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

I. EDITOR COMMENTS

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**).

Q17. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sigma-Aldrich, Velcro, flexiVent, etc.

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.