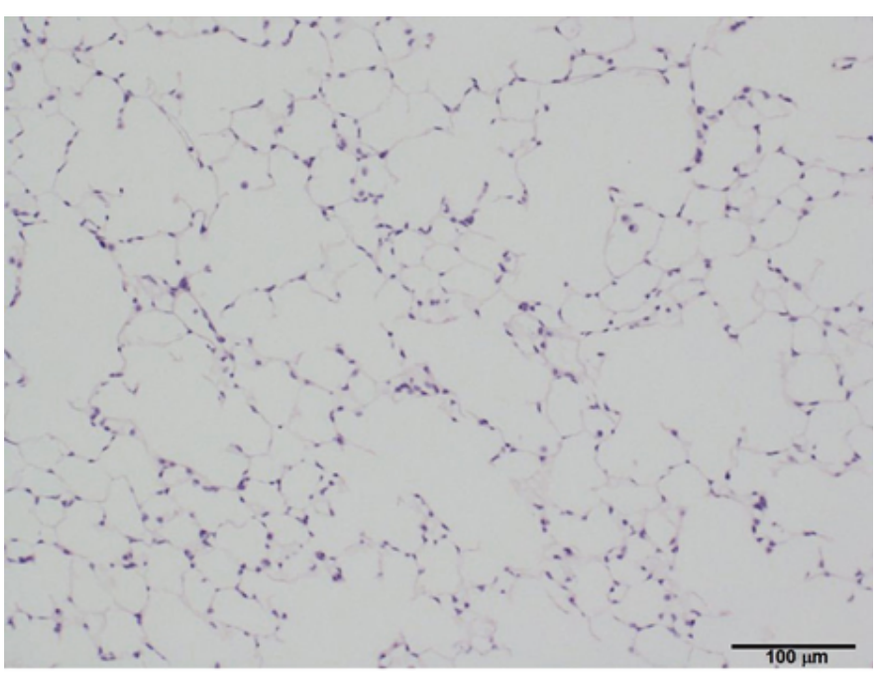
Figure5



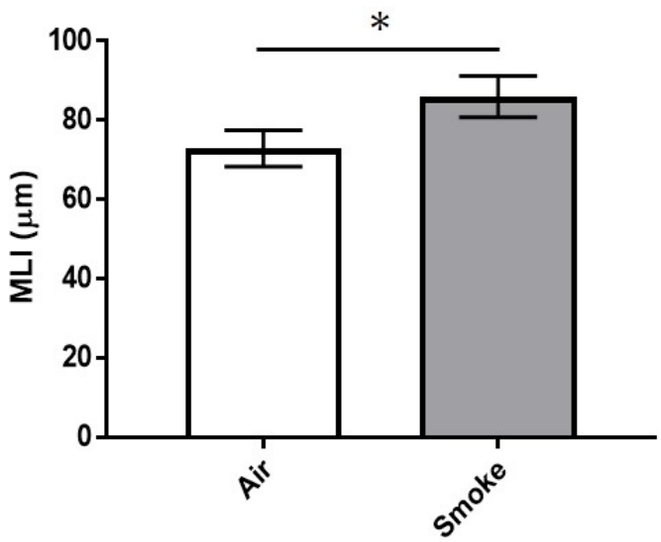
A. Air-exposed lung



B. Cigarette smoke-exposed lung



C. Mean linear intercepts (MLI)



D. Destructive index (DI)

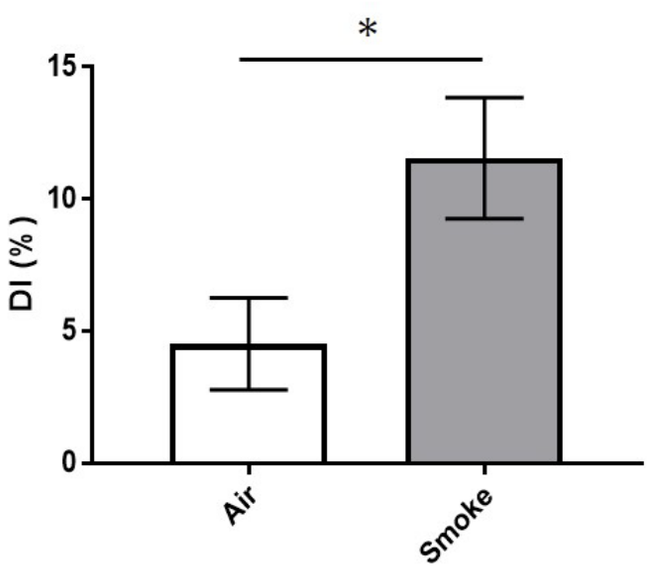


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_KK201810.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10% formalin (formalin neutral buffer solution)	Wako	060-01667	
Bent forceps	Hammacher	HSC187-11	
Cannula, size 20G	Terumo	SR-FS2032	
Cannula, size 22G	Terumo	SR-OT2225C	Cannula to exsanguinate lung
Forceps	Hammacher	HSC184-10	
Kimtowel	Nippon Paper Crecia (Kimberly Clark)	61000	
Kimwipe	Nippon Paper Crecia (Kimberly Clark)	62011	
Lower container (acrylic glass material)	Tokyo Science	Custom-made	Pressure equipment component
Roller pump	Nissin Scientific Corp	NRP-75	Pump machine to exsanguinate lung
Roller pump RP-2000	Eyela (Tokyo Rikakikai Co. Ltd)	160200	Pressure equipment pump
Silicone tube Ø 9 mm	Sansyo	94-0479	Pressure equipment component
Somnopentyl (64.8 mg/mL)	Kyoritsu Seiyaku	SOM02-YA1312	Pentobarbital Sodium
Surgical scissor	Hammacher	HSB014-11	
Suture thread, size 0	Nescosuture	GA01SW	
Syringe, 1 mL	Terumo	SS-01T	

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Title of Article:	Lung Fixation under Constant Pressure as a Method to Evaluate Emphysema in Mice
Author(s):	Keiko Karasutani, Hario Baskoro, Tadashi Sato, Naoko Arano, Yohei Suzuki, Aki Mitsui, Naoko Shimada, Yuzo Kodama, Kuniaki Seyama, Yoshinosuke Fukuchi, Kazuhisa Takahashi

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CORRESPONDING AUTHOR

Name:	Tadashi Sato
Department:	Department of Respiratory Medicine
Institution:	Juntendo University Graduate School of Medicine
Title:	Lung Fixation under Constant Pressure as a Method to Evaluate Emphysema in Mice
Signature:	Tadashi Sato
Date:	22/Oct/2018

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Details of the changes made to the revised manuscript (JoVE58197R1)

Dear Dr. Bing Wu,

Thank you for reviewing our manuscript. We appreciate your kind remarks. Our point-by-point responses to the reviewers' comments are provided below.

Point-by-point response to the comments of Reviewer #1:

This revision is disappointing. The authors try to defend their approach despite the reviewers´ comments. They make no serious efforts to take these comments into consideration and thereby improve the quality of their work. As an example, the authors now cite the ATS / ERS guidelines, but inappropriately. They cite them for stating that there is no gold standard for lung fixation. Although this is correct, the ATS/ ERS guidelines do contain very detailed information about silver standards. They are not mentioned by the authors. By doing so, the authors knowingly not only ignore these standards, but they violate them. This is scientifically inacceptable.
I challenge the statement in the response to reviewers that "We have reported several evidences wherein the fixed lungs did not shrink". A formalin-fixed lung does shrink. See e.g. Oldmixon et al. J Appl Physiol 1985;58:105-113 or Schneider & Ochs Am J Physiol Lung Cell Mol Physiol 2014;306:341-350 (cited here as ref. 21, but inappropriately).
Taken together, the method described here is not suitable for any proper evaluation of emphysema in mice.

Response: Our sincere apologies for not being able to satisfy the reviewer. We did not violate the silver standards. We have eliminated the statement "We have reported several pieces of evidence wherein the fixed lungs did not shrink". We have confirmed that glutaraldehyde can be adopted for the current system. We have revised the manuscript to emphasize this point. We appreciate your leading comments.

Point-by-point response to the comments of Reviewer #4:

Manuscript Summary:
This manuscript described a new setting for murine lung inflation. It meets the urgent need to improve drawbacks of current method.

Response: We appreciate the kind remarks.

Major Concerns:
1. Fixation of 48 hours seems too long and it will probably make the immunohistochemical staining for certain antibodies difficult. Suggest to show IHC data with a few antibodies in lung slides after extended fixation of 48 hours.

Response: Thank you for your comments. As mentioned, in our lab, lung specimens usually proceed to embedment after 48 hours of fixation. We agree that there is no rationale as to whether this length of fixation time is proper or not. The Japanese Society of Pathology recommended fixation times of no longer than 1 week to produce consistent immunohistochemical slides, although this is an analysis using human specimens (Sato M et al. Pathology International 2014; 64:209-216). We think that it is difficult to make a comparison between the current system and other methods. However, we have tried to fix lung tissues for 24 hours using the current system and confirmed that lungs can be reasonably fixed for morphologic evaluations.

2. how do you evaluate the inflation efficiency of lung specimens placed in the middle of the lower container vs. samples placed in the edge of the container?

Response: Thank you for your comment. We also think that this is an important point. We have compared mean linear intercepts (MLI) between the samples placed in the middle of the lower container and samples placed in the edge using control (no treatment) lungs and confirmed that the MLIs were not different.

Minor Concerns:
Will be helpful to include a few sentences of discussion to indicate how feasible to do more than 5 lungs. For example, are two peoples required to collaboratively use this setting for inflation or one person is enough?

Response: We agree with the reviewer. We usually use this system for inflation alone. Therefore, we cannot start fixing many samples at the same time. This issue may be inconsiderable for relatively long periods of fixing time (at least 24 hours), however, we agree this is a limitation of the current system and have revised the manuscript.

Point-by-point response to the comments of Reviewer #5:

Manuscript Summary:
Karasutani and colleagues improve the lung fixation histological specimens of lungs from a mouse model of emphysema for consist processing of many mouse lung samples under constant pressure. They describe specimens obtained following exposure to chronic cigarette smoke and fixation performed with specialized equipment of constant pressure (25 cmH2O). This process is a major improvement on current methodology processes in animal COPD models. The manuscript is well written and clearly presented with many helpful visual aids. An example of the analysis post fixation would be good, such as MLI.

Response: We appreciate the kind remarks.

Minor Concerns:
Can authors show an example of histology analysis with this excellent fixation method?

Response: Yes, we can. We have added the data of MLI and DI as Figure 6C and 6D.

Point-by-point response to the comments of Reviewer #6:

Manuscript Summary:
Karasutani and colleagues present a method to inflate and fix mouse lungs at a constant pressure using a specialized device. Two main advantages of this system they claimed are 1) multiple samples can be fixed under a same constant pressure at the same time 2) A constant pressure can be kept for a various period of time. As the author mentioned in the manuscript, there are no gold standard how to fix the lung samples to evaluate emphysema, several other ways exist. It is certainly good system to fix the lungs, however, it is not sure if this present complicated system can provide two claimed benefits over other cited works which can be done much simpler and they are unlikely that someone would need to try to build this complicated system.

Response: We appreciate the kind remarks. Our point-by-point responses to the reviewer's concerns are provided below.

Major Concerns:
1. It is not clear why other systems cannot fix many samples at the same time. Also,

for the present system, the authors failed to describe how lung sample placement box can be inserted into the lower container? Is it just dropped into the lower container? My concern is how we can have all lungs samples fixed at the same time using this device since it takes some times to dissect lungs from the animals, especially when the experiments contain a lot of mice. Do you connect the first dissected lung directly to the system and drop into the lower container? In this mean, you have to remove the sample placement box from the container to connect the second, third, … lungs again and again? Is there any effect from taking it out from the formalin container and put it back multiple times (especially for the very first samples compare to latter samples)? Or you just wait for all lung samples are dissected out and connect them to the system at the same time. With this way, how can you keep the sample while dissecting the other tissues.

Response: We appreciate your comments and suggestions. We agree that the fixation procedure presented in the current manuscript is relatively more complex when compared with other conventional systems. However, we would like to emphasize the advantages of the current system; 1) it can fix many lungs (maximum 20) with the same conditions at one time. 2) it can fix lung tissue at a constant pressure for various periods of time.
When we place or remove lung samples to the lower container, we do so individually while wearing long rubber gloves. As you indicated, we cannot start fixing for many samples at the same time. This issue may be inconsiderable for relatively long periods of fixing time (at least 24 hours), however, we agree that this is a limitation of the current system and have revised the manuscript accordingly (line 231-233 in the cleaned manuscript).

2. It is clear that this fixation system can be used to fix lungs for various time point and the authors decide to keep the lungs in the system for 48 hours. The authors also cited the guideline from Hsia, 2010 (ref. 14) describing the silver standard for airway for airway instillation fixation saying that "After fixation, airway inflation pressure must be maintained for at least 24 hours, by tying off the trachea or the tubing without leaks." However, the authors might have to discuss how this method with 48 hour immersion of lungs in the formalin would be beneficial over other methods suggesting by the silver standard guideline that inflate lungs at a certain pressure for a short period of time (like 10-20 minutes), tie off and maintain the trachea+lung in fixative for at least another 24 hours. With the latter way, you can also vary the period of time that specimens would be immerse in the fixative as long as there is no leak.

Response: Thank you for your comments. As mentioned, in our lab, lung specimens are usually moved to embedment after 48 hours of fixation. We agree that there is no rationale whether this length of fixation time is proper or not. The Japanese Society of Pathology recommended fixation times of no longer than 1 week to produce consistent immunohistochemical slides, although this is an analysis using human specimens (Sato M et al. Pathology International 2014; 64:209-216). We think that it is difficult to compare between the current system and other methods, however, we have tried to fix lung tissues for 24 hours using the current system and confirmed that lungs can be reasonably fixed for morphologic evaluations. We have discussed this point as a limitation of the current system.

3. It is ambiguous why and how the specimens need to be inserted into 10 mL syringe with formalin and inflate the lung with vacuum in step 3.5 -3.6 and Figure 2 before the trachea is inserted with cannula (step 3.7) and connected to the sample placement box. And insertion of cannula is done while the lung is still in the 10ml tube? Also, after cannula insertion, lung will be inflated again with formalin using 1 ml syringe? How can you control the pressure of inflation of these steps to avoid over extension of the lungs? The authors need to explain more details of these steps before validation if the methods are appropriate.

Response: We apologize for this confusion. At step 3.5 and 3.6, the lungs are deaerated and inflated with fixing agents. After that, samples are taken out from 10 mL syringe and inserted into a 20G cannula (3.7). Next, lungs will be inflated again with a little volume of fixing agents to check with or without leaks (3.8). We have revised the manuscript to clarify these points.

4. The representative results show only the histological sections of air-exposed vs cigarette smoke-exposed lungs. This is somewhat not gold standard for assessing emphysema since we can select any tiny random areas of the lungs to show. To validate that this method can provide the good evaluation of emphysema particularly with cigarette smoke model which results in such a mild emphysema, the fixed lung measurement has to be measured and stereological assessment results like mean linear intercept should be at least shown in the results.

Response: We completely agree with the reviewer. We have added the MLI and DI data as Figure 6C and 6D, respectively. We appreciate your suggestions.

Minor Concerns:
1. The authors did not describe the step after fixation 48 hours. How to detach the lung from the system? Do we need to tie off the left bronchus or we can simply remove it and process it to the next step (embed?)

Response: We apologize that we missed out in describing the step after fixation. When we remove lung samples from the lower container, we need to tie the trachea off with a knot. We have added the statement to the Protocol (3.10).

2. Step 3.4, the right lungs are separated for frozen sections. Is there any reason for this particular manuscript? Can we inflate whole lungs with this system? My guess is the separation would be optional but might have to state it clearly in manuscript.

Response: The reviewer is correct. The separation is optional. We can inflate whole lungs with the current system. We have revised the manuscript to clarify this.

3. Step 3.7, insert cannula size 20G into the trachea, is this the same cannula as 22G cannula described in representative results (line 157).

Response: Thank you for carefully reading through. We have made the needed correction (line 166 in the cleaned manuscript).

4. Line 162 - 163, are these Figure 3A and 3B instead of 4A and 4B.

Response: Thank you for carefully reading through. We have made the needed correction (line 170 and 172 in the cleaned manuscript).

Point-by-point response to the comments of Reviewer #7:

Manuscript Summary:
In this paper, Karasutani et al. report a protocol for lung fixation that can be useful for evaluating emphysema in mice. A research paper can be used for recognizing technical issues and this is a good technical paper to read for beginners.
However it needs some changes before its publication.

Response: We appreciate the kind remarks.

Major Concerns:

-Please add at pag. 7 line 251 (….. including the mean linear intercepts, THE INTERNAL SURFACE AREA and the destructive index.).

It is well known that mean linear intercepts per se (without lung elastin determination) measure only alveolar wall enlargements (and not emphysema),and that destructive index measures an alveolar ongoing lesion, but not emphysema. On the other hand, Internal surface area (ISA) measures the amount of the residual surface in which the gas exchange takes place. This value documents the loss of alveolar septa and thus the presence of emphysema (Martorana A, Cavarra E, Lucattelli M, Lungarella G. "Models for COPD involving cigarette Smoke". Drug Discovery Today: Disease Models 3, 225-230, 2006).

Response: We appreciate your comments and suggestions. We agree with the reviewer and we have added the ISA as suggested.

-Please modify the title of the paper accordingly: "Lung Fixation under Constant Pressure as a Method Useful for Evaluating Lung Emphysema in Mice"

Response: Thank you for your comment. We have modified the title as suggested.