**JoVE - JoVE58437**

**Generation of human lung tissue slices for disease modeling**

Dear Dr. Bajaj,

Thank you very much for the expedite review of our submission JoVE58437 to the Journal of Visualized Experiment (JoVE). Attached, please find our revised manuscript entitled “Generation of human lung tissue slices for disease modeling". As required, all changes in the manuscript are marked in red.

We have substantially revised our original submission as suggested by the editor and reviewers of the initial submission, and responded to all editor and review comments. We thank the editor and the reviewers alike, especially for their time and valuable comments, all of which have significantly improved the quality of our initial manuscript.

Best regards,



Dr. Gerald Burgstaller.

Prof. Dr. Melanie Königshoff

Point by Point response to the editor’s and reviewers’ comments:

***Editor:***

1. 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.

***We proofread the manuscript and corrected typographical errors.***

2. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

***We removed the title and figure legends, and provide the figures as .tiff files at 300 dpi.***

3. Figures 4A-C: Please define the scale bar in the figure legend.

***We added the following sentence to the figure legend:*** Scale bars indicate 1000 µm.

4. Figures 4D and 4E: Are they reprinted? If so, please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

***The image was not used in previous publications. Alsafadi et al (2017), Am J Physiol Lung Cell Mol Physiol 312: L896–L902, shows different images acquired using the same technique.***

5. Figure 4E: Please include a space between number and its corresponding unit (i.e., 58 kDa and 42 kDa).

***We changed these units accordingly.***

6. Please remove the embedded Table between lines 160 and 161 from the manuscript and upload the table separately to your Editorial Manager account in the form of an .xls or .xlsx file. Refer to the Table in step 3.2. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

***We separated the table into an xls-File. We provide the table legend in the manuscript. We corrected an error in 3.3.***

7. Please number the figures in the sequence in which you refer to them in the manuscript text.

***With the changes in the revised manuscript the figure numbers match their sequence of appearance in the text. We are convinced that the suggested order of figures is the most intuitive to the reader.***

8. Please shorten the figure legend it possible. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

***We shortened the legend of Figure 2. And added the following methodology to step 4.1.11:***

4.1.11) If high resistance while filling or agarose leaking from the tissue is observed, retry the whole procedure with a different bronchus from step 4.1.4. Troubleshooting can be performed as follows:

4.1.11.1) Note that the degree of agarose filling is highly dependent on the position of the catheter in the tissue and deep penetration of the catheter results in agarose filling of small cone like regions (\*) of the lung tissue (**Figure 2C**). In case of high resistance, try positioning of the catheter leads to proper filling of most regions of the tissue (#) (**Figure 2D**)

4.1.11.2) As plugs of early solidified agarose in the proximal bronchi or other airway obstructions (arrow) can lead to an incomplete filling of the tissue (**Figure 2E**), do not force agarose filling, this might lead to defects in the filled area, but not in a filling of the obstructed tissue parts.

4.1.11.3) Note that if the respiratory tree derived from the cannulated bronchus is damaged during resection and the agarose filling results in a constant leaking of the liquid agarose (arrow in **Figure 2F**). Here, insert the catheter into a more peripheral part of the airway system to fill at least a minor part of the tissue (\*) (**Figure 2G**). Additionally, seal the damaged peripheral airway with a surgical clamp (arrow) (**Figure 2H**).

9. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

***We rephrased the short abstract accordingly.***

10. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

***We rephrased the long abstract in order to clarify the goal of the protocol and to focus on the agarose filling of surgically resected tissue, as suggested by reviewer 3.***

11. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

***We revised the introduction, as we did with the long abstract.***

12. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

***We changed all SI abbreviations accordingly. We believe that using the non SI units “degree” (angle) and “degree Celsius” (temperature) instead of the SI abbreviations radian (angle) and Kelvin (temperature) makes the protocol more accessible for the general reader.***

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

***We formatted all units accordingly.***

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

***We changed this in the protocol part.***

15. 3.1, 3.1.1, 3.2, 3.3, 4.1.13, 5.1.3, 5.1.6, 7.1.2, 7.2.1, and 7.3.2.: Please revise the protocol to contain only action items that direct the reader to do something. 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.”

***We changed these expressions accordingly.***

16. 6.1.3: Please specify the cell culture medium used and the culture conditions.

***Culture medium has been specified in 1.1.1. To be even more clear, we referenced this step in 6.1.3.***

17. 7.3.2: Please spell out PFA.

***We changed step 7.3.1. and 7.3.2 to:***

7.3.1) For histological analyses, wash the PCLS and punches three times with PBS and fix them with 4% paraformaldehyde by incubating for 30 min at 37 °C. Finally, store the PCLS in PBS at 4 °C for further downstream staining.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.

***We changed this accordingly.***

19. There is a 2.75 page limit for filmable content. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

***We highlighted (yellow) 2.5 pages of the protocol from page 6 to 8.***

20. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. 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.

***We modified the protocol, so the highlighted text (yellow) contains actionable items in imperative tense only.***

21. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

***We referenced figure 1-3 in the protocol.***

22. References: Please do not abbreviate journal titles.

***We used the JoVE Citation Style for Endnote from the JoVE website.***

**Reviewer #1:**  
Manuscript Summary:  
This is a well-written protocol designed to allow researchers to generate precision cut lung slices from diseased lung resectates. The authors provide documentation of the steps from receipt to the start of experiments with this tissue. Importantly, the generation of these slices provides a substantial advantage to human lung research as the tissue is generated from the correct host in the correct site of disease. The applications are numerous as documented in the introduction. This reviewer does have a few questions for the authors.

We thank the reviewer for the valuable and constructive comments to our manuscript. Within the revised version, we addressed the questions of the reviewer, which we feel resulted in an improved manuscript.  
  
Major Concerns:  
1. The authors mention cold ischemia as, a problem for generation of slices; however, they do not mention reperfusion injury. Are there any specific interventions that one should consider regarding reperfusion injury (glutathione?)?

***Thank you very much for raising this important point. We agree that storage and ischemia time remain crucial in ex-vivo culture preparation. The tissue storage is based onhypothermic organ storage, which is decreasing the metabolic rate and energy requirement of the tissue, which altogether is focused on donor lung preservation1. However, our model clearly lacks vascular reperfusion. Therefore, we cannot relate tissue injury to the clinical reperfusion of lung injury.***

2. Have the authors attempted to improve cold ischemia by slightly increasing storage temps? Is there a better storage temp than 4 degrees?

***We did not attempt to do so yet due to limited amount of tissue available, however, we agree that this would be an important step to consider for future studies.***

3. The authors mention inspection and selection criteria prior to making PCLS; however, does this correlate with alive tissue once the slices are made? Are there other criteria that could be considered when thinking about whether the tissue is viable (i.e., ciliary motility, contractility of airways)?

***The tissue quality scoring is an attempt to apply objective criteria to accept or reject tissue for agarose filling. These criteria have derived from our accumulated experience over the years. Tissue that fail in agarose filling might otherwise be used for human tissue sampling and experiments, therefore it is an ethical imperative to us, to predict tissue performance in agarose filling. At this stage, the score is not suitable to be directly predictive for tissue viability. We have assessed tissue culture viability extensively in our system using metabolic assays (such as MTT-Assay, Calcein-Staining) as well as functional readouts, such as cilia movement and surfactant secretion 2.***

4. The authors mention that slices are only viable for 120hrs post-creation. Is this true? This reviewer has heard of other labs that are able to keep slices in culture for months.

***We have observed consistent viability of cultured PCLS for up to 120h post-creation2, however, we did not intend to imply that there is a general time limit for culture as the choice of media and media supplements might extend or alter persistence of the tissue’s native cell composition and viability2. We have adapted our manuscript accordingly.***

5. Do the authors find problems with contamination with fungus or yeast? If so, can they comment on treatment options and how they decide which slices get specific antibiotic/antimycotic interventions?

***The tissue is obtained surgically sterile and lung tissue with known bacterial and mycotic infections, such as pseudomonas, fungi etc is not used for PCLS. However, to further prevent potential contamination, the culture media is supplemented with penicillin and the broad-spectrum antibiotic streptomycin, as well as an antimycotic Amphotericin B as outlined in the manuscript (XX). In our hand bacterial or mycotic contamination did not occur.***

6. How long before experiments is FBS removed from the slices?

***The PCLS are continuously cultured in 0.1% FBS. FBS is removed for protein/RNA extraction by washing of the PCLS prior to snap-freezing.***

Minor Concerns:  
Please consider citing the following articles:  
Cooper, P. R., Lamb, R., Day, N. D., Branigan, P. J., Kajekar, R., San Mateo, L., … Panettieri, R. A. (2009). TLR3 activation stimulates cytokine secretion without altering agonist-induced human small airway contraction or relaxation. American Journal of Physiology-Lung Cellular and Molecular Physiology, 297(3), L530-L537.  
Kennedy, J. L., Koziol-White, C. J., Jeffus, S., Rettiganti, M. R., Fisher, P., Kurten, M., … Kurten, R. C. (2018). Effects of rhinovirus 39 infection on airway hyperresponsiveness to carbachol in human airways precision cut lung slices. The Journal of Allergy and Clinical Immunology.  
An SS, Wang WC, Koziol-White CJ, Ahn K, Lee DY, Kurten RC, et al. TAS2R activation promotes airway smooth muscle relaxation despite beta(2)-adrenergic receptor tachyphylaxis. Am J Physiol Lung Cell Mol Physiol 2012; 303:L304-11  
Cooper PR, Kurten RC, Zhang J, Nicholls DJ, Dainty IA, Panettieri RA. Formoterol and salmeterol induce a similar degree of beta2-adrenoceptor tolerance in human small airways but via different mechanisms. Br J Pharmacol 2011; 163:521-32.

**We thank the reviewer for bringing these interesting articles to our attention and have included the studies by Kennedy et al (2018) and Cooper et al (2011) to highlight the state-of-the-art work that has been performed in human PCLS with respect to modulation of human airway function.**

**Reviewer #2:**   
Manuscript Summary:  
The manuscript „Generation of human lung tissue slices for disease modeling by Gerckens et al. Deals with the preparation of human PCLS as versatile tool for various purposes. The use of human ex vivo tissues allows much better extrapolation to the human in vivo situation than the use animal tissue. Furthermore, this method successfully replaces animal experiments and is in line with current EU directives. The methods are thoroughly described and the methods for tissue slicing i.e. Krumdieck Slicer and vibratome are well balanced. Analysis of RNA, immunofluorescence and the use of fibrotic cocktails allow a plethora of application. It was a pleasure to read the manuscript and I can recommend publication without any changes.  
***We thank the reviewer for the positive feedback***

**Reviewer #3:**  
Manuscript Summary:  
The work by Gerckens et al is an important contribution to lung biology. Specifically, this work contributes to emerging knowledge regarding preparation, storage, and utility of human lung slices. The work should therefore be well received and highly cited. However, the general techniques of preparing human lung slices from donated lung have been previously published by multiple groups; the authors should make this clear and avoid the repetitive description, such as slicing the lung tissue. Instead, the distinguishable advantage of the presented technique should be emphasized, which is to prepare slices from surgically resected lung sample so that the accessibility to diseased lung is significantly increased. On the same token, the challenge to implementing the technique should also be clearly pointed out, i.e. to retain the agarose during the lung inflation when the pleural membrane was not intact any more.  
We thank the reviewer for the insightful and thoughtful comments to our manuscript. We agree with the important points to clarify the unique focus of this protocol and have adapted our manuscript accordingly.  
Major Concerns:  
1. Ideally, the lung tissue samples should have an intact pleural surface or surgically sutured pleural membrane around the tissue except the resection surface, otherwise it is extremely hard to maintain the sealing during inflation. It is hard to interpret "intact pleural surface at a least two sides of the tissue" in the manuscript (how many sides are we expecting here?).

***This is a good point, the wording we used was misleading. We clarified that we refer to an intact pleural surface of the tissue piece. We changed the sentence in the Lung Agarose Filling Score accordingly.***

2. There is also confusion regarding the filling process as it pertains to multiple bronchial openings on the section surface. Our personal experience is to cannulate all of the big openings, instead of doing one by one, for 2 reasons: 1) leaking can be from any of the airway openings and there is no way you will know that before injecting agarose; 2) Once the tissue is partially inflated, the tissue distortion and the concern of agarose leakage from the inflated part make very difficult to cannulate some other airways to inflate the lung. Can the authors please clarify.

**We agree that the occlusion of the other airways in the tissue is beneficial to prevent agarose leaking, possible through collateral ventilation within the tissue. However, in our experience the cannulation of a second or third airway after filling of the first airway does not represent a problem. In small tissue parts however the cannulation of several airways in the first place can hamper the overview over the tissue and tissue manipulation. Therefore, we added a step to the protocol recommending the occlusion of the other airways without prior cannulation.**

4.1.7) Seal the bronchus around the cannula by compressing the bronchial wall around the cannula with a forceps, ideally clamping any adjacent pulmonary artery at the same time.

4.1.8) Occlude other additional airways with a surgical clamp to prevent agarose leaking through these airways.

3. The authors should show the slices that contain the airways, not only because lung slices are usually used to study airways diseases, but also because it reflects the quality of a good preparation.

**We agree that airways are an important anatomical structure studied in PCLS, however the manuscript focuses on studies of lung alveolar parenchyma. We have previously published micrographs of small airway containing PCLS in a cited publication3. Here is an example from this research article:**

**F:\collagen snapshots\COL_CC_120_DAPI_003.tif**

**This micrograph shows a PCLS punch after 120h with Collagen type 1 immunofluorescence staining (yellow), DAPI nuclei staining (blue). In the upper quarter of the punch, an airway is visible.**

4. It is noted that 3% agarose is used in preparing the slices. This concentration is quite high which make it easy to solidify when temperature dropped and may cause problem while filling small airways. The authors should also consider using lower concentration when inflating COPD lung sample. Furthermore, lower concentrations will enable better characterization of airway narrowing studies.

***We thank the reviewer for this valuable suggestion and agree that titration of agarose concentration is an important issue. Low concentrations of agarose will prolong the solidification process, lower the melting temperature of agarose and result in less rigid agarose filled tissue, as higher concentrations result in the opposite. In our experience obstruction of airways by early solidified agarose clots occurs in very large tissue parts only. As the reviewer noted, we describe filling of smaller tissue parts, here we found 3% (w/v) agarose concentration optimal. In personal communication with other research groups in the field, we found consistently that higher concentrations of agarose clearly facilitated slicing with the vibratome, however, we agree that lower concentration could be beneficial for agarose filling of emphysematous lungs.***

***We added the following sentence to the discussion:***

Emphysematous tissues as found in diseases such as COPD or alpha-anti-trypsin deficiency might not withstand the pressure of agarose filling, and thus will result in rupture of the alveoli and tissue architecture artefacts. Here, the usage of a lower agarose concentration, e.g. 1% (w/v) might prevent this problem by decreasing pressure and speed during agarose filling.

**Reviewer #4:**  
Minor Concerns:  
2.1: If the tissue is stored at 4°C and submersed in ice-cold medium, the question arises whether this would impair the filling process with the agarose solution by causing premature polymerization of the polymer within the cooled tissue. Additionally to this: Is the excess media that might still reside within the tissue after submerse storage drained? If not, isn't this risking additional dilution of the introduced agarose solution by an unknown dilution factor (albeit potentially small) just like excess blood would as depicted in the inclusion criteria for tissue?

***We thank the reviewer for bringing up this important point. Excess media drains spontaneously from the tissue when lifting it from the medium and placing it on the cell culture dish. To clarify this for readers we added the following sentence to the protocol:***

4.1.1) Lift the tissue from the storage medium and drain excess media from the tissue. Transfer the lung tissue into the 15 cm culture dish prepared in 1.1.2.

***The problem of premature agarose solidification in the 4°C cold tissue seems plausible, however our experience shows that filling at an appropriate pace prevents this problem. The excess media is not explicitly drained to avoid tissue trauma (squeezing, additional atelectasis), however when taking the tissue out of the media, minor amounts of media drain spontaneously from the airways. Excess of blood in our opinion is less a problem due to dilution of the agarose solution but rather a sign of tissue trauma during surgery and the introduction of tissue hematoma. Tissue hematoma compromise tissue and pleural integrity and are therefore a predictor of “bad” agarose filling performance.***

4.1.9: As agarose introduction is described in great detail, is it controlled manually or through a pump? How is the speed of agarose introduction controlled?

***This is also a very important point, which we addressed in the revised version. Agarose application is controlled by hand. While a pump would be ideal for an automated process, we strongly feel that manual filling of the tissue allows for a better assessment of each individual tissue section. This is of particular importance as human tissue from lung resection exhibits a high degree of heterogeneity and thus needs adaptation as outlined in this protocol. For example, if very low resistance is observed when filling with the syringe, this is a strong hint for a leak in the tissue. High resistance could be a sign for wrong anatomical localization of the cannula. To clarify this is the protocol we changes step 4.1.10:***

4.1.10) Manually pour the agarose with the syringe not faster than 0.3 mL/s. (Note: speed of agarose filling might vary between approximately 0.05 and 0.3 mL/s due to the heterogeneous resistance of airways and/or atelectasis.

5.1.3: Has the degree of diffusion of the glue been checked? Are there changes in viability or responsiveness to treatments when comparing more "central" and more "subpleural" slices generated from a single tissue block despite discarding the last 2-3 mm of the tissue as described in 5.1.6?

***We are using cyanoacrylate-based glues that are commonly used in surgery and have not systematically analyzed potential diffusion. Cyanoacrylate polymerizes within seconds and the polymerized cyanoacrylate becomes inert.***

PCLS cultivation: Has there been any signs of fibroblast outgrowth/differentiation due to usage of (albeit minor) concentrations of serum in the culture medium?

***This is an important point. Fibroblast outgrowth is certainly a concern, when using serum concentrations in ex-vivo culture and we have observed an increase in fibroblasts when using higher concentrations of FBS and this is much reduced in our current protocol, in which we use 0.1% FBS to still support cell survival2, but prevent significant fibroblast outgrowth and overgrowth.***

Literature:

1 de Perrot, M., Liu, M., Waddell, T. K. & Keshavjee, S. Ischemia-reperfusion-induced lung injury. *Am J Respir Crit Care Med.* **167** (4), 490-511, (2003).

2 Uhl, F. E. *et al.* Preclinical validation and imaging of Wnt-induced repair in human 3D lung tissue cultures. *Eur Respir J.* **46** (4), 1150-1166, (2015).

3 Alsafadi, H. N. et al. An ex vivo model to induce early fibrosis-like changes in human precision-cut lung slices. Am J Physiol Lung Cell Mol Physiol. 312 (6), L896-L902, (2017).