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TITLE:

Direct Intrabronchial Administration to Improve the Selective Agent Deposition Within the Mouse Lung

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KEYWORDS:

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SUMMARY:

Intratracheal (IT) administration of experimental agents in mice often results in asymmetric delivery to the distal lungs. In this report, we describe a direct intrabronchial (IB) approach to cannulate each lung in living mice non-operatively. This approach can be used to selectively administer agents to one lung or may be adapted to improve the symmetric agent delivery to both lungs.

ABSTRACT:

Intratracheal (IT) administration of experimental agents is an essential technique in murine models of diffuse lung diseases, such as bleomycin-induced pulmonary fibrosis. However, distribution of intratracheally-administered agents to the distal mouse lung is often asymmetric, with lung parenchymal concentrations increased in the smaller (but equally accessible) left lung of the mouse. Described in this report is a novel intrabronchial (IB) approach to cannulate the left and/or right lungs of living mice non-operatively. It is also demonstrated how this approach can be used to selectively administer agents to one lung or adapted (*via* dose-adjusted IB delivery) to improve the left-right symmetry of lung delivery of experimental agents, thereby improving models of diffuse lung disease such as bleomycin-induced pulmonary fibrosis.

INTRODUCTION:

Direct pulmonary administration of experimental agents in mice allows for the study of lung immune responses, acute lung injury, and lung fibrosis. Direct pulmonary administration is

typically performed *via* intratracheal (IT) instillation, as described previously[1-3]. However, this approach is nonselective, affecting both lungs in a nontargeted and often asymmetric fashion. Experimental modeling of lung injury may benefit from the ability to selectively target one specific lung, allowing for use of the contralateral lung as a control. Conversely, accurate modeling of human diffuse lung diseases benefits from symmetric distribution of experimental agents to the bilateral lung parenchyma.

The overall goal of this report is to describe a method for selective delivery of experimental agents to the left or right lung of a mouse (**Figure 1**). This intrabronchial (IB) administration approach allows for unilateral treatment of a mouse lung and can be easily adapted to ensure equal delivery of an agent to the bilateral mainstem bronchi. By using IB administration to deliver larger doses of experimental agents to the larger right lung and smaller volumes to the smaller left lung (i.e., dose-adjusted IB administration), demonstrated in this report is an improvement in the homogeneity of pulmonary delivery of experimental agents, optimizing the model of diffuse lung injury in mice. As such, this report may hold value for investigators seeking to either unilaterally administer experimental agents to mice or improve the symmetry of drug deposition in both lungs.

PROTOCOL:

All animal protocols have been approved by the University of Colorado Denver Institutional Animal Care and Use Committee (IACUC). All procedures described below (sections 4–7) have been optimized using both male and female C57BL/6 mice. This approach has been validated using mice ranging from 19–40 g in body weight.

1. Creation of platform for IB administration

1.1 Bend the bookend from the original 90° angle between the basal wing and standing wing to 70° (**Figure 2A**).

1.2 Drill a hole at the center-top of the standing wing of the metal bookend (**Figure 2A**).

1.3 Drill a hole of identical size at the corresponding position of the plastic board. Drill two smaller holes inferiorly and laterally (**Figure 2A**).

1.4 Drape a 4:0 silk suture between these small holes in the plastic board (**Figure 2A**).

1.5 Place the hook and loop tape on the edge of the plastic board (**Figure 2A**).

1.6 Assemble the plastic board to the metal bookend with the screw (**Figure 2B**). Ensure that the screw-nut is sufficiently tight to hold the board in position while allowing for adjustment of angle, if necessary.

1.7 Ensure that rotation of the plastic board is clockwise and counterclockwise, moving freely.

NOTE: Clockwise motion is represented in this report as a (+) degree rotation, and counterclockwise is represented as a (-) degree rotation.

1.8 Use an angle protractor to position the plastic board at +30°, +86°, -30°, and -74° and mark them on the bookend, respectively.

2. Creation of extended catheters for IB agent administration

2.1 Make a right angle cut with a sharp blade on the tip of an original 22 G catheter (25 mm, see **Table of Materials**) (**Figure 3,** step 1a).

2.2 Bevel (~50°–60°) the tip of the other original catheter (25 mm) with the blade, then cut off at right angle from the hub (**Figure 3,** step 1b).

2.3 Glue the two catheters at their blunt ends with a slightly less than 180° angle (**Figure 3,** step 2).

2.4 Blunt the beveled tip by melting with a low temperature cautery (see **Table of Materials**).

2.5 Polish the extended catheter with "0" size sandpaper on the glued area and the beveled tip of the extended catheter (**Figure 3,** step 3).

2.6 Mark on the extended catheter with different colors at 25 mm, 30 mm, and 35 mm (**Figure 3,** step 3).

2.7 Indicate the bevel side of the extended catheter by labeling its hub with marker.

2.8 Rinse the extended catheter with DI water, following by flushing inside of the catheter with 70% ethanol. Airdry the catheter.

2.9 Sterilize with UV light for 10 min before use.

3. Pre-procedure preparation

3.1 Make all administered agents in a biological safety hood under sterile technique.

3.2 Clean the workplace with 70% ethanol.

3.3 Sterilize all surgical tools with 70% ethanol.

3.4 Fix the base of the work platform to the bench immediately in front of the researcher by affixing C-clamps to the basal wing of the bookend.

3.5 Generate several makeshift spirometers, which are devices that will allow for detection of tidal airflow in mice. Briefly, deposit 60 µL of sterilized saline into a 1 mL syringe (plunger removed) with a gel loading tip.

NOTE: The deposited drop of saline occludes the barrel and moves upwards and downwards when exposed to tidal ventilation[3].

3.6 Loosely attach the hub of a 22 G extended catheter to the makeshift spirometer.

3.7 Place each of the glass droppers to each side of the platform for ease of access.

3.8 Connect the isoflurane induction chamber to the rodent anesthesia machine (see **Table of Materials**) in an isoflurane-compatible biological safety cabinet.

4. Non-operative IT intubation approach

4.1 Anesthetize a C57BL/6 mouse (male or female, 8–10 weeks, ~25 g) with oxygen (2 L/min) and 5% isoflurane (see **Table of Materials**) in an induction chamber for 4 min.

4.2 Aspirate the experimental agent to be delivered (e.g., Evans blue dye or FITC-dextran, as demonstrated in **Figure 4**) into two pipettes, then place them to each side of the platform during sedation.

4.3 Ensure a respiratory rate of approximately 24–30 breaths/min before removing the mouse from the anesthesia induction chamber.

NOTE: Isoflurane anesthesia typically lasts for ~4 min, sufficient for all IB procedures. If the operator is not proficient with the technique, ketamine/xylazine (80 mg/kg and 10 mg/kg intraperitoneally, see **Table of Materials**) may be used for more prolonged anesthesia.

4.4 Suspend the mouse by its incisors on the draped suture line in the supine position. Secure the mouse with two to three pieces of hook and loop the tape loosely, avoiding restriction of ventilation.

4.5 Turn on the LED fiber optic illuminator (see **Table of Materials**, **Figure 2C**).

4.6 Position the operator behind the platform (dorsal to the mouse).

4.7 Orient the gooseneck of the illuminator so that it illuminates the larynx area through the skin. The distance between the mouse and light source is 2–3 cm (**Figure 2C**).

4.8 Confirm the depth of anesthesia with a toe/paw pinch before performing all procedures below.

4.9 Hold the sterile forceps with the dominant hand, then draw the tongue out of the oral cavity with the forceps.

4.10 Hold the sterile depressor with the nondominant hand, then flatten the root of the tongue with the depressor to expose the oropharynx widely. The forceps can then be released, freeing the dominant hand.

4.11 Use the dominant hand to intubate the extended catheter into the trachea *via* the oral cavity (**Figure 2C**).

4.12 Confirm placement by observing if the bubble in the syringe moves up and down with each breath.

4.13 Additional details of IT intubation have been published previously[3]. Total procedure time, excluding anesthesia, lasts 10–15 s for a well-trained operator.

5. Non-operative IB intubation and delivery approaches

5.1 IB approach to selective lobar cannulation of the distal right lung

5.1.1 After performing IT cannulation (step 4.11), rotate the plastic board +30° (**Figure 4A**).

5.1.2 Hold the hub of the catheter and guide it naturally in parallel to the mouse midline, extending it to weight-based depths as described in **Table 1**.

NOTE: The resistance at these depths should be noted. At this point, the mouse will become slightly tachypneic, as explained in the representative results. For an experienced operator, approximately 90% of attempts will successfully cannulate the right lung (with tachypnea noted).

5.1.3 Deliver 20 µL of 0.3% Evans blue dye (EBD, see **Table of Materials**) with a gel loading tip.

5.1.4 Dispense 1–2 aliquots (0.1 mL each) of air by using the glass dropper.

NOTE: This ensures clearance of the residual EBD solution (or experimental agents) from inside of the catheter.

5.1.5 Withdraw the catheter, then maintain the mouse position for 30 s.

5.1.6 Place the animal on a warming blanket until it regains consciousness. Recovery is typically complete within 2 min.

5.2 IB approach to selective segmental cannulation of the distal left lung

5.2.1 After performing IT cannulation (step 4.11), rotate the plastic board -74° (**Figure 4B**).

5.2.2 Hold the hub of the catheter and apply gentle pressure to advance the catheter into the left mainstem bronchus, while placing modest pressure both downwards (90°) and towards the bookend. At depths noted in **Table 1**, the operator should note resistance as the lower segments of the left lung are engaged. If tachypnea occurs, withdraw the catheter to the 20–25 mm position, and reattempt.

5.2.3 After cannulating the left lower lung segments, a change in position is required to allow gravitational assistance for agent administration. Rotate the plastic board -30° (**Figure 4B**).

5.2.4 Deliver 40 µL of 0.3% EBD with a gel loading tip.

NOTE: It is feasible to deliver a larger volume of agent because the left lung has only one lobe.

5.2.5 Dispense 1–2 aliquots (0.1–0.3 mL each) of air using the glass droppers.

NOTE: This ensures clearance of any residual EBD (or experimental agents) from inside of the catheter.

5.2.6 Withdraw the catheter, then maintain the mouse position for 30 s.

5.2.7 Place the animal on a warming blanket until it regains consciousness. Recovery is typically complete within 2 min.

5.3 Adaptation of IB administration to allow delivery of agent to entirety of left or right lung

NOTE: If the operator seeks to deliver agents not to a specific right lung lobe or left lung segment, but rather to the entire lung (right or left lung), the catheter should be slightly withdrawn to the respective mainstem bronchi, as follows.

5.3.1 Right entire lung administration

5.3.1.1 After step 4.11, rotate the plastic board +30° (**Figure 5A**).

5.3.1.2 Hold the hub of the catheter and guide it naturally in parallel to the mouse midline, reaching it to depths necessary for right sided distal lobar cannulation (**Table 1**).

5.3.1.3 Confirm appearance of the tachypnea sign.

5.3.1.4 Rotate the mouse -74° to enable gravity assistance for agent delivery (**Figure 5B**).

5.3.1.5 Withdraw the catheter to a position that corresponds to the takeoff of the right mainstem bronchus (**Table 1**). Ensure that the bevel of the catheter faces downward (**Figure 5B**).

5.3.1.6 Deliver 30 µL of 0.3% EBD with a gel loading tip to the right lung.

5.3.1.7 Dispense 1–2 aliquots (0.1–0.3 mL each) of air using a glass dropper.

5.3.1.8 Withdraw the catheter, then maintain the mouse position for 30 s. Place the animal on a warming blanket until it regains consciousness. Recovery is typically complete within 2 min.

5.3.2 Left entire lung administration

5.3.2.1 After step 4.11, rotate the plastic board -74° (**Figure 6A**). Alternatively, rotation may occur after step 5.3.1.8 by withdrawing the catheter to the trachea, enabling bilateral IB agent administration.

5.3.2.2 Hold the hub of the catheter and apply gentle pressure to advance the catheter into the left mainstem catheter, while placing modest pressure both downwards (90°) and towards the bookend. Depth of intubation is guided by **Table 1**.

5.3.2.3 Confirm the no tachypnea sign.

5.3.2.4 Rotate the mouse +86° to allow for gravity assistance with agent administration.

5.3.2.5 Withdraw the catheter to the left mainstem bronchus (the same distances as the right lung are sufficient, **Table 1**) and rotate the bevel of the catheter faces downward (**Figure 6B**).

5.3.2.6 Deliver 30 µL of 0.3% EBD with a gel loading tip to the left lung.

5.3.2.7 Dispense 1–2 aliquots (0.1–0.3 mL each) of air using a glass dropper.

5.3.2.8 Withdraw the catheter, then maintain the mouse position for 30 s. Place the animal on a warming blanket until it regains consciousness. Recovery is typically complete within 2 min.

6. Use of sequential IB cannulation approaches to deliver dose-adjusted volumes of agent to each lung

6.1 IT administration group

6.1.1 Perform IT cannulation as described in steps 4.1–4.11.

6.1.2 Deliver 60 µL of 0.05% FITC-dextran (see **Table of Materials**) with a gel loading tip (**Figure 1B**).

6.1.3 Dispense 1–2 aliquots (0.1–0.3 mL each) of air using the glass droppers.

6.1.4 Keep the position for 60 s and allow for mouse recovery as described above.

6.2 Symmetric bilateral IB administration

6.2.1. Perform steps 5.3.1.1–5.3.1.8 (right lung) and steps 5.3.2.1–5.3.2.8 (left lung).

6.2.3. Administer equal volumes (30 µL) of 0.05% FITC-dextran (or an experimental agent) to each side of the lung.

6.3 Dose-adjusted bilateral IB administration

6.3.1. Perform step 5.3.1.1–5.3.1.8 (right lung) and steps 5.3.2.1–5.3.2.8 (left lung).

6.3.2. Administer larger volume (40 µL) of 0.05% FITC-dextran to the larger right lung, and a smaller volume (20 µL) of 0.05% FITC-dextran to the smaller left lung. In lieu of FITC-dextran, an experimental agent can be administered.

7. Use of Dose-Adjusted IB administration to improve symmetry of single dose bleomycin (BLM)-induced lung injury

7.1 BLM administration groups

7.1.1 Dose-adjusted IB-BLM (1.2 mg/kg, see **Table of Materials**) administration group: 60 µL (20 µL for left lung and 40 µL for right lung, respectively) of BLM solution were delivered to mice (n = 5). Controls (n = 5) received similar volumes of saline.

NOTE: Refer to steps 5.3.1 and 5.3.2.

7.1.2 IT administration group: 60 µL of BLM solution were delivered to mice with IT administration techniques.

NOTE: Refer to steps 6.1.1–6.1.4.

7.2 Lung function measurement

7.2.1 On day 21 after BLM or saline, anesthetize mice with an intraperitoneal (IP) injection of ketamine (160 mg/kg) and xylazine (32 mg/kg).

7.2.2 After confirming depth of anesthesia by paw/toe pinch, perform a tracheostomy with an 18 G cannula (see **Table of Materials**).

7.2.3 Connect mice to the ventilator and measure respiratory mechanics as previously described[4].

7.3 Lung tissue collection and processing

7.3.1 Following measurement of pulmonary mechanics, euthanize the anesthetized mice by cardiac puncture.

7.3.2 Open the chest wall and induce bilateral pneumothoraces.

7.3.3 Inflate lungs with 1% low melt agarose (40 °C)[5] in PBS at a consistent pressure (42 cm H_2O).

7.3.4 Cut four to five pieces of the lung along the long axis transversely, fix in 10% formalin, and embed in paraffin.

7.3.5 Cut 5 µm sections and stain with Masson's trichrome to visualize collagen deposition.

8 Post-procedural care

8.1 At the end of survival procedures, place the animal on a warming blanket until it regains consciousness. Recovery is typically complete within 2 min.

REPRESENTATIVE RESULTS:

Selective IB intubation targets specific lobes (right lung) or basilar segments (left lung).
IB administration of EBD to the right lung was performed as described in section 5.1. After completion of the experiment, mice were administered a lethal dose of intraperitoneal ketamine/xylazine, and lungs were harvested for demonstration of EBD distribution (**Figure 4A**, right). Gross appearance of the lung demonstrates that 90% of attempts cannulated the small posterior lobe of the right lung, while 10% of attempts targeted the inferior lobe. It is speculated that the small volumes of these lobes explains the compensatory tachypnea of the mouse during distal cannulation (to maintain minute ventilation through the catheter).

IB administration of EBD to the left lung was performed as described in section 5.2. 100% of attempts target the inferior segments of the left lung (**Figure 4B**). In contrast to right-sided intubation, no tachypnea occurs with this engagement, reflecting intubation (and ventilation) of the larger left lung segments.

Adaptation of the selective IB cannulation technique can target the entire left or right lung.
Once IB cannulation is performed, withdrawal of the IB catheter (and changes in mouse positioning, as detailed in section 5.3) can be used to improve delivery of agents to all lobes of the right lung (and all segments of the left lung). Instillation of EBD solution to the right lung

(section 5.3.1) successfully targeted all right lobes, as demonstrated in **Figure 5C**. Instillation of EBD solution to the left lung (section 5.3.2) successful targeted all left segments (**Figure 6C**).

IT administration or symmetric IB administration yields asymmetric lung parenchymal agent concentrations, which can be corrected by IB dose-adjustment.

Mice underwent bilateral administration of 30 µL of 0.05% FITC-dextran to the left lung and 30 µL of 0.05% FITC-dextran to the right lung as described in section 6.2. Alternatively, mice received 60 µL of 0.05% FITC-dextran intratracheally as per section 6.1. At the end of the experiment, mice were euthanized via terminal anesthesia overdose (ketamine/xylazine). Lungs were immediately harvested and homogenized. FITC-fluorescence (quantified by optical density) was measured with 96-well plate reader. Date were analyzed with student's t-test for two-group comparisons.

As detailed in **Figure 7**, both IT (**Figure 7A**) and symmetric IB administration (**Figure 7B**) of FITC-dextran led to asymmetric lung parenchymal FITC fluorescence, with greater relative concentrations (normalized to weight) noted in the left lung. This suggests that asymmetric lung delivery of experimental agents after IT administration is not a consequence of asymmetric presentation of these agents to each mainstem bronchus. Rather, it was hypothesized that equal mainstem delivery (as ensured by symmetric IB administration) was diluted by differences in lung weights/mass, as observed in **Table 2**.

To overcome these differences in symmetric delivery, 40 µL of 0.05% FITC-dextran was administered to the larger right lung and 20 µL to the smaller left lung, as per section 6.3. This "dose-adjusted IB administration" improved the symmetry of lung parenchymal agent delivery (**Figure 8A**). Despite this correction, however, we observed persistent heterogeneity within different lobes of the right lung (**Figure 8B**).

BLM-induced lung injury in different delivery systems:

To demonstrate that dose-adjusted IB administration of experimental agents can improve modeling of diffuse lung disease, we administered BLM (a mouse model of fibrosing lung injury) either intratracheally or via dose-adjusted IB administration, as per section 7. As expected with this model of injury, both IT and IB injections of BLM led to lung injury and systemic illness (with loss of weight). This systemic illness resolved in 7 days. 21-day mortality was 20% (1/5) in the IT group and 0% (0/5) in the dose-adjusted IB group.

21 days after IT- or IB-BLM administration, mice were harvested for lung histology. As demonstrated in representative histologic images (**Figure 9A**), IT-BLM induced fibrotic lung injury predominantly in the left lung, with less injury notable in the right lung. Conversely, dose-adjusted IB administration (which delivered a larger dose to the right lung) slightly decreased injury to the left lung and increased injury to the right lung, with greatest injury notable in the middle and superior lobes. It should be noted that this finding slightly differs from the EBD approach (**Figure 8B**), which found relatively less EBD deposition in the middle lobe.

To determine if this improved left-right homogeneity of fibrotic lung injury is physiologically relevant, it was observed that dose-adjusted IB administration of BLM imparted a more consistent loss of inspiratory capacity (IC) and respiratory system compliance (Crs), as well as a concordant increase in respiratory system elastance (Ers) (**Figure 9B)**.

FIGURES AND TABLE LEGENDS:

Figure 1: Anatomy of mouse airway cannulation. (**A**) A mouse airway cast was made by inflating a mouse lung (harvested from a 25 g mouse) with silicon elastomer. (**B**) Catheter placement for standard IT administration. (**C**) Catheter placement for IB administration.

Figure 2: Setup for the work platform. (**A**) A metal bookend (90° angle) is bent to 70°. A screw hole is placed in the top midline to anchor a movable (80 mm x 150 mm). Hook and loop tape and a suspending suture are placed to allow positioning of an anesthetized mouse on the board. (**B**) The plastic board is anchored with a screw on the metal bookend. The screw is sufficiently loose to allow rotation of the board in a clockwise (+) or counterclockwise (-) direction. (**C**) An anesthetized mouse is positioned using with hook and loop tape (0.75" W) for IT/IB agent administration. A suture is passed under the mouse incisors to allow head stabilization. The operator is positioned at the dorsal aspect of the mouse, and the neck is illuminated *via* a goose neck lamp.

Figure 3: Creation of customized catheters for IB administration. (**Step 1**) To enable sufficient catheter length to engage the mainstem bronchi, two catheters are combined. (**Step 2**) Catheters are connected at a slight angle, facilitating selective intubation to the mainstem bronchi. (**Step 3**) Furthermore, the distal catheter tip is beveled, allowing better directional control of airway instillation.

Figure 4: Approach for selective right/left lung lobar cannulation and administration. (**A**) To target the right lung, the plastic board is rotated +30°, improving ease of selectively engaging the right mainstem bronchus. The catheter is advanced (per distances proposed in **Table 1**) to selectively engage right sided lobes. 20 µL of 0.3% EBD was administered. In ~90% of attempts, the posterior lobe is cannulated. The remaining 10% of attempts engage the inferior lobe. (**B**) To target the left lung, the plastic board is first rotated -74° for left mainstem engagement. After successful intubation of the catheter, rotation is then decreased to -30° to allow for gravity to assist with agent delivery. To prove selective engagement of the left side, 40 µL of 0.3% EBD was delivered. This approach consistently (100% of attempts) targeted left lung basilar segments.

Figure 5: Symmetric administration approach to unilaterally deliver agents to the entire right lung. (**A**) Right-side IB intubation was performed at +30°, identical to selective right lung lobar cannulation (**Figure 4A**). (**B**) The plastic board was then rotated to -74° to allow for gravity assistance during agent administration. The catheter tip is then withdrawn to depths detailed in **Table 1**, corresponding to the right mainstem bronchus. The bevel of the tip is positioned downwards by rotating the catheter hub. (**C**) 30 µL of EBD was delivered at -74°, proving diffuse

right lung administration of EBD.

Figure 6: Symmetric administration approach to unilaterally administer agents to the entire left lung. (**A**) Left-sided IB intubation was performed at -74°, identical to selective left lung lobar cannulation (**Figure 4B**). (**B**) After a successful intubation, the plastic board was then rotated +86° to allow for gravity assistance during agent administration. The catheter tip is then withdrawn to depth detailed in **Table 1**. The bevel of the tip is shifted downwards by rotating the catheter hub. (**C**) 30 µL of EBD was delivered with gel loading tip, proving diffuse left lung administration of EBD.

Figure 7: IT administration of experimental agents is equally delivered to mainstem bronchi, yet leads to different lung parenchymal concentrations. (**A**) IT administration of 0.05% FITC-dextran (60 µL) imparted higher fluorescence in the left lung, suggesting uneven lung concentrations of delivered agent. (**B**) This unequal lung parenchymal fluorescence persists even when equal volumes of 0.05% FITC-dextran (30 µL) are administered to each mainstem bronchus. This persistent parenchymal imbalance, despite equal right/left mainstem delivery, suggests that differences in lung agent concentrations reflect dilution in the larger right lung (n = 10 per group).

Figure 8: Improved homogeneity of agent deposition by dose-adjusted IB administration. (**A**) Asymmetry of lung parenchymal delivery is improved when a greater proportion of agent (40 µL of 0.05% FITC-dextran) is administered to the larger right lung and a lesser proportion of agent (20 µL of 0.05% FITC-dextran) to the smaller left lung. (**B**) Despite this improved left-right symmetry, there remains lobar heterogeneity of agent deposition (black: superior lobe; yellow: middle lobe; blue: inferior lobe; green: posterior lobe; red: left lung).

Figure 9: Improvement of mouse BLM-induced lung fibrosis model using dose-adjusted IB administration. (**A**) IT administration of BLM (1.2 mg/kg in 60 µL solution) induces left-side predominant lung injury/fibrosis 21 days later, consistent with higher lung concentrations of agent in this smaller lung. Left-right symmetry improves by adjusting the volume of BLM to each side of lung: 40 µL of the solution is administered to the larger right lung and 20 µL of the solution is administered to the smaller left lung. I: inferior lobe, M: middle lobe, S: superior lobe, P: posterior lobe. Images represent lobes from a single, representative mouse. (**B**) Consistent with improved symmetry of distribution, the dose-adjusted IB administration of BLM improves the physiologic modeling of lung fibrosis, with more representative increases in respiratory system elastance (Ers) and decreases in inspiratory capacity (IC) and dynamic respiratory system compliance (Crs).

Table 1: **Suggested depth of catheter insertion.** Predicted catheter depths necessary to selectively cannulate the distal and proximal lungs were empirically determined using C57BL/6 mice of various weights (total = 79 mice).

Table 2: **Right:left lung weight ratios.** Differences in lung weights observed in 81 C57BL/6 mice demonstrate rationale for corrected IB drug administration. Lungs were dissected and weighed

after a lethal dose of ketamine and xylazine.

DISCUSSION:

Lung injury has been classically modeled in rodents using IT administration of injurious agents such as BLM[6]. Such IT administration, however, only leads to patchy injury, reflecting the nontargeted nature of lung delivery with this approach[7]. These limitations of modeling lung injury are instructive challenges faced when attempting the IT delivery of non-injurious experimental agents, such as drugs, siRNA, or cellular therapies.

In this report, we describe the direct IB administration of experimental agents. This approach offers two distinct benefits over classic approaches to IT administration. Firstly, the approach allows for selective unilateral administration to one lung, allowing for sparing of the contralateral lung. This approach is useful for the selective administration of drugs into a unilaterally injured lung (e.g., ischemia-reperfusion injury[8]), avoiding nonspecific effects in the uninjured lung. Furthermore, directed administration of tumor cells can be used to distinguish primary tumor growth from contralateral, metastatic spreading[9,10].

Secondly, the report details a previously unrecognized benefit of IB administration. As detailed in **Figure 7A**, IT administration relatively concentrates experimental agents within the smaller left lung. This asymmetry can be corrected by administering a relatively larger volume of agent to the larger right lung (**Table 2**), while delivering a smaller volume to the smaller left lung (**Figure 8A**). The relevance of this dose-adjusted IB administration to BLM-induced fibrotic lung injury was demonstrated here. Dose adjustment mitigates the injury to the left lung (which received less BLM), while increasing injury to the right lung (**Figure 9A**). This increased symmetry coincides with decreased variability of lung injury, as quantified by measurements of pulmonary function (**Figure 9B**).

There are several critical steps in the protocol, including the need to have a stand capable of easily and repeatedly altering animal positioning (i.e., rotation). More critical is the ability to determine when selective lung cannulation has been achieved. As described in section 4.12, use of a spirometer (in which a water column demonstrates tidal ventilation) ensures successful tracheal cannulation[3]. Observation of mouse tachypnea is consistent with distal right lung segment cannulation, while the absence of dyspnea (despite feeling resistance with catheter insertion) suggested cannulation of the left lung. Using these non-operative localization techniques, the operator should be able to accurately guide IB cannulation and experimental agent deposition.

This approach has several limitations. The IT model of agent delivery is attractive in its simplicity. It requires a moderate degree of practice and technical skill, although a skilled operator can still rapidly perform this technique within the window of isoflurane anesthesia. The additional technical skill/practice required, however, may be easily offset by the benefit of this approach in experiments that prioritize either selective agent/siRNA/cell delivery or increased homogeneity of agent deposition. An additional limitation to this method is the

uncertainty regarding length of catheter insertion. As detailed in **Table 1**, 79 male and female mice were measured to estimate the depth of catheter insertion necessary for selective IB cannulation. These data serve as a resource to guide the operator to perform our protocol. However, we cannot confidently extrapolate our resource to other mouse strains (including knockout mice) or morbidly obese mice. In addition, we have not measured if there are differences in airspace volume (lobar, segmental) that vary based on weight. As such, it is possible that large mice may be able to accommodate larger instillation volumes with IB administration. Thus, the operator should perform an initial optimization/troubleshooting step (using EBD instillation) to ensure that our technique is well-adapted to the desired mouse model.

In summary, this report describes a novel IB technique that can be used to selectively administer experimental agents to a single lung or adapted to ensure symmetric distribution throughout both lungs. These benefits justify the marginal increase in complexity in comparison to standard IT techniques.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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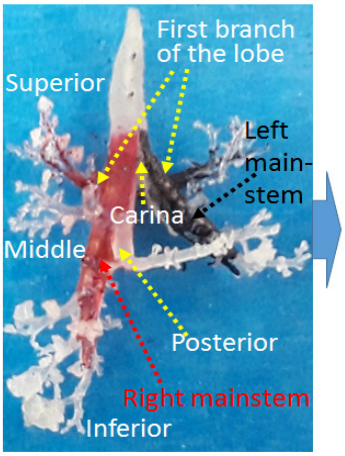
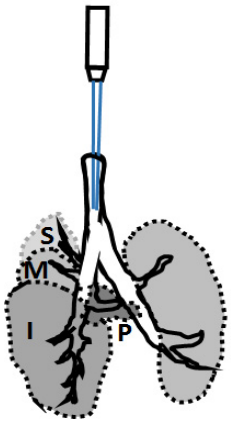
Figure 1

A. Mouse bronchial anatomy



B. Agent administration to the lung via intratracheal (IT) injection



C. Agent administration to the lung via intrabronchial injection bilaterally

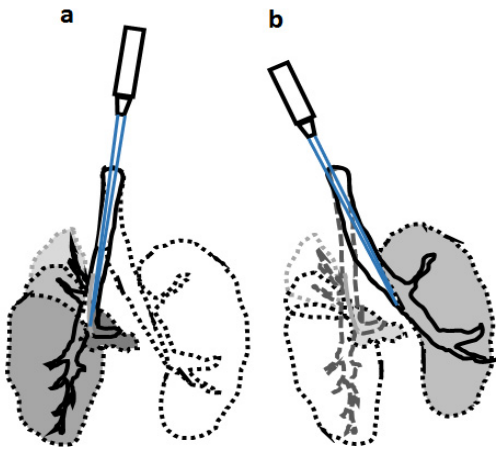


Figure 2

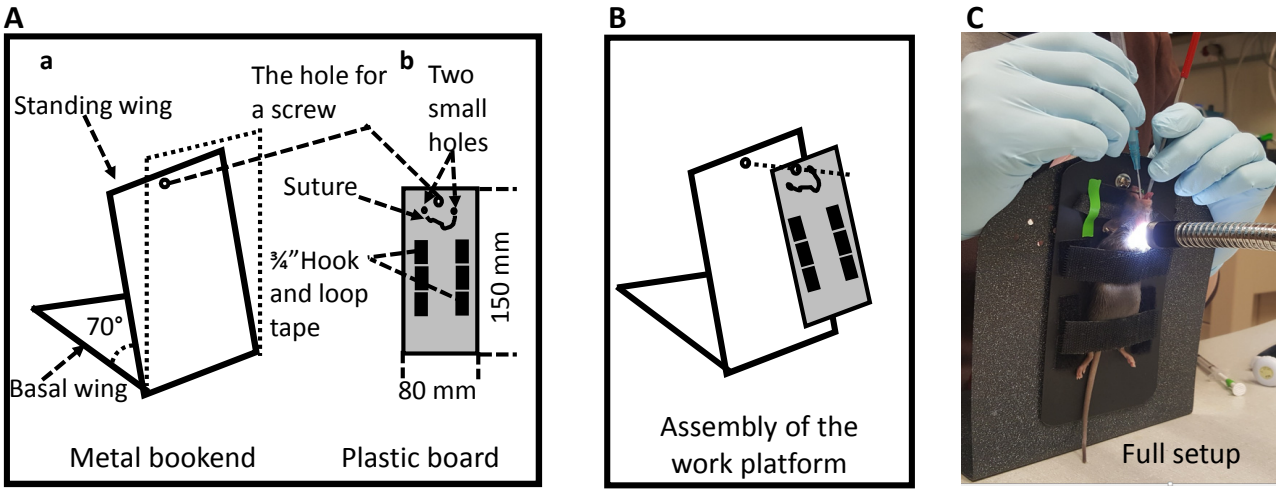


Figure 3

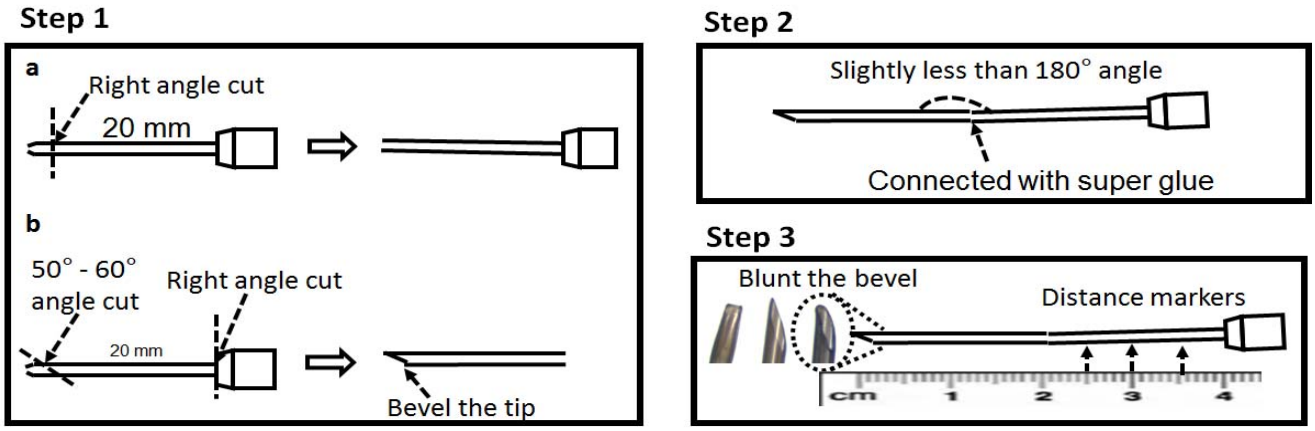


Figure 4

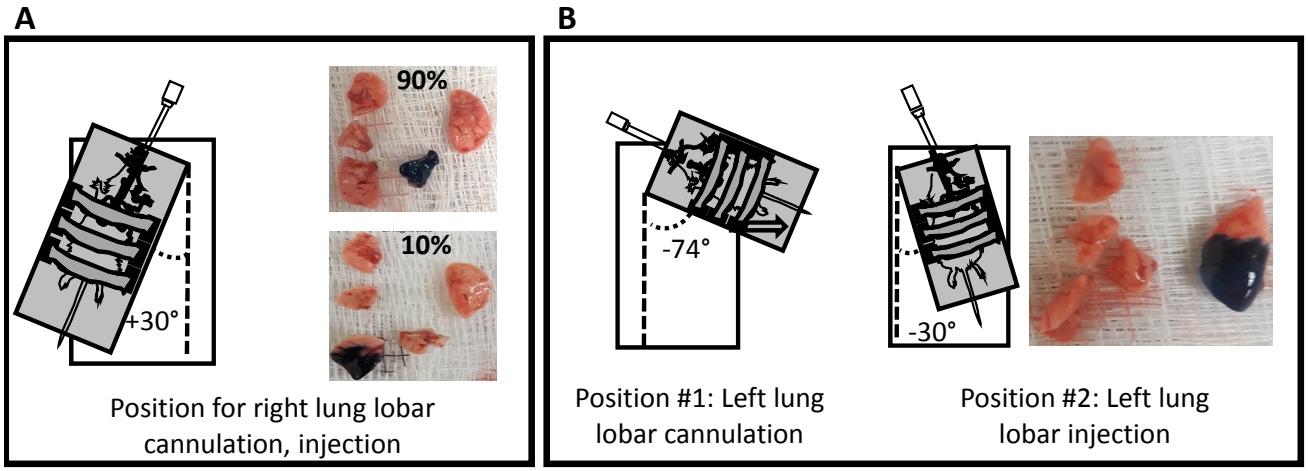


Figure 5

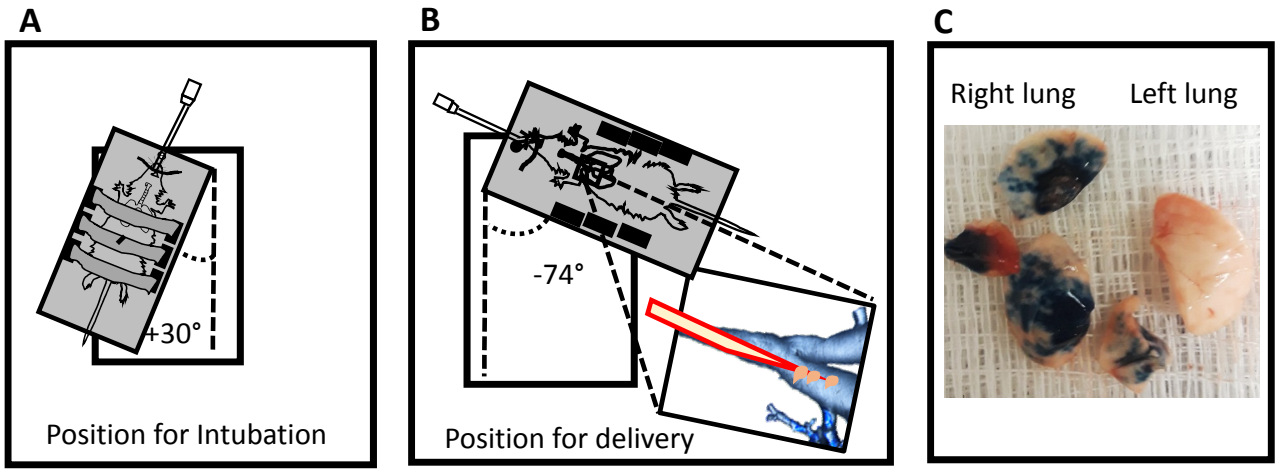


Figure 6

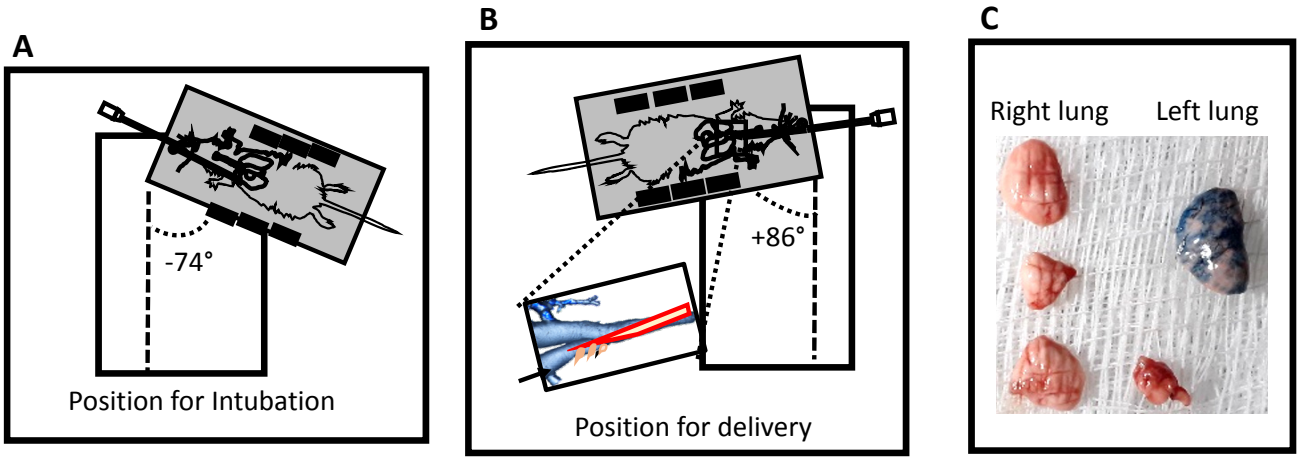


Figure 7

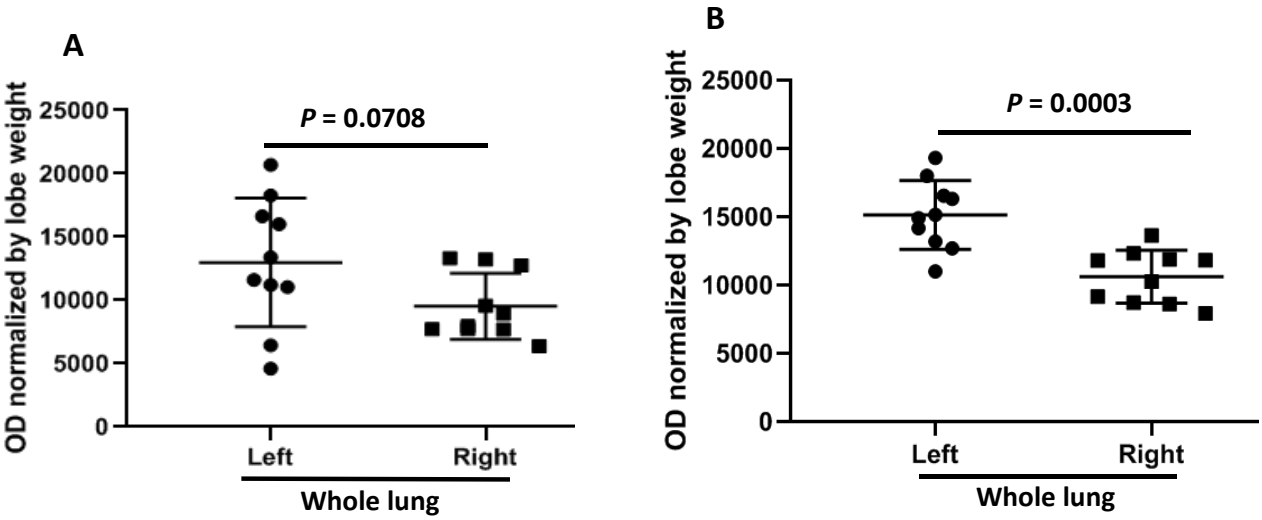


Figure 8

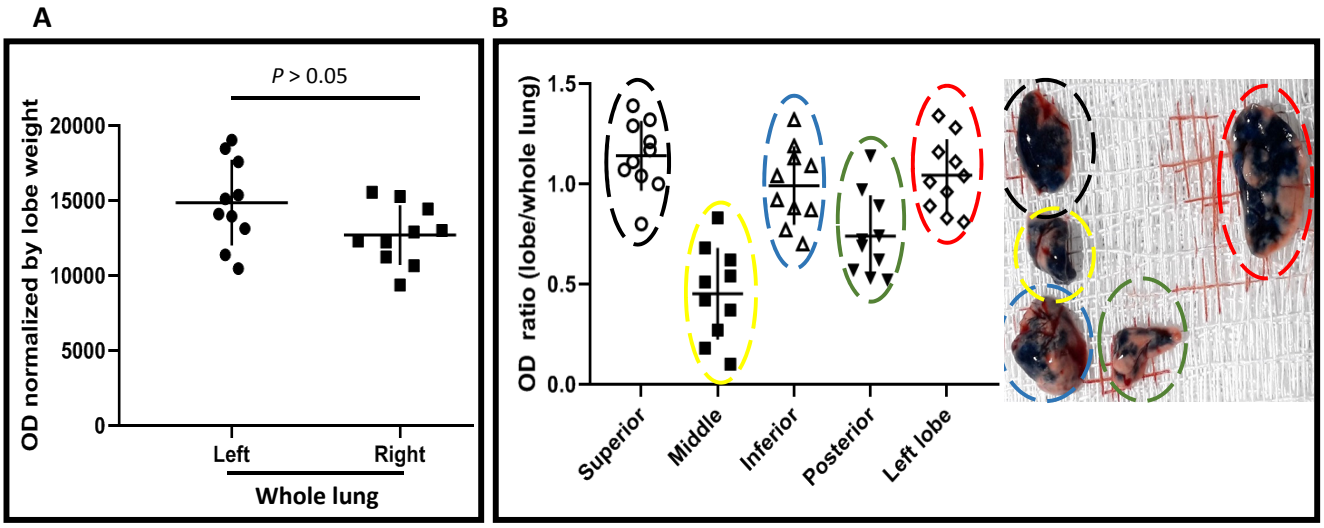


Figure 9

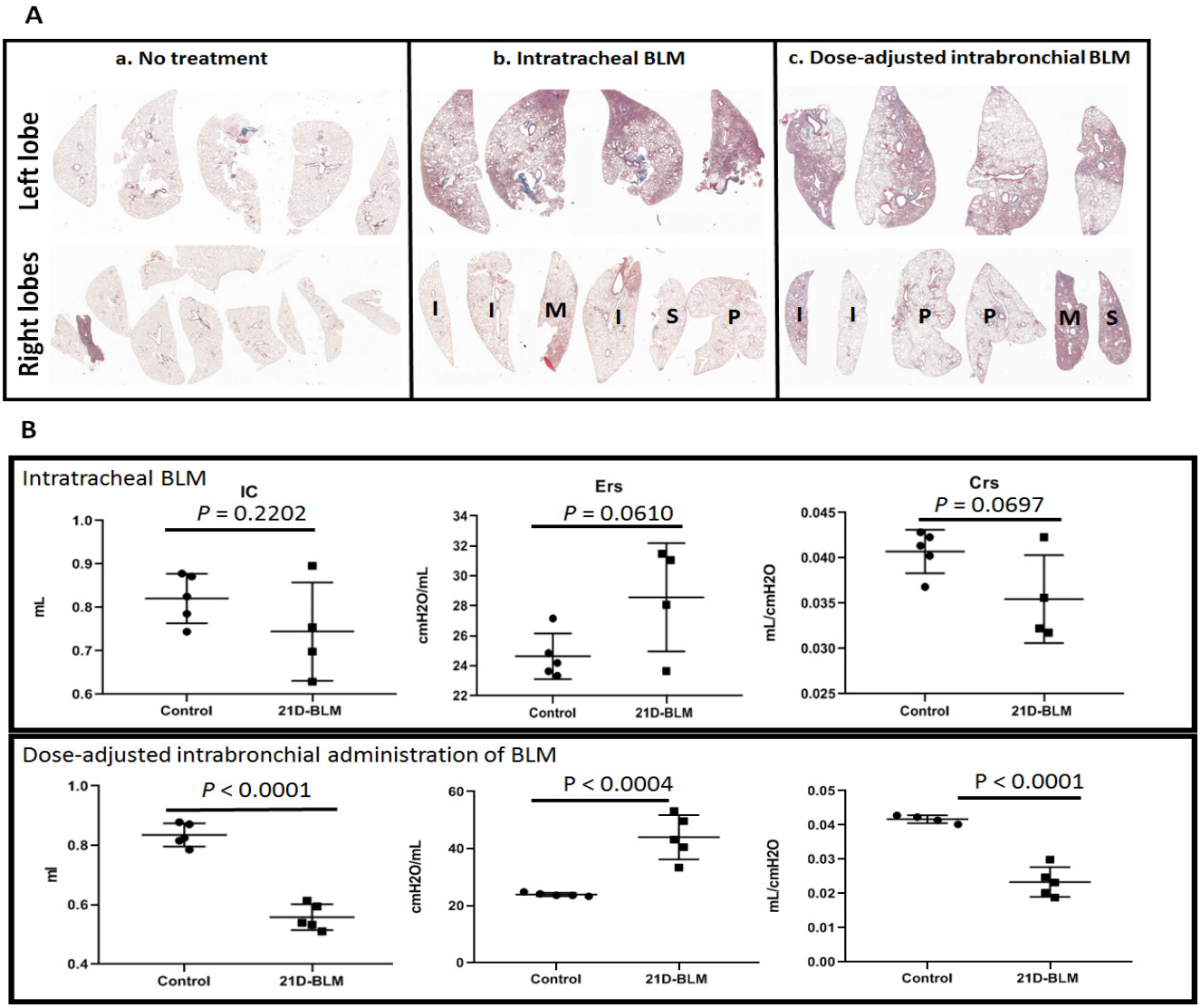


Table 1: Suggested depth of catheter insertion				
Body weight (g)	Number of mice tested	Catheter depth (mm) for Selective cannulation		Catheter depth (mm) for whole lung cannulation
		Right lung	Left lung	
15 - 19	17	37	38	26
20 - 25	22	38	39	27
25 - 30	29	39	40	28
> 30	11	40	41	31

Table 2: Right:left lung weight ratios		
Body weight (g)	**Number of mice tested**	**Ratio of lung weights**
14 - 10	25	2.01 ± 0.16
20 - 25	35	1.88 ± 0.27
25 - 30	15	1.88 ± 0.27
> 30	6	2.03 ± 0.09

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials (3).xlsx

Name of Reagent/ Equipment	Company	Catalog Number	Country
22 G shielded IV Catheter	BD	381423	USA
Bleomycin	Enzo life sciences	BML-AP302-0010	USA
Compact Mini rodent anesthesia machine	DRE Veterinary	9280	USA
Evans blue dye	Sigma-Aldrich	E2129	USA
FITC-dextran	Sigma-Aldrich	FD150	USA
Isoflurane	Piramal Critical Care	NDC	India
LED-30W Fiber Optic Dual Gooseneck Lights Microscope Illuminator	AmScope	LED-30W	USA
Low temperature cautery with fine tip	Bovie	AA02	USA
Precisionglide needle, 18G x 1"	BD	305195	USA
Xylazine	AKORN	NDC 59399-110-20	USA
Zatamine	VetOne	NDC 13985-702-10	USA

Comments/Description

Beveled tip, 12 mm in length

Ketamine

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Author(s): Sumei Liao, Oliver Eickelberg, Eric P Schmidt & Yimu Yang

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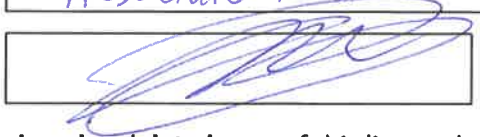
Signature:	
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We appreciate the careful consideration by the Editors and Reviewers. Accordingly, we have responded to all concerns as described below:

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Query 1 (Q1). Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response 1 (R1). We have proofread the manuscript to ensure grammar and spelling accuracy.

Q2. Please revise lines 228-230, 242-247 to avoid previously published text.

R2. Our apologies for this oversight; this has been corrected.

Q3. Please upload each Figure individually to your Editorial Manager account as .a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file. Please remove the title of figure from all uploaded figures.

R3. All figures and tables have been made into PDF format; titles have been removed from Figures.

Q4. Figures 1, 2: Please describe different panels in the figure legend.

R4. Our apologies; this error has been corrected.

Q5. Figure 3: Please include a space between all numbers and their corresponding units (25 mm, 30 mm, 35 mm).

R5: This has been corrected.

Q6. Figure 4: Please describe the left panel in the figure legend. Please rephrase the title of the table to be grammatically correct and upload the table separately in the format of an .xls or .xlsx file. Please include a space before and after the "±" symbol.

R6. Figure 4 has been deleted; all information is now provided in a revised Table 1, with data presented in an .xls file.

Q7. Figure 5: Please describe the three inset pictures in the figure legend.

R7. This has been corrected (now listed as "Figure 4").

Q8. Figures 6 and 7: Please describe the inset picture in the figure legend.

R8. These have been described in newly-created Figures 5 and 6.

Q9. Figures 6 and 8: Please consider submitting the tables separately in the format of an .xls or .xlsx file. Please include a space before and after the "±" symbol.

R9. Because of Figure 4 has been deleted, Table 1 and Table 2 have been combined, called Table 1. The Tables have been separated and are now included in an .xls file ("Tables.xls").

Q10. Figures 8-10: Please describe what different bars/colors represent in the figure legend.

R10. These have been described in the figure legends.

Q11. Figure 10: Please combine all panels of one figure into a single image file. Please describe different panels/images/graphs in the figure legend.

R11. Figure 10 has been combined into one PDF.

Q12. Please provide an email address for each author.

R12. These have been added to the title page.

Q13. Keywords: Please provide at least 6 keywords or phrases.

R13. SIx keywords have been added to the title page.

Q14. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion.

R14. These have been corrected.

Q15. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

R15. These have been corrected.

Q16. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

R16. This statement has been added prior to the first use of animals (introduction to **Section 4**).

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R17. All commercial language has been removed. For example, we have now described Velcro as "hook and loop tape".

Q18. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

R18. We have avoided personal pronouns in the protocol text as requested. There remains some rare use in the Representative Results section as we describe data interpretation.

Q19. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should

be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.
R19. We have made the necessary edits.

Q20. Lines 71-86: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.), or move the solutions, materials and equipment information to the Materials Table.
R20. We have made the requested edits.

Q21. 1.3, 2.1-2.8, etc.: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "NOTE".
R21. We have ensured imperative tense, and we have offset other language as "Notes".

Q22. 3.1: Please specify the age, gender and strain of mouse, and mention how proper anesthetization is confirmed.
R22. These descriptive data have been added (now in **Section 4**).

Q23. Lines 227-247: Details of the methodology should not be in the Representative Results section. Please move them to the Protocol.
R23. The methodology has be removed and rewritten in session 7.

Q24. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
R24. We have limited the numbers of statements per protocol step.

Q25. Please include single-line spaces between all paragraphs, headings, steps, etc.
R25. This has been completed.

Q26. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
R26. These essential steps have been highlighted in yellow.

Q27. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.
R27. We have highlighted complete, imperative sentences as instructed.

Q28. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

R28. This has been completed.

Q29. Discussion: Please discuss any limitations of the technique.
R29. We have completely revised the discussion to detail the precise strengths of this approach, as well as the weaknesses.

Q30. References: Please do not abbreviate journal titles.
R30. Our apologies; the JoVE Endnote plug-in lists references by their PubMed name. We have edited the references to include the entirety of the journal name.

Q31. Table of Materials: Please revise the Table of Materials to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software in separate columns in an xls/xlsx file, and sort the items in alphabetical order according to the name of material/equipment.
R31. This has been completed.

II. REVIEWER 1:

Query 1 (Q1): The manuscript by Liao et al describes a new technique that is used to evenly distribute a molecule of interest to each lobe of mouse lung. There is always a problem to obtain equal distribution of materials and the resulting disease progression in the lungs when intratracheal intubation is used to deliver materials of interest. However, the manuscript is somewhat confusing and needs more clarity, especially for the protocol section, which should allow the readers to have an idea for the procedure without seeing the video.
Response 1 (R1): We appreciate the Reviewer's consideration, and we apologize for the confusing nature of our first version of the manuscript. We have extensively revised the manuscript to better clarify the protocol and benefits of our approach, as detailed below.

Q2. One problem that arises upon reading the manuscript is whether the newly proposed technique is really superior to those currently routinely used. It depends on the purpose of experiments using this technique. Since this is a protocol paper, I would not argue against this manuscript being published, however, the authors need to more clearly demonstrate the differences obtained by the new technique and which aspects are superior to the others for what types of experiments.
R2: We have completely rewritten the discussion section to detail the benefits of our approach over pre-existing (i.e. intratracheal) approaches. This includes the selective delivery of intrabronchial agents (for example, siRNA delivery or adoptive transfer of tumor cells) or dose-adjusted intrabronchial approaches to improve homogeneity of alveolar deposition (as described in our protocol).

Q3: Please split "Material Required" into two sections; 1) lists all materials that one needs to carry out the procedure from the beginning to the end, which also includes chemicals, anesthesia, etc. and 2) provides specific and clear instructions of what one needs to do with the materials prepared. Also because of protocol description, all sentences are better described in the present tense…
R3: These changes have been made. We appreciate the Reviewer's guidance.

Q4. Line 63: Please explain what is the "dose-corrected"? This sentence could be as follows;
"By performing dose-corrected intrabronchial administration, which we term "corrected

intrabronchial administration" since it is a maneuver to improve drug overall distribution in the lungs, this asymmetry can be corrected, improving the modeling of diffuse lung injury in mice.

R4. We apologize for the confusion regarding our explanation of our approach. Based upon Reviewer 1 and Reviewer 2's findings, we have now simplified the introduction to focus on (1) the ability of our technique to unilaterally deliver an experimental agent to a lung and (2) the ability to modify this technique to improve symmetry of delivery to both lungs. We have rephrased this modification as "dose-corrected intrabronchial administration", clarifying this approach as "…administering larger volumes to the larger right lung, and smaller volumes to the smaller left lung". We hope that these changes improve the clarity of our manuscript.

Q5. Protocol. Please put the methodology (Instructions) in the exact order of what happens and what needs to be done specifically.

R5. We have revised the protocol to clearly delineate the order of what occurs during our procedure.

Q6. Need to provide concentration of solutions used. Bleomycin dose (what doses?) and how many times administered, etc.

R6: These data are now provided.

Q7. Did the authors administer the same volume for a 20 g and for a 40 g mouse, and had different results with that? Since mouse weight is critical for intubation, the more detailed information needs to be provided although Figure 6 describes some of this. With the current format, it is not easy to grasp the whole picture of what are exactly required to carry out the procedure.

R7. We apologize for the confusion. The use of weights was only to guide the length of the catheter and the dose of bleomycin—but not the volume of agent administered. We have not measured if and/or how airspace volumes vary based upon mouse weight. The limitations of our weight-based approach are now discussed in the Discussion.

Q8. Line 104: Is sterilization with UV light is good enough to completely sterilize? UV light would not reach inside the catheter.

R8. This is an excellent point that we had not considered. We have added a step in which the catheters are flushed with 70% Ethanol to allow intraluminal sterilization.

Q9. 133 to 138: Specify if whoever wants to use the technique described need to measure the length of carina or can use the measures as provided as a reference?

R9. We have revised the manuscript to refer to Table 1 as a reference of length of the catheter needed. In addition, we reviewed the strengths and limitations of using Table 1 as a reference in the Discussion.

Q10. 140: Remove (Figure 5,6). Please mention "figure" in the main text, not in the titles. Do this to the rest of manuscript as well.

R10. We have removed "Figure" from all titles.

Q11. 147: Which drug? Please specify. Or mention drug of interest. Since a smaller area is reached, is it necessary to use a smaller volume than the usual 50 ul? Need to detail this in the methodology.

R11. We have clarified this in the protocol by detailing use of either 0.3% Evans Blue Dye or 0.05% FITC-dextran or "other experimental agent" to be used by the operator for their experiments.

Q12. 160: Remove figure number
R12. This has been completed, as per R9.

Q13. 172: How much drug was given? Volume? Dose? Please detail more. Isn't it better to use a syringe to dispense air in the lungs, since you can measure the volume more precisely than the glass dropper?
R13. Additional details have been provided throughout the manuscript. We have been using a dropper to "flush" the catheter with air, but a syringe can be used as well. This has been added to the manuscript (Line 133).

Q14. 176: "… left sided segment, but rather to the entire left lung…" is very confusing. Left side presents only one lobe. What did you mean by this? Rewrite it to make it clear.

R14. We apologize for the confusion. As above, we have extensively rewritten the protocol. We take care in sections 5.1 and 5.2 to distinguish lobar delivery to the right lung from segmental delivery to the left (since there is only one left lobe). We provide additional explanation as well:

> ***5.3. Adaptation of intrabronchial administration to allow delivery of agent to entirety of left or right lung***
> *If the operator seeks to deliver agents not to a specific right lung lobe or left lung segment, but rather to the entire lung (the right and left lung), the catheter is slightly withdrawn to the respective mainstem bronchi, as follows….*

Q15. 184: How long the anesthesia with isoflurane lasts? Is it enough time? It may be better to state somewhere that until you have mastered the procedure, the techniques should be practiced with ketamine and xylazine (provide the doses).
R15. We have now included the following statement:

> *4.4 Ensure respiratory rate approximately 24 - 30 breaths/min before removing the mouse from the anesthesia induction chamber. Note: isoflurane anesthesia typically will last for ~ 4 min, sufficient for all intrabronchial procedures. If the operator is not proficient with the technique, ketamine/xylazine (80 mg/kg and 10 mg/kg intraperitoneally) may be used for more prolonged anesthesia.*

Q16. 228: Please mention the exact day where lung function analysis was carried such as 21 days after bleomycin administration, instead of "on the day of the experiment" because this is in the results section.
R16. These details have been provided.

Q17. 230: Instead of "the trachea was exposed", use "mice were tracheostomized with an 18G canula and connected to the FlexiVent".
R17. We have changed this protocol step to the "imperative" tense as recommended by the Editor.

Q18. 239: All data were analyzed using flexiVent software (version8).
R18. Unfortunately, per editorial guidance, we are unable to list any trademarked name. As such, we cannot describe the software used (other than general terms).

Q19. 241: Lung tissue collection and processing section,
Why use 1% low melt agarose to inflate the lung instead of formalin? It is nice to briefly explain the reason for this. Also what the pressure the authors used to inflate lung needs to be mentioned.
R19. Previous studies from our institution have demonstrated that 1% low melt agarose yields superior sectioning and (in our experience) superior histological quality. A reference is now provided (reference #5) and additional details are provided.

Q20. 253: … but only minor changes in the right lobes… I assume that this observation was obtained with intratracheal intubation. The authors apparently used 3 different techniques to deliver material (for instance bleomycin) to mouse lungs; intratracheal, intrabronchial, and corrected intrabronchial administration.
R20. We have clarified Figure 9 (previously Figure 10) to demonstrate that we are comparing intratracheal and dose-corrected intrabronchial administration. In addition, details are described in the Representative Results section.

Q21. These 3 techniques are not clearly described in differences, superiority and/or inferiority to each other, which causes confusion to the readers. Figure 8 through 10 are therefore not very easy to understand. The authors need to differentiate these 3 techniques more clearly and explain why your newly described technique is better than the others in what aspects (see below).
R21. In addition to the clarifications throughout the protocol and representative results, we not explicitly define the benefits of the different approaches in the Discussion.

Q22. -Overall: place the figures in the order that they appear in the main text, and in the exact order of the events.
R22. We have made the necessary edits; our apologies for the confusion.

Q23. -Did you use an optical fiber system to guide you through the trachea? If so, please mention it.
Q23. Our technique does not require fiberoptic guidance, other than illumination of the neck itself. This is now described in Section 4.

Q24. -Figures and table legends are supposed to be placed after references unless the journal format says so.
R24. JoVE instructions for authors requests that references follow the figure legends.

Q25. The authors need to provide details of figure legends what are shown in the figure. For instance, in Figure 3 legend: no explanation for 3 marks on the catheter.
-Compile Figure 1 through 4, 5 through 7, and 8 and 9 if possible because they describe for the same thing.

-Figure 4 needs detailed legend. Same for Figure 6C, 8B, and 10B. Please provide figures and figure legends that the readers not from the field can understand.

R25. We have clarified, simplified, and consolidated our figures to improve readability.

Q26. By the way, <u>why is the right to left lung weight ratio important? Please provide more detailed explanation.</u>

R26. The right-left lung weight ratios are important as they provide a potential explanation for the observed heterogeneity of drug deposition with equal (30 µL per lung) volumes of EBD. These data are now integrated into our "representative results" and ensuing interpretation.

Q27. -In the Figures where statistical analyses were carried out, please mention which statistical analysis method was used.

R27. These data have been provided.

Q28. -Figure 9B: The results of a "normal" intratracheal administration for the distribution pattern of FITC-dextran to compare with those obtained with the CibA method is necessary to determine if there is a real better distribution with the latter.

R28. It is technically difficult to measure FITC-dextran deposition after histologic processing, as much of the (soluble) FITC-dextran is washed out with agarose inflation and sectioning (we have observed it "seeping" from the sectioning). As such, we only quantified FITC-dextran in fresh, homogenized lungs, allowing for a precise quantification of global left-right lung delivery. To determine geographic differences in distribution within a lung (e.g. lobar on right, segmental on left), we used a more effective marker of regional delivery: EBD staining. EBD irreversibly stains tissues, allowing for the measures of regional lung delivery as provided in **Figures 4, 5, 6, and 8.**

Q29. -Figure 10A: Please describe how these sections were obtained; whether each image is from a different mouse lung or from same lung sliced at different positions, and on the right lobes row, which one corresponds to superior, middle, inferior, or posterior lobe in the right lobes.

R29. Our apologies for this confusion. The images provided are from one representative mouse. The entire lung is embedded; as such, sectioning randomly slices through multiple different lobes. These lobes are now labeled and described in **Figure 9A**.

Q30. -Sections shown in Figure 10A do not seem to be reflecting the results in Figure 9B since there are 3 sections that present very low distribution of damage. Because of this, it is extremely important to show the pictures <u>of immunofluorescence of FITC</u> to check if the initial delivery was homogeneous.

R30. Please see discussion in R28 above. FITC-dextran provides excellent quantification of bilateral (left vs. right) delivery, while we used EBD staining to provide information on regional selectivity (for example, **Figure 4** vs. **Figures 5, 6**). As described above, EBD provides a better regional indicator of delivery than FITC-dextran.

Q31. Discussion:

I am wondering if the authors ever notice any tachypnea by cannulating the left side lung. If not, this

is strange, because when a catheter is introduced, it still blocks the air flow and the animal should feel some discomfort.

R31. We have consistently noted increased dyspnea only during distal cannulation of small right lobes, and we have not noticed it with cannulation of the left. Please note that this only occurs during deep (distal) cannulation, as detailed in **Sections 5.1** and **5.2**. This abates as we withdraw the catheter to access the entire right lung (**Section 5.3**). While discomfort is a possibility, the mouse is deeply anesthetized during this procedure. We thus speculate that the tachypnea that occurs during deep right sided cannulation (and not deep left sided cannulation) is from differences in the distal airspaces engaged (as demonstrated in the EBD staining in Figure 4). We acknowledge that this is "speculation" in the Representative Results section.

Q32. In the right lung, where the authors noticed tachypnea, the manuscript says that "…catheter tips has passed the two upper lobes (line 348)……" and this is responsible for difficulty in breathing. However, this can be a problem with the technique because it can also be responsible for the low distribution seen in image 10A; CibA bleomycin in the middle sections. Again the initial FITC immunofluorescence image should be able to demonstrate the better distribution throughout the lungs.

R32. As discussed above, bleomycin is administered to the entire lung (i.e. after withdrawing the catheter after deep cannulation, as detailed in **Section 5.3**). As such, we do not anticipate that deep cannulation prevented access of bleomycin to the entire left or right lung.

Q33. General comments:

I do not see that much better distribution between intratracheal intubation and CibA method in Figure 10.

R33. We consistently observed that there is less fibrosis in the left side with dose-adjusted administration, and there is more fibrosis (particularly in the middle and superior lobes) on the right side. To confirm these observations, we performed quantitative analyses of lung physiology that are sensitive to diffuse lung disease (IC, Ers, Crs). These data, coupled with the EBD and FITC-dextran quantitative data, support our assertion that our dose-adjusted intrabronchial approach improves delivery symmetry in a manner that impacts experimental results.

Q34. It is known that in bleomycin-induced pulmonary fibrosis model, animals lose considerable amount of weights during the experimental period before their weights start coming back. In this regard, the authors need to show mortality of bleomycin-treated mice using the newly described method. The mortality also depends on dose of bleomycin used in the experiments. Please provide the bleomycin dose and with this same dose of bleomycin, the authors need to compare the differences in weight loss and mortality between normally used intratracheal intubation and CibA method. In general, the more diffused the injury is, the higher the weight loss is and so as the mortality. I assume that if bleomycin is evenly distributed to all lung lobes, mice having received bleomycin would not survive for that long. Depending on the purpose of experiments, this could be the worst scenario in which the experiment fails.

R34. This is an excellent point that has been discussed in the Representative Results. We observed equal mortality in the intratracheal and dose-corrected intrabronchial groups.

Q35. The CibA method is useful if targeting one particular lobe to produce a lesion of interest. The author may want to emphasize this.
R35. This is an excellent point. We have emphasized this use of our approach in both the introduction and discussion.

Q36. Another indication that intubation was done properly is that the solution added to the catheter is promptly sucked into the lungs by the animal. Did this happen in the newly described technique or the solution has to be forced into the lungs (with a syringe or the glass dropper)? The quick suction of added solution may happen only when catheter is located before bifurcation point in the trachea. This needs to be addressed.
R36. This is another excellent point. We rely upon this "suction" phenomenon with our spirometer approach as a marker of successful endobronchial intubation. The syringe/glass dropper is used to promote clearance of the catheter, ensuring complete drug delivery independent of the degree to which the animal "sucks" on the catheter.

Reviewer #2:
Query 1 (Q1): Manuscript Summary:
This is an interesting paper showing how to implement delivery of agents to the left or right lungs. While such a method might be useful, there are some issues with the reproducibility of the approach as well as the rationale for the study.
Response 1 (R1): We thank the Reviewer for his/her careful review of our manuscript.

Q2: Although you do find some variation in agent delivery between left and right lungs, it isn't clear how significant this would be.
R2: A major impetus for our attempt to publish this approach is that it seems to produce a significant impact on the degree of bleomycin-induced lung injury, as assessed by pulmonary function measurements (**Figure 9**). Thus, we believe that optimization of lung delivery is of importance in modeling diffuse lung disease.

Q3. Also, it is not clear how consistently you can get into the left or right lungs. How many times will you end up in the wrong lung if you were aiming for one? And is there any way to tell in advance of killing the mouse? This is critically important and it affects again the whole rationale for the study.
R3. This is an excellent point that has prompted further clarification in the manuscript. We note nearly 100% first-pass success at left lung cannulation, and approximately 90% with right lung cannulation. In our experience, the tachypnea sign is the most reliable measure of successful distal right cannulation, and this can be performed without killing the mouse. Indeed, reliance upon such "physical exam" findings has been necessary to guide other measures of lung delivery (for example, if the mouse "coughs" after intratracheal delivery").

Q4. Overall, the procedure seems very difficult and cumbersome, with reliability uncertain. While a video might resolve these issues and concerns, it would be nice if they could be addressed in the document.

R4. As noted above, one of the reasons we decided to publish this technique is that it is surprisingly not difficult to do, and it yields improved modeling of lung injury. We have attempted to be as clear as possible in the protocol to provide nuance for performing this approach, as well as identifying potential pitfalls.

Q5. Introduction, and especially lines 53-57. This rationale for the paper makes no sense. Why wouldn't any drug delivery not follow the same pattern as air delivery, i.e., more going to the larger lung. Surely within a lung, do you imagine a similar heterogeneity between lobes?

R5. While ventilation patterns help dictate the distribution of gas to the lung, other factors influence the distribution of liquids. For example, in humans an aspirated liquid goes to the superior segment of the right lower lobe because of (a) gravity and (b) the relatively straight take-off of the right mainstem. Mice, which have unique anatomy and body positioning, make have specific factors that guide deposition of an intratracheally-administered liquid. Our work is novel in that it demonstrates that there are additional factors (such as lung mass) that influences distal lung concentration—specifically, we used our approach to show that these differences persist even with equal presentation of a labeled agent to both mainstem bronchi. We additionally provide data showing that adjusting doses based upon different lung sizes can offset observed left-right asymmetry. Taken together, our work therefore provides not only a new technique but may offer insight into mouse lung drug deposition. Of note, for purpose of this method paper we have emphasize the techniques, acknowledging that much of our explanations are speculative.

Q6. Tracheal delivery of agents has been shown many times to result in a reasonable uniform distribution across most lobes and left or right lungs. While there may be a very good reason to have a method of delivering agents to the left or right lungs, your rationale is not one of them.

R6. We and others have noted that intratracheal administration of injurious agents have induced patchy, asymmetric lung injury (Matute Bello et al, reference 7). As such, we endeavored to use our model to see if we can improve the distribution of injury (and improve physiologic modeling of diffuse lung injury, Figure 9). However, we agree with the reviewer that an additional benefit of our approach is the local administration of agents. We have now rewritten the introduction and discussion to emphasize the use of our technique to locally administer experimental agents to better model unilateral disease (lung cancer, ischemia-reperfusion injury, etc.).

Q7. Also I don't think anybody was doing mouse intubation in 1985, so ref 1's date seems incorrect.

R7. Our apologies for the confusion. The Journal Title (in Endnote) for Journal of Applied Physiology is "Journal of Applied Physiology [1985]"—the reference is indeed 2009 (last section of the reference). We deleted "1985" from the reference. Of note, rat intratracheal cannulations date as far back as 1979, which has now been cited in our manuscript.

Q8. And you might want to cite 9 Vertrees et al, J. Investigative Surgery. 13:349-358, 2000) who delivered tumor cells consistently into the left lung via intubation to result in left lung tumors.

R9. Thank you for this reference; we added to illustrate a potential role for our technique in unilateral lung administration.

Q9. Line 241. It isn't clear why you used agarose to inflate the lung. It would seem impossible to

control a consistent and reproducible pressure during fixation since the agarose is steadily cooling and stiffening

R9. This is an excellent question raised also by Reviewer #1 (Question #19). Our standard approach in our laboratory has been to use this technique, first reported by Rubin Tuder and colleagues at our institution in 1994. Use of agarose improves both inflation and sectioning of the lung, allowing for better histological images. A reference has been added to this effect (Halbower et al, Ref #5).

Q10. Line 269. It is not clear why you needed to bevel the intubation cannula. Making a sharp point would seem to introduce a level of danger as it would make penetration of an airway wall more likely. And your explanation makes little sense-increasing the cross-sectional area over just the tip of a long catheter would have minimal effect on the overall resistance or regional pressures.

R10. We have added additional clarifications. The beveled edge is softened by use of a cautery tip; this has been added to the protocol. We have corrected the rationale for beveling; we agree that it is unlikely to change resistance (which is likely to be governed by the length of the catheter). Rather, beveling allows for directional administration of the agent, as described in our protocol.

Q11. Figure 4. While this is useful for your mice, it is not clear that mouse body weight is always very useful. Surely if one were doing an obesity study, the cannula length should likely be independent of body weight. And this would of course vary considerable with different mouse strains or knockouts.

R11. This concern was also raised by Reviewer #1; we have dedicated a paragraph in the Discussion section to the use of Table 1 (and its limitations). While we intend to use these data to help guide the operator, we acknowledge that it may not be informative in every mouse strain or weight.

Q12. Figures 6&7. It is not exactly clear why you rotate the mice laterally for the intubation. Is this for ease of the person doing the intubation, or are you trying to utilize gravity to reposition the lungs? Given the small mass of the lungs and very small size, how much of an effect would gravity have?

R12. When we first developed this procedure, we frequently noted immediate resistance when mice mice were suspended by its upper incisors vertically. This led to frequent (~40%) failures of intrabronchial intubation. However, at the optimized position (at + 30 ° for the right lung, -74 °for the left lung), cannulation occurred without resistance to at least ~ 35 mm without resistance. We speculate that that this reflects the effect of gravitational shifts on airway anatomy, not only of the lung but also of the mediastinum and chest wall.

Q13. Line 345. This tachypnea is interesting, although I'm not sure of your explanation. However, how common is this, and does it matter if the animals is rotated laterally? If it happens as soon as there is some obstruction it is not likely to be a result of loss of ventilation in a small region.

R13. We consistently note tachypnea with right-sided intubation, as described in R3. While we speculate it is due to the small airspace being cannulated, we cannot prove that this is the mechanisms (such proof is beyond the scope of this methods paper). We have acknowledged that this explanation is speculative in our manuscript. We have noted tachypnea with distal right cannulation regardless of positioning.