Reviewer Responses:

We greatly thank both of the reviewers and the editor for their suggestions and their critical evaluation of this manuscript; your comments were hugely helpful in improving the manuscript overall. We have made a number of changes to our manuscript in response to these valuable recommendations, as detailed in a point by point response below. For convenience, new additions to the text are included in blue font, corresponding to the track changes sections in the attached manuscript. Page and Line reference numbers indicate locations in the tracked-changes version of the document.

Editorial Comments

***Protocol Detail**: Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.*

1. *1.1, 1.2: mention button clicks and menu selections*

We have added a more detailed description of the process for preparing lung models from CT scans.

2. *1.4.1: 100% IPA?*

We have specified that the IPA used in the protocol is ≥99% purity.

3. *1.4.2: mention UV intensity*

We have added the UV oven specifications to the Table of Materials as well as modifying step 1.4.2 below:
Page 5, Line 179: "…cure in UV oven (365 nm light at 5-10 mW/cm^2)…"

***Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*

We have updated the highlighted sections to reflect changes made to the protocol.

***Discussion:** Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

Thank you for this comment. We have revised the discussion section of our manuscript and believe we have addressed all five of these points. Please advise if we have not sufficiently addressed any of these areas.

***Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language…Examples of commercial sounding language in your manuscript are Materialise Mimics, Meshmixer, Solidworks, etc.*

Throughout the manuscript, we have replaced all instances of "Materialise Mimics" with "CT scan software," "Meshmixer" with "mesh editing software," and "Solidworks" with "3D modeling software." The Table of Materials has also been updated to reflect these changes.

Reviewer Comments

Reviewer #1:

1) *Authors have followed the work of Sul et al., but at most of the places authors have just cited the paper and not provided necessary information e.g., flow rate, aerosols diameters and other properties (See NOTE given at Line no 226). It would have good to define all the details in the manuscript as well in addition to the citation.*

Thank you for this feedback. The distribution of air flow to each lobe of the lung was calculated by Sul et al. by comparing the volume of each lobe at full inspiration and full expiration as determined by thin-slice computed tomography images of patient lungs. As a result, this method could be extended to specific patients to generate unique flow profiles that, when used in conjunction with a patient-specific lung model, have a greater capacity to predict regional distribution of a therapeutic for the specified patient. The indicated NOTE in the manuscript has been modified to clarify these details:

Page 6, Line 256: "…calculated by Sul et al. using thin-slice computed tomography images of patient lungs at full inspiration and expiration, comparing the relative changes in the volume of each lung lobe. Results are presented for two distinct flow conditions, both at an overall inlet flow rate of 1 L/min. The healthy lung lobe outlet flow profile is distributed to each outlet by the following percentage of the inlet flow: LL-23.7%, LU-23.7%, RL-18.7%, RM-14.0%, RU-20.3%. The COPD lobe outlet flow profile is distributed between each outlet by the following percentage of the inlet flow: LL-10.0%, LU-29.0%, RL-13.0%, RM-5.0%, RU-43.0%."

2) *Figure 3 is provided without mentioning the details of experimental conditions/parameter values.*

Thank you for this comment. The images presented in this figure were collected during an experiment targeting the left lung using 1 micron particles and the endotracheal (ET) tube attachment depicted in Figure 5 at a flow rate of 1 L/min. The Right Upper (RU) Lobe and Left Lower (LL) Lobe filters were used for the low and high deposition examples, respectively. To demonstrate visually a "good" threshold, a range of 43 to 255 was used, producing images identical to those used in our analysis for that experiment. In comparison, the "poor" threshold images were produced using a range of 17 to 255. Reducing the lower limit of the threshold range detects fluorescent signal from the adjacent filter fibers in addition to the particles' signal, leading to an overestimation of particle deposition on the filter. The figure caption was modified to include these details as indicated below:

Page 10, Line 422: "The raw images presented were collected during an experiment to target the left lung using 1 µm fluorescent polystyrene particles at 1 L/min under a healthy breathing profile. The "high" and "low" deposition images depict the LL (23.7% outlet flow) and RU (20.3% outlet flow) Lobe filters, respectively. The "good" threshold, applied with a range of 43 to 255…The "poor" threshold, applied with a range of 17 to 255…"

3) *The results and discussion sections do not provide much information on the flow physics and reasoning for the specific deposition behaviour.*

Thank you for this feedback. We have enhanced our discussion with a number of points raised by both reviewers. Specifically, we have added a discussion paragraph to highlight the importance of geometry and targeting position in the resultant deposition profiles, as shown in the following:

Page 11, Line 461: "As shown in **Figures 4 and 5**, lobe-level deposition can be accurately and rapidly measured for both targeted and non-targeted inhalation aerosols. In the absence of a targeting device, particles in this size range (1-5 µm) and flow conditions (1-10 L/min) follow the fluid stream lines and total airflow profile diverted to each lobe (**Figure 4**). Notably, various inhaler devices and ET tube attachments can be developed to concentrate inhaled medicines to controlled lobe locations. As described in our recent work and those of others, many features of the inhaler device, flow profile, and airway geometry contribute to targeted deposition behavior. In general, efficient regional targeting as demonstrated by our unique *in vitro* models requires a narrow aerosol size distribution and low inhalation flow rates to avoid airway turbulence specifically found within the trachea. Inclusion of the full upper airway within our *in vitro* model allows for accurate recreation these airflow patterns that are known to influence downstream lobe-level distribution. Because of these complex flows, recent work has demonstrated increased targeting from below the glottis. Our results in **Figure 5** specifically highlight the benefit using an ET tube adaptor to regionally target individual lobes from release below the glottis, with efficient lobe-specific targeting shown for lobes of both the right and left lungs at efficiencies ranging between 62-74% of the total dose. This represents an increase over previously experimentally reported mouth-release regional targeting efficiencies and is an important avenue for clinical implementation of this approach. Importantly, the protocol allows for experimental lobe distribution measurements of a complete pharmaceutical dosage from a wide range of potential regional targeting devices beyond those demonstrated here."

4) *No conclusion has been given in the manuscript. Manuscript is written in a very simple way and provides very limited details regarding the experimental outcomes.*

We appreciate this feedback and have tried to enhance our protocol discussion to also reflect the experimental outcomes where appropriate. We have added additional experimental discussion points (see responses to additional reviewer comments) and have augmented the concluding statements to include the following:

Page 13, Line 539: "Our protocol demonstrates the first *in vitro* experimental setup with the ability to quantify lobular pulmonary deposition in a patient-specific lung geometry. Achieving controlled lobe-level distribution is expected to increase therapeutic efficacy of inhalation therapeutics, which will only be achieved through advances in *in vitro* whole-dose measurements. With the growing interest in personalized medicine, this protocol has the potential to spur the development of new targeted lung therapies by allowing for more accurate predictions of potential treatment efficacy."

Reviewer #2:

This work shows a novel methodology based on 3D printing to create patient-specific bronchial tree models to test deposition patterns of pharmaceutical aerosols. The text and figures are illustrative and well structured. Some more details are required, in particular for the 3D printing of the models

and how to perform some quality control over them in terms of accuracy and reproducibility and integrity of the resins used after each experiment.

Further details are required in terms of how the models are 3D printing and testing the integrity of them during the experiments with the aerosols. More details with regard to how to go from CT patient images to the models are needed and also about certain decisions taken for the models such as stopping them at the 2-3 bifurcation instead of going beyond in the bronchial tree.

Thank you for this helpful feedback. We have made multiple additions to the manuscript (detailed below) to touch on these points. Specifically, in our procedure, we have added additional information about the 3D printing method used and the process of preparing models for 3D printing. In our discussion, we have emphasized some additional key limitations of the current lung models used and the current work ongoing in our lab to ameliorate these points, as described in the following.

1) *Page 2, section 1.1.1 and Fig. 1A. Fig 1A is not the CT scan, please modify the title over that particular image, it is a model. Please add to Fig 1 a sagittal slice of the actual thorax scan that was used for that particular model.*

Thank you for this feedback. The suggested change was made to the title in Figure 1A. The particular model used in this study was a generous gift from our collaborator and an in-depth geometrical analysis of this particular model has been previously published, which includes a sagittal slice in addition to other views[1]. Please refer to Figure 8 of the referenced Feng et al. paper, for additional views of the original CT scan and lung model, in addition to a more detailed discussion of geometrical features. We have added the following statement to the manuscript to direct readers to this additional information:

Page 2, Line 86: "NOTE: For a more detailed discussion of the geometrical features of the lung model used in these studies, refer to Feng et al."

2) *Page 3, line 89. Why was the wall extended 2 mm? Is this related to the selected 3D printing technique to guarantee that the printed models will be robust/strong enough or is it linked to the actual wall thickness of the bronchii? Please explain in the text.*

The thickness of the lung model walls was chosen to be 2 mm to adhere to recommended feature sizes for the 3D printer (Carbon M1 Printer) and resin (PR25 resin) used to produce the models. As a result, this thickness could be varied by researchers depending on which instruments and materials are available. Since the protocol uses a rigid model, the wall thickness can be adjusted for the requirements of various 3D printing methods as long as the interior geometry and dimensions of the model are maintained. This does not impact the airspace topography at all. The following statement has been added to the text to clarify this:

Page 3, Line 104: "The thickness of 2 mm was chosen based on the acceptable feature sizes specified by the manufacturer of the 3D printer listed in the Table of Materials. This thickness can be adjusted based on the specifications of the 3D printer available as long as interior geometry of the model is maintained."

3) *Page 3, line 91. In your method, the modelling of the bronchial tree stops at the generations 2 or 3, at the entrance of each of the lobes. Please comment in discussion if this is realistic enough to test the deposition of the pharmaceuticals or if it could be better in the future to generate more vessels in your model to measure more accurately the distribution.*

Thank you for this comment. Our method was developed specifically to address the need for a tool to assess regional deposition at the lobe level. Modeling the bronchial tree up until the lobe entrances allows for the identification of regional differences in delivery while remaining high throughput. Additionally, the clinical data used to validate the flow profiles generated in the model are reported at the lobe level. Since this clinical data was collected *in vivo,* it includes the full lobes and incorporates aspects of the geometry relevant to our analyses. Nevertheless, this is a limitation of our method which warrants further development. There is ongoing work in our research group on developing a method of modeling further generations of the lung without adding a great deal of complexity to the protocol. The following statement has been added to address these points:

Page 12, Line 507: "While these models currently can only replicate patient geometry up to the second or third generation to adequately measure lobe-level distribution, work is ongoing to develop modifications that can incorporate the lower airways for more detailed analysis."

4) *Is there such a big inter patient variation related to sex and race (I can imagine differences due to age, specially for pediatrics), if you only consider bronchial models till the entrance of the lung lobes? Please, give some details in discussion.*

Thank you for this point. Regions of the upper airways including the pharynx, larynx, and trachea can have a significant impact on regional deposition, which varies among subject age, race, and sex. The following addition has been made to the discussion to expand on these points:

Page 12, Line 493: "Differences in lung flow profiles and geometries due to characteristics such as age, race, and sex are well documented in literature[16-18] and can be readily incorporated into our modelling approach. Specifically, geometric variations in the larynx, pharynx, and trachea of lung models can have a significant impact on airflow and subsequent regional deposition patterns[14], which this protocol is well-equipped to detect."

5) *Page 3, section 1.3.1. Please provide more details about the printing materials and the printer used and the printing method (the printer is not mentioned in the Materials table, I believe, just the resins). It is important to mention that the chemical properties of the selected 3D printing materials are relevant, specially in terms of possible interactions with the aerosols or the rinsing liquids used in between experiments.*

Thank you for this feedback. Per JOVE's statement on commercial language, we have kept the protocol to be generalizable to a wide range of 3D printers but have elaborated on your points in the following. The 3D printer and resins used in this protocol are products of Carbon® and use a form of stereolithography (SLA) termed Continuous Liquid Interface Production™ (CLIP). The hard resin used is PR25, a proprietary mixture of photoinitiator and reactive diluents meant for rapid prototyping. It can be exposed to IPA for

up to one hour before impacting the structural integrity of the cured resin. The soft resin used is an elastomeric polyurethane (EPU40), another proprietary composition that allows for the printing of the elastic cap components. Due to this property, EPU40 has a solvent exposure time of only one minute during post processing. However, the parts printed with this resin are not patient-specific and can be easily replaced in the event of wear-and-tear due to stretching or solvent exposure. This protocol follows all manufacturer instructions with respect to these resins and can be easily adapted for compatibility with other brands or classes of materials. Links to manufacturer information on both of these resins was added to the Table of Materials. In addition to the printer and resins, other post-processing tools, including the convection and UV ovens used, were added to the Table of Materials. In the text, we have included additional references to the Table of Materials and added the following note:

Page 4, Line 166: "NOTE: All post-processing steps described below are specific to the 3D printer listed in the Table of Materials. When utilizing alternate printers or materials, adjust these steps to reflect manufacturer instructions."

6) *Did you test the accuracy and/or reproducibility of the 3D printing process and your models? It would be interesting to scan the model with a protocol similar to the one used for the patient and compare the images. Even though the attenuation properties of the resin will be different, this experiment can give insight about how similar is your final product to the actual patient.*

Thank you for this comment, the suggestion of imaging the model to quantify the accuracy of the printing process will be incorporated into this paper and future projects. The process of using CT scans to generate a 3D rendering to be printed using the Carbon M series printers has been FDA approved[6] for medical use and, thus, we did not previously investigate the accuracy of the print due to the proven accuracy in these specific prints. However, we recognize the benefit of confirming that this process is as accurate as we assumed it to be, and would recommend the use of a µCT (Micro-Computed Tomographic scan) to perform this characterization due to the small size of the replica. This has been included in the following statement:

Page 5, Line 182: "Note: To evaluate the accuracy of the 3D printed replica, we recommend the use of µCT scanning the printed part and using CT scan software to compare, quantitatively, variations between the original 3D rendering and the 3D printed replica."

7) *Page 7, line 280 (Note): In between experiments, it is stated that the 3D printed tree is rinsed with isopropyl alcohol. Plastics tend to be quite sensitive to light exposure and subject to chemical interactions. Have you tested the durability of your models and their integrity after several experiments? Have you considered designing a sort of quality control procedure to check one model cannot be used anymore?*

Thank you for this value comment. We agree with the potential sensitivity to plastic parts to various solvents, including IPA. Per the manufacturer, the Carbon resin used for the airway replicas is PR25 which has a maximum IPA exposure time of one hour per manufacturer instructions. However, only a few minutes of exposure is required to complete the initial post-processing cleaning, and the exposure time during experiments referenced in the note on Page 7 Line 280 is 10-15 seconds. Additionally, more than

one replica was created for this work. Therefore, we estimate that the total exposure time of any one replica is less than 10 minutes, which does not approach the IPA limit. We recommend use of an exposure log to track the use of a model and ensure the exposure limit is not approached. This information has been included in the below statement:

Page 8, Line 318: Note: "...Additionally, a log is kept to ensure all replicas used have been minimally exposed to IPA to maintain part integrity and visual part inspection is recommended prior to use."

8) *Page 8, line 325. Please add the size range here.*

We have added the size range (1-5 µm) on page 8.

9) *Page 8, line 336. Your method reproduces the distribution of the air flow till the entrance of the lung lobes, not on all the lungs. Please modify the statement.*

We have altered the specified statement as shown below:

Page 9, Line 383: "…distribution of air flow to each of the lung lobes."

10) *Figure 3. Please give some details about what is a "good" threshold and a "bad" threshold, even for the particular case you show. If someone tries to reproduce your experiment they would need this information.*

Thank you for this feedback. The critical difference between a "good" threshold and a "poor" threshold is the amount of signal detected from the filter paper fibers rather than the particles themselves. Although the filter paper does not have any inherent fluorescence, the fibers can diffract light, obscuring the borders of large deposits of fluorescent particles. To clarify this distinction and aid in identifying a proper threshold range, we have added more detail to the figure caption and added an additional note as shown below:

Page 10, Line 426: "The 'good' threshold, applied with a range of 43 to 255, maintains defined edges between individual particles and avoids detection of filter paper fibers. The 'poor' threshold, applied with a range of 17 to 255, obscures individual particle borders and overestimates the fluorescent area of the filter."

Page 9, Line 352: "NOTE: For filters with high levels of deposition, a 'corona' of fluorescence, caused by the diffraction of light by the filter paper fibers, may be observed around large groupings of particles. When thresholding these images, a range that is too large displays small dots or 'feather-like' shapes around these groupings, as observed in the 'poor' threshold images in Figure 3. This can be improved by gradually increasing the lower limit of the threshold until the signal from the filter paper fibers is minimized without obscuring the signal from the particles themselves."

11) *Page 10, line 404. It is stated that the proposed protocol can be used to create patient-specific models and thus test the influence of anatomical differences in age, race, sex in the deposition of the aerosols. Nonetheless, the proposed models only expand for a few bifurcations in the bronchial tree. Please explain the validity/limitations of your approach and also if in some studies it would be better to have a larger range of bronchii to simulate a more realistic air flow and aerosol deposition.*

Thank you for this comment. Please see our responses to comments 3 and 4 above.

12) *Page 10, line 410. Please, comment on discussion about the costs of the 3D printed models that are used in your work and how feasible would it be to do it routinely for clinical trials.*

Each lung model requires about 130 mL of PR25 resin to produce. At $0.12/mL of PR25 resin, a lung model can be produced for only $15. The most significant cost associated with the production of these lung models is the 3D printer itself and its associated software; however, 3D printers are becoming increasingly prevalent in hospitals worldwide for diagnostic and surgical planning purposes[7]. In the United States and Canada alone, over 113 hospitals have 3D printers on-site. For hospitals with an existing 3D printing infrastructure, producing a large number of lung models for a clinical trial would be relatively low cost. We have added the following statement to touch on this topic:

Page 12, Line 485: "This protocol will not only provide an experimental lab-scale approach to support design of new inhaler devices, but also create opportunity for on-demand personalized inhalation devices in clinical practice. The hard resin used in this protocol costs ~$0.12/mL; therefore, hospitals with existing 3D printing infrastructure could print a lung model for as little as $16 in materials[15] and assemble a personalized airway in under a day. Notably, printing times and material costs in additive manufacturing continue to decrease rapidly, increasing the overall feasibility of this approach."

13) *Page 10, line 426. The authors explained nicely the possible differences in the behavior of the selected resin and the actual tissues in patients. Please add a sentence of the potential possibility in the future to use 3D printed materials that actually mimic patient tissue, such as has been already done with skin. 3D printing is a fast moving field and new materials are continuously released. Please add some sentences about the properties (chemical, mechanical...) that a material would require ideally to create the best possible phantom for your application.*

Thank you for this comment. Bioprinting has great potential to incorporate further patient-specificity into the lung models; however, the lung epithelium is highly complex, with great diversity in cell types, making it very difficult to develop an accurate bioprinted mimic. Most pulmonary bioprinting work, thus far, has been focused on small structures such as organoids or grafts[8]. Nevertheless, we are investigating other methods of incorporating a cellular response into our analysis such as culturing cells on 3D printed materials. The following statement has been added to the discussion:

Page 12, Line 523: "Other techniques such as bioprinting and culturing cells on 3D printed models are being investigated for their ability to incorporate a cellular response into the protocol"

14) Page 1, line 32. Typo: "This protocol has the potential promote..." Please add "to" in front of promote

We have made this change on page 1.

References in this Response Document

1. Feng, Y., Zhao, J., Chen, X. & Lin, J. An In Silico Subject-Variability Study of Upper Airway Morphological Influence on the Airflow Regime in a Tracheobronchial Tree. *Bioengineering*. **4** (4), 90, (2017).

2. Feng, Y. *et al.* An in silico inter-subject variability study of extra-thoracic morphology effects on inhaled particle transport and deposition. *Journal of Aerosol Science.* **123** 185-207, (2018).

3. Martin, S. E., Mathur, R., Marshall, I. & Douglas, N. J. The effect of age, sex, obesity and posture on upper airway size. *European Respiratory Journal.* **10** (9), 2087, (1997).

4. Jiang, Y.-Y., Xu, X., Su, H.-L. & Liu, D.-X. Gender-related difference in the upper airway dimensions and hyoid bone position in Chinese Han children and adolescents aged 6–18 years using cone beam computed tomography. *Acta Odontologica Scandinavica.* **73** (5), 391-400, (2015).

5. Weber, P. W., Price, O. T. & McClellan, G. E. Demographic Variability of Inhalation Mechanics: A Review. (Defense Threat Reduction Agency 2016).

6. "KeySplint Soft™ Clear by Keystone Industries®, a Unique 3D Printing Resin for Night Guards and Splints Available Exclusively on the Carbon® Digital Manufacturing Platform, Cleared for Sale by U.S. FDA." Carbon, 18 November 2019. Press Release.

7. Pietila, T. *How Medical 3D Printing is Gaining Ground in Top Hospitals*, <https://www.materialise.com/en/blog/3D-printing-hospitals> (2019).

8. Galliger, Z., Vogt, C. D. & Panoskaltsis-Mortari, A. 3D bioprinting for lungs and hollow organs. *Translational Research.* **211** 19-34, (2019).