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TITLE:

Precision Cut Lung Slices As an Efficient Tool for *Ex Vivo* Pulmonary Vessel Structure and Contractility Studies

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SUMMARY:

Presented here is a protocol for preserving the vascular contractility of PCLS murine lung tissue, resulting in a sophisticated three-dimensional image of the pulmonary vasculature and airway, which can be preserved for up to 10 days that is susceptible to numerous procedures.

ABSTRACT:

The visualization of murine lung tissue provides valuable structural and cellular information regarding the underlying airway and vasculature. However, the preservation of pulmonary vessels that truly represents physiological conditions still presents challenges. In addition, the delicate configuration of murine lungs result in technical challenges preparing samples for high-quality images that preserve both cellular composition and architecture. Similarly, cellular contractility assays can be performed to study the potential of cells to respond to vasoconstrictors *in vitro*, but these assays do not reproduce the complex environment of the intact lung. In contrast to these technical issues, the precision-cut lung slice (PCLS) method can be applied as an efficient alternative to visualize lung tissue in three dimensions without regional bias and serve as a live surrogate contractility model for up to 10 days. Tissue prepared using PCLS has preserved structure and spatial orientation, making it ideal to study disease processes *ex vivo*. The location of endogenous tdTomato-labeled cells in PCLS harvested from an inducible tdTomato reporter murine model can be successfully visualized by confocal microscopy. After exposure to vasoconstrictors, PCLS demonstrates the preservation of both vessel contractility and lung structure, which can be captured by a time-lapse module. In combination with the other procedures, such as western blot and RNA analysis, PCLS can contribute to the comprehensive understanding of signaling cascades that underlie a wide variety of disorders and lead to a better understanding of the pathophysiology in pulmonary vascular diseases.

INTRODUCTION:

Advances in the preparation and imaging of lung tissue that preserves cellular components without sacrificing anatomical structure provide a detailed understanding of pulmonary diseases. The ability to identify proteins, RNA, and other biological compounds while maintaining physiological structure offers vital information on the spatial arrangement of cells that can broaden the understanding of the pathophysiology in numerous pulmonary diseases. These detailed images can lead to a better understanding of pulmonary vascular diseases, such as pulmonary artery hypertension, when applied to animal models, potentially leading to improved therapeutic strategies.

Despite advances in technology, obtaining high-quality images of murine lung tissue remains a challenge. The respiratory cycle is driven by a negative intrathoracic pressure generated during inhalation[1]. When traditionally obtaining biopsies and preparing lung samples for imaging, the negative pressure gradient is lost resulting in the collapse of the airway and vasculature, which no longer represents itself in its present state. To achieve realistic images reflective of current conditions, the pulmonary airways must be reinflated, and the vasculature perfused, changing the dynamic lung into a static fixture. The application of these distinct techniques allows preservation of structural integrity, pulmonary vasculature, and cellular components, including immune cells such as macrophages, allowing lung tissue to be viewed as close to its physiological state as possible.

Precision cut lung slicing (PCLS) is an ideal tool for studying the anatomy and physiology of pulmonary vasculature[2]. PCLS provides detailed imaging of the lung tissue in three dimensions while preserving structural and cellular components. PCLS has been used in animal and human models to allow for live, high-resolution images of cellular functions in three dimensions, making it an ideal tool to study potential therapeutic targets, measure small airway contraction and study the pathophysiology of chronic lung diseases such as COPD, ILD, and lung cancer[3]. Using similar techniques, the exposure of PCLS samples to vasoconstrictors can preserve lung structure and vessel contractility, replicating *in vitro* conditions. Along with preserving contractility, prepared samples can undergo additional analysis such as RNA sequencing, Western blot, and flow cytometry when prepared correctly. Finally, reporter color labeled cells marked with tdTomato fluorescence after lung harvest can preserve labeling after preparing microslices, making it ideal for cell tracking studies. The integration of these techniques provides a sophisticated model preserving the spatial arrangement of cells and vessel contractility that can lead to a more detailed understanding of the signaling cascades and potential therapeutic options in pulmonary vasculature disease.

In this manuscript, PCLS murine lung tissue is exposed to vasoconstrictors, demonstrating preserved structural integrity and vessel contractility. The study demonstrates that the tissue prepared and handled appropriately can remain viable for 10 days. The study also demonstrates the preservation of cells with endogenous fluorescence (tdTomato), allowing samples to provide high-resolution images of the pulmonary vasculature and architecture. Finally, ways to handle and prepare tissue slices for RNA measurement and Western blot to investigate underlying mechanisms have been described.

PROTOCOL:

All animal care was in accordance with the guidelines of Boston Children's Hospital and the Institutional Animal Care and Use Committee approved protocols. The mice used in this study are wild type C57/B6 mice and Cdh5-CreERT2 x Ai14 tdTomato crossed mice.

1. Preparation of solutions

1.1. Prepare phosphate buffer solution (1x PBS) and 2% agarose solution required during the experiment in advance.

1.1.1. Mix 2 g agarose powder into 100 mL of autoclaved water. Heat it in the microwave a few seconds at a time until the solution is clear.

1.1.2. Place the solution in a water bath at 42 °C until use.

NOTE: Both PBS and 2% agarose can be stored at room temperature for weeks.

2. Extraction of the mouse lung

2.1. Euthanize the mouse by isoflurane overdose. Achieve isoflurane overdose by placing a small amount of isoflurane on tissue paper in the lower level and placing the mouse in the upper level in a desiccator. The mouse remains in the desiccator until unconscious.

2.2. Ensure death of the mouse by applying inferior pressure on the neck and pulling caudal on the tail. Bring the mouse to the dissecting surface.

2.3. Place the mouse in the supine position and secure it into place taping paws and nose to table with adhesive tape.

2.4. Spray the ventral surface of the mouse with 70% ethanol and wipe off the excess ethanol.

2.5. Lift the abdominal skin of the mouse with forceps at the location of the bladder and cut at the midline with surgical scissors, moving superiorly to the cervical region, exposing the trachea.

2.6. Lift the underlying fascial layer with tweezers and cut with surgical scissors to expose visceral organs and mediastinal compartment, again cutting inferiorly to superior.

2.7. Cut the sternum at the midline to expose the heart and the lung.

2.8. Using blunt dissection, detach the diaphragm from the liver and abdominal compartment.

2.9. Locate the inferior vena cava (IVC) under the intestines and cut the IVC.

2.10. Locate the right ventricle.

NOTE: The right ventricle will have a lighter appearance compared to the left ventricle and the intraventricular septum should be visualized, separating the right ventricle from the left ventricle.

2.11. Inject 0.5 mL of 1% fractionated heparin to the right ventricle with a 25–30 G butterfly needle and then flush slowly but steadily with 10–15 mL of 1x PBS (**Figure 1A**).

2.11.1. Use caution not to insert the needle through the heart and inject heparin into the mediastinal cavity.

NOTE: The injection of 1x PBS turns the lung tissue white. The fluid extravasating from the abdominal aorta should be clear and colorless and the liver may become white in appearance after successful flushing.

2.12. Locate the larynx and dissect the surrounding fascia and tissue using blunt tip tweezers.

2.13. Place the surgical suture under the trachea and loosely tie a surgical knot.

2.14. Place a small hole in the trachea superior to string and canulate with 20 G blunt-ended needle.

2.15. Tighten the suture around the needle and trachea using a surgical knot.

2.16. Inject 2.5–4 mL of agarose into the trachea and monitor for inflation of the lung.

2.17. After agarose is instilled, pour cold, chilled 1x PBS over the lung to solidify agarose.

2.18. Cut the trachea superior to surgical suture and dissect the lung and heart from the mediastinum by removing any adhesions and fascia.

2.19. After solidification, place in 1x DMEM (enough to submerge tissue) in a Petri dish to keep on ice. Immediately slice one lobe using a vibratome machine (**Figure 1B**).

3. Precision cut lung slices

3.1. Attach the sample to the platform with superglue, keeping the medial side of the sample facing up.

3.2. Fill the sample container with 1x PBS, ensuring that the sample is completely submerged.

3.3. Fill the container surrounding the sample container with ice to keep surrounding PBS and sample cold.

3.4. Turn on the vibratome and adjust to the desired settings below.

3.4.1. Set the thickness to 300um.

3.4.2. Set the frequency to 100 Hz.

3.4.3. Set the amplitude to 0.6 mm.

3.4.4. Set the speed to 5 µm/s.

3.5. Using an Allen wrench, turn the blade holder into the safe position and open the jaws to insert a blade.

3.6. Tighten the jaws with an Allen wrench and turn the blade holder into the appropriate position for slicing.

3.7. Place the sample and the platform onto the box and slide in front of the blade.

3.8. Fill the box around the sample platform with 1x PBS until the sample is submerged.

3.9. Bring the vibratome blade up to the edge of the block and manually lower the blade until it is even with the top of the sample.

3.10. Confirm the appropriate settings and run the vibratome (**Figure 2**).

3.11. As fresh slices are cut, remove the samples from the PBS and place them into a sterile Petri dish with 10 mL of 1x DMEM containing 1x antibiotic-antimycotic.

3.12. When finished, retract the blade entirely and then use the Allen wrench to raise the blade into a safe position.

3.13. Store the samples in 1x DMEM in an incubator at 37 °C with viability preserved for up to 10 days (see viability experiment below).

4. Example vasoconstrictor experiment

4.1. Place a vibratome slice on a microscope slide.

4.2. Before placing it on the slide, use a cleaning wipe to get rid of the excess medium (PBS).

4.3. Place the sample under phase-contrast microscopy and use 10–20x magnification to identify a vessel.

4.4. Put 500 µL of vasoconstrictors, such as 60 mM KCl or 1 µM Endothelin-1 to completely submerge the slide.

4.5. Observe and capture the video by recording for 30 s–60 s (**Video 1**).

5. Preparing the tissue for RNA or protein lysis on PCLS

5.1. Before beginning the experiment, fill a small polystyrene foam box with 1 L of liquid nitrogen.

5.2. Place two small microcentrifuge tubes (1.5 mL) in the container with liquid nitrogen before starting.

NOTE: Liquid nitrogen should cool outside of the tubes, however, not be inside. Be sure to handle liquid nitrogen with care and do not expose the skin to liquid nitrogen. Use forceps and gloves to place and remove tubes from the box.

5.3. Place 4–5 fresh vibratome slices in a mortar bowl.

5.4. Pour 5–10 mL of liquid N2 on top of the slices in the mortar bowl.

5.5. Quickly use the pestle to grind the flash freeze slices into powder before liquid nitrogen evaporates.

NOTE: Tissue should condense into a fine powder. If not, add additional liquid nitrogen until achieved.

5.6. Using a steel laboratory scooper (prechilled with liquid nitrogen), scoop the powder into the two chilled microcentrifuge tubes.

5.7. Lyse the powder by adding 500 µL of RNA extraction reagent to proceed with RNA isolation or 500 µL of RIPA buffer (contains 1x protease inhibitors) to proceed with protein lysis.

5.8. Store the samples at -80 °C until additional RNA isolation, BCA measurement, and western blot.

6. Determining viability

6.1. After vibratome preparation, place two lung slices into a 24-well culture plate with 450 µL of DMEM media and 50 µL of cell viability reagent (ensure that the tissue is submerged entirely).

6.2. Place the samples back into 5% CO_2 at 37 °C incubator overnight with minimal exposure to light.

6.3. In the morning, observe the solution for color change. The blue solution turns pink when the tissue is viable.

7. Preservation of cell labeling

7.1. Treat a transgenic Cdh5-CreERT2 crossed with Ai14 tdTomato mouse with an IP injection of Tamoxifen (2 mg/day) for a total of 5 days (total dose 10 mg Tamoxifen) to induce tdTomato label Cre positive cells.

7.2. One week after injection, prepare the lungs using the above steps 1 to 3.

7.3. After preparation of the precision-cut lung slice, place the tissue onto a microscope slide and place a coverslip on top of the tissue.

7.4. Use a confocal microscope to detect tdTomato staining (using excitation wavelength 555 nm).

REPRESENTATIVE RESULTS:

When added to cells or tissue, the viability reagent is modified by the reducing environment of viable tissue and turns pink/red, becoming highly fluorescent. The representative color changes detected from day 0–1 and day 9–10 are demonstrated in **Figure 3**. As noted, the solution started blue and turned pink overnight, demonstrating viability. Color change typically occurs within 1–4 h; however, a longer time may be necessary. To assay for viability, a plate reader was used to determine the absorbance of a vibratome-prepared sample and a 4% PFA fixed lung slice, serving as a control. Solutions in which samples were incubated were read at an absorbance of 562 nm and 630 nm following the manufacturer's recommendation. The daily difference between absorbance at 562 nm and 630 nm wavelength for the vibratome-prepared tissue and comparison to the control tissue are shown in **Figure 3**.

To demonstrate contractility of vibratome-prepared lung tissue, a fresh piece of tissue was placed under the bright field microscope at 400x and treated using KCL or Endothelian-1 until the tissue was submerged. A video taken over 30–60 s of the tissue reveals constriction of the vasculature (**Video 1**).

To demonstrate preservation of cell labeling 1 week after tamoxifen injection, the lung slices obtained after vibratome sectioning using the above protocol were observed under a confocal microscope. The tdTomato labeling in the lung slices is demonstrated in **Figure 4**.

FIGURE AND TABLE LEGENDS:

Figure 1: Murine lung tissue preparation for vibratome sectioning. (**A**) The RV is cannulated with a 30G butterfly needle to facilitate switching solutions from Heparin to PBS. (**B**) The whole lung lobes after agarose inflation

Figure 2: Lung slices using vibratome sectioning. Thickness: 300 µm.

Figure 3: Viability experiment for PCLS slices from Day 1 to Day 10. (**A**) Representative images of reagent color change of PCLS-prepared tissue versus 4% PFA-fixed tissue. Fresh PCLS tissue and 4% fixed PFA tissue were placed into 450 µL of tissue media and 50 µL of viability reagent. Solution color initially purple, as can be seen on Day 0 and Day 9. After incubating overnight, the solution containing freshly prepared PCLS tissues changed to purple/pink color (Day 1 and Day 10) due to the reducing environment of live tissues. (**B**) Quantification of PCLS viability from Day 1 to Day 10 by the ratio of the absorbance at 562 nm and 630 nm with a plate reader.

Figure 4: Demonstration of preserved tdTomato positive cells labeling after harvest. The PCLS preparation is from a Cdh5-tdT transgenic mouse. Scale bar = 20 µm

Table 1: Characteristic and uses of various vasoconstrictors. (*used in this study)

Video 1: Constriction of vessels using Endothelin-1 on PCLS. Wildtype C57/B6 mice are inflated with 1.5 mL of 1.5% agarose and sectioned into 130 µm using a vibratome. A fresh piece of tissue was placed under the bright field microscope at 400x and treated using KCL or Endothelian-1. Magnification: 400x.

DISCUSSION:

In this manuscript, an enhanced method to produce high-resolution images of murine lung tissue that preserves the vascular structure and optimizes experimental flexibility is described, specifically using the application of PCLS to obtain microslices of lung tissue that can be viewed in three dimensions with preserved contractility of the vasculature. Using the viability reagent, the protocol demonstrates that carefully prepared and preserved slices can retain viability for more than a week. Preserved viability of the microslices allows the possibility for multiple and prolonged experiments to be performed on vasculature and airway structures, making it an ideal sample to test the *ex vivo* response of multiple drugs, evaluate underlying molecular mechanisms, and test reagents. This makes prepared samples the ideal platform to study potential therapeutic effects on vascular tone prior to *in vivo* trials[4]. The preservation of the pulmonary airway and vasculature allows prepared samples to be treated with additional techniques such as contractility testing described in this article and summarized in **Table 1**. This allows for the opportunity to provide high-resolution images detailing the spatial arrangement of the pulmonary vasculature and signaling cascades, contributing to the pathogenesis of pulmonary vascular diseases.

The preparation of tissue microslices for experimentation is a technique that uses a vibratome, or vibrating blade, to produce precision-cut organotypic slices. This allows for increased accuracy and reproducibility compared to traditional techniques[5]. Microslices of the lungs obtained with

PCLS maintain the physiological structure of lung tissue to a cellular level, making it a useful tool to study a variety of pulmonary vascular diseases. The technology has been used in both animal and human models to study the complex lung anatomy and pulmonary pathology, with a specific focus on toxic exposures, infectious diseases, and immunological studies[6–8]. Depending on the underlying pathophysiology, microslices can be obtained of the specific lobes of interest or one/all lobes to compare disease heterogeneity. Slices too thin result in difficulty preserving structural integrity, while thicker slices may be more difficult to perform additional staining or deep in tissue imaging.

Specific to vascular disease, PCLS has been utilized to study pulmonary artery hypertension (PAH), allowing a sophisticated model to analyze the effects of potential therapies[9–11]. The preservation of vascular contractility in murine lung samples has numerous therapeutic and investigational benefits. By incorporating a technique preserving both the structure and contractility of the pulmonary vasculature, prepared samples can model pulmonary vascular disease *ex vivo*. This has been demonstrated in the past by Rieg et al., who with PCLS samples were able to demonstrate that the pulmonary veins were more sensitive with a1-agonists and b2-agonists, suggesting the use of these medications in left heart failure may cause increased pulmonary edema[12]. Using this model to study the effects of drugs, *ex vivo* helps identify the potential therapeutic agents for possible clinical trials. In this manuscript, the protocol describes methods to handle and store the tissue with viability preserved for 10 days, allowing the monitoring of tissues after potential interventions and offers greater experimental flexibility. Previous studies have applied similar methods to preserve airway contractility in asthma models, with the IL-13 effect persisting for up to 15 days[3]. Immunostaining RNA and cytokines can lead to a better understanding of the underlying pathogenesis responsible for disease progression and development in various vascular diseases. The prepared samples can retain cell labeling after preparation, demonstrated in this manuscript with the preservation of tdTomato labeled Cdh5+ endothelial cells. The preservation of such labeling in vibratome-prepared samples allows the structure to be preserved along with cell labeling, making it an ideal tool to perform cell tracking experiments. Finally, the prepared samples are susceptible to further investigation such as RNA lysis or western blot described in this protocol to further study the underlying pathophysiology and mechanisms causing disease progression.

Despite its advantages to other techniques, there are limitations to PCLS-prepared tissue. Performing PCLS turns the dynamic lung into a static fixture, providing a snapshot in time of the cells and molecules present within lung tissue at the time of biopsy. Unless numerous samples are obtained, it does not account for the vast heterogeneity commonly seen in many pulmonary diseases such as chronic obstructive pulmonary disease (COPD). Tissues prepared with PCLS are mainly limited to small airway and vessel disease. This is due to the technical challenges with tissue harvesting, as the trachea is typically incised for lung inflation and identifying and isolating large bronchi is challenging. These technical limitations make using PCLS tissue suboptimal to study larger bronchi and the conducting airways. Dynamic testing is also limited, making it a less than ideal platform to study ventilator-associated lung disease and barotrauma. Finally, the application of PCLS to the field of pulmonology has focused primarily on murine lung tissue, and

further testing and protocols are needed before being applied to human tissue in a clinically relevant scenario.

In summary, the manuscript provides a complementary method preserving the vascular contractility of PCLS murine lung tissue, resulting in high-resolution images of the pulmonary vasculature for up to 10 days that contains endogenous fluorescent cells and numerous other procedures.

[Place **Table 1** here]

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DISCLOSURE:

The authors have no conflicts of interest to disclose.

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Figure 1

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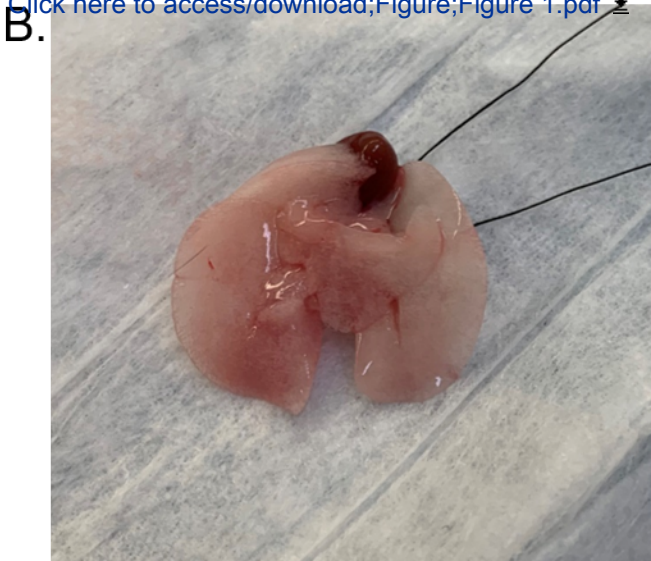

Figure 2

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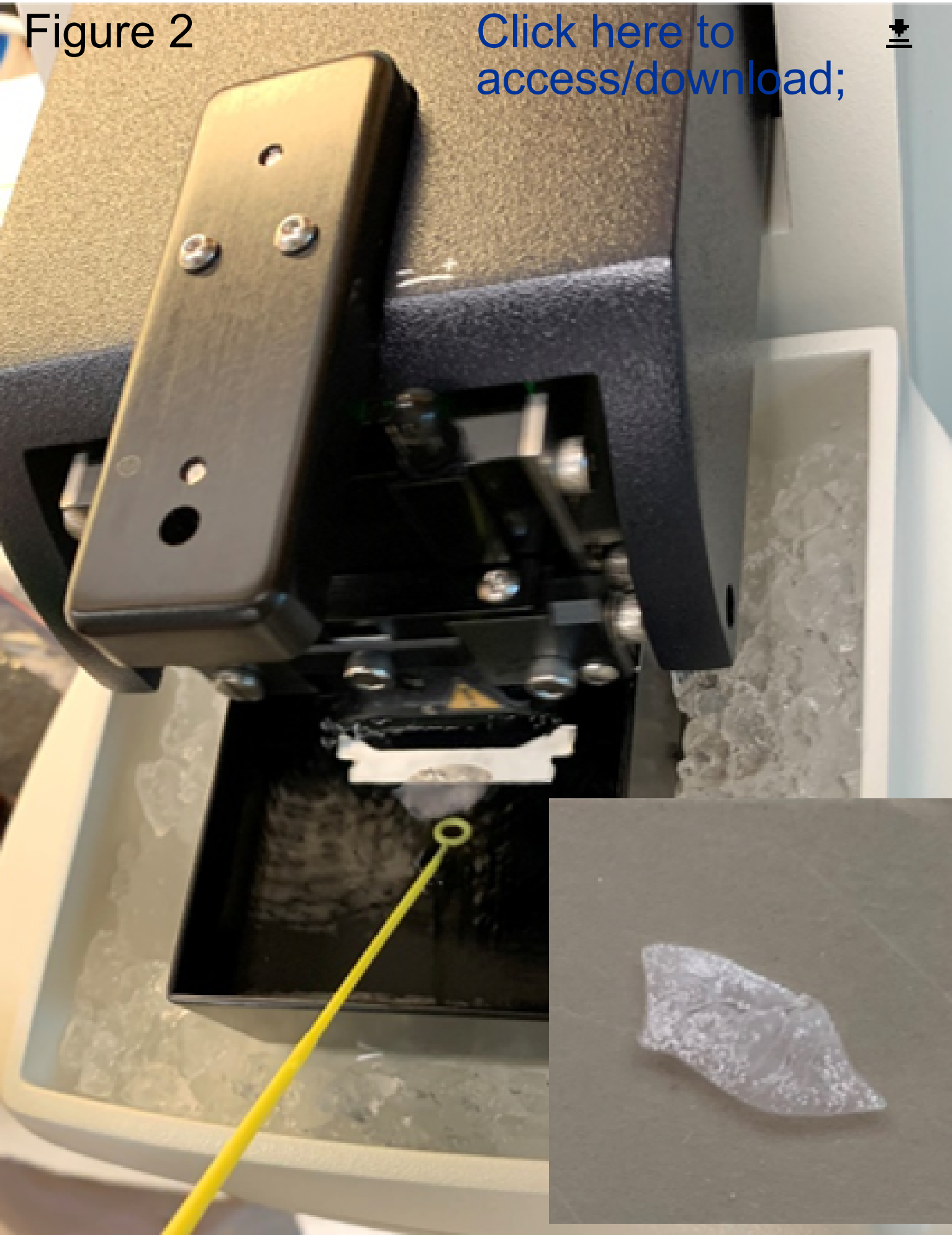
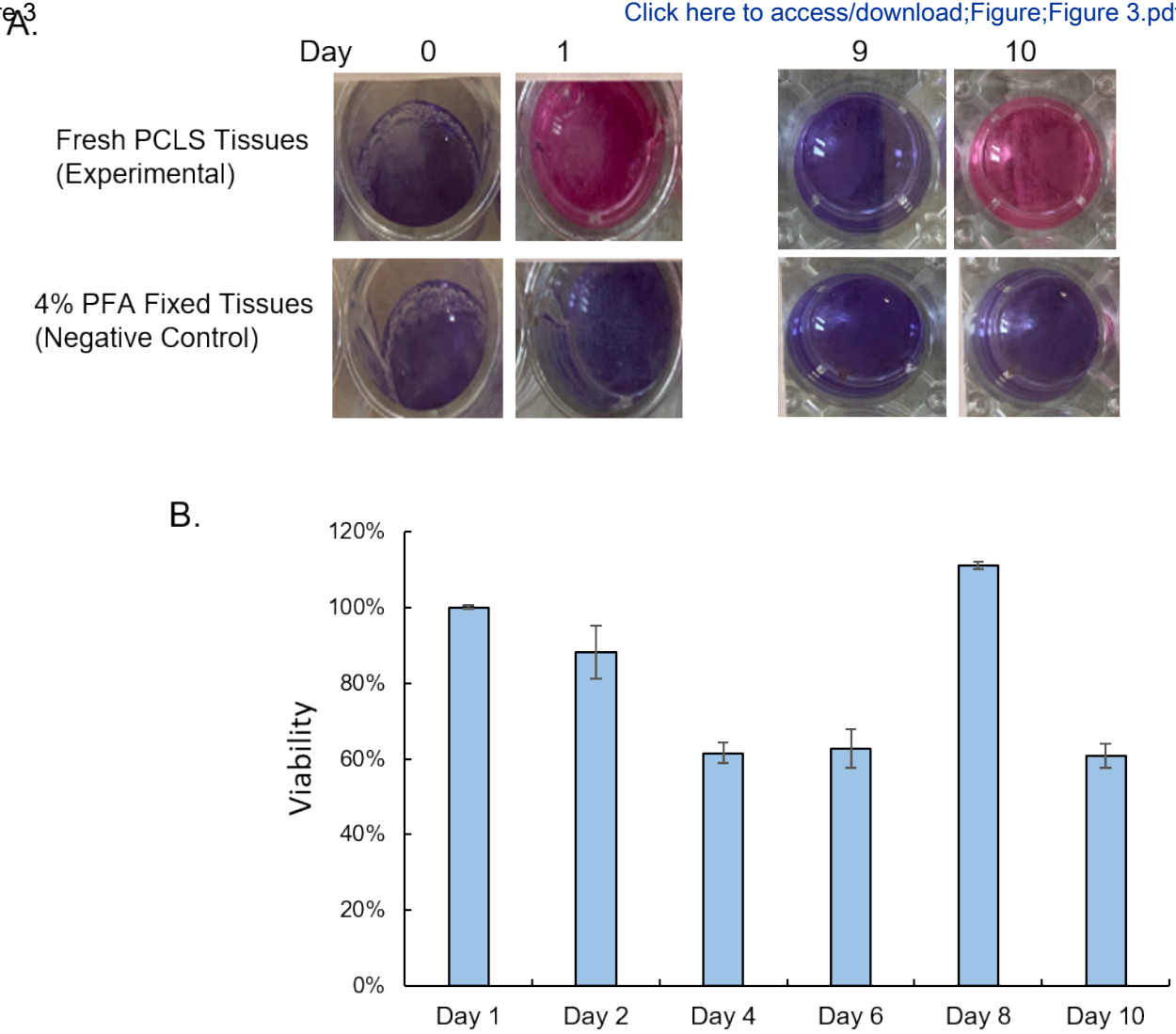
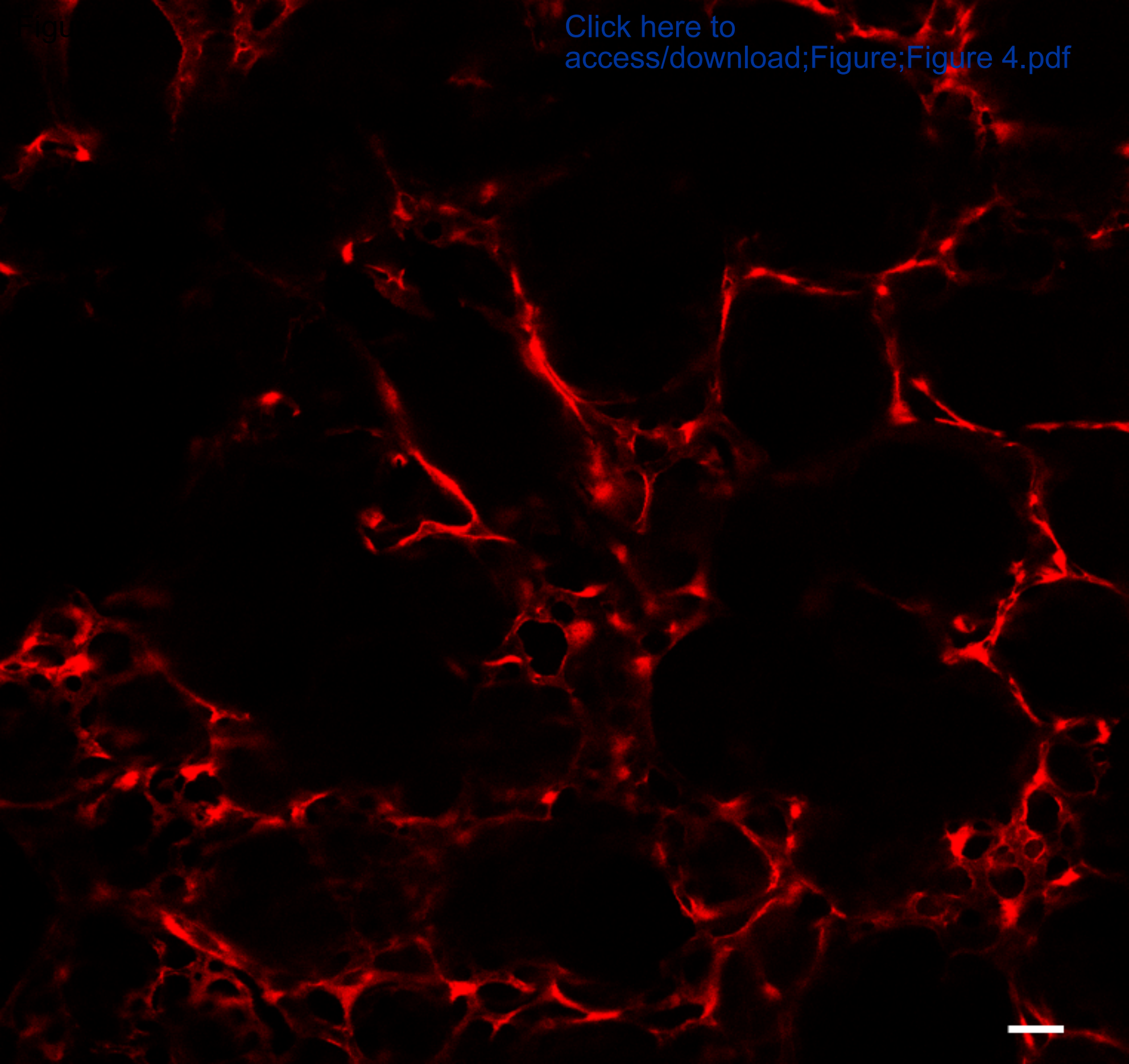


Figure 3





Video or Animated Figure

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With Endothelin-1

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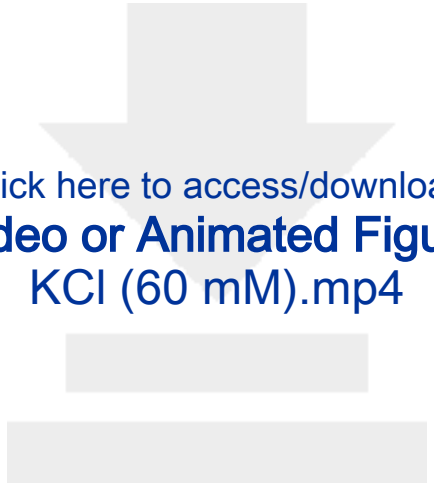
with KCl



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Control without vasoconstrictor

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Table 1

Vasoconstrictor	Dosages	Expected response and usages	REF
Acetylcholine	0.1~10 μM	- Airway contraction with reduced lumen area by 60% within 2 min. -After 5 min, the average decreased airway luminal area was 57%. - Upon washout, the airway relaxed to ~90% of its initial size within 3 min. - The arterioles had no contractile response.	[13]
5-hydroxytryptamine	0.1~10 μM	- Induce contraction of both the airway and arteriole. - Reduces the luminal area by 41% after 5 min, and arteriole up to 80% within 2 min. - Upon washout, the airway relaxed quickly, whereas the arteriole relaxation occurs more slowly.	[14]
KCl*	10~100 mM	- A large reduction of the arterial lumen - A small reduction of the airway lumen - After 5 min, a luminal reduction in airways of 14% and arterioles of 64%. - Removal resulted in arteriole and airway relaxation although some airways continued to display transient contractions. - Mostly used as a positive control in research.	[15-18]
Methacholine	0.2~10 μM	- Induces a sustained concentration-dependent contraction of the airway without a change in the $[Ca^{2+}]$. - The airway displayed a large reduction in lumen area in 5 min - Removal of Methacholine, the $[Ca^{2+}]$ oscillations stop and the airways immediately relax.	[3, 19]
Endothelin-1*	1 nM~1 μM	-A potent powerful endogenous vasoconstrictor, mainly secreted by endothelial cells - Vasoconstricting both the afferent and efferent arterioles. - Submucosal arterioles have a high affinity. - Constriction occurs up to 79%.	[20, 21]
Angiotensin II	0.1~10 nM	- Induces a significantly blunted vasoconstriction in PI3Kγ-deficient vessels - Acts systemically to cause vasoconstriction as well as constriction of both the afferent and efferent arterioles of the glomerulus.	[22, 23]

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
0.5cc of fractionated heparin in syringe	BD	100 USP units per mL
1X PBS	Corning	21-040-CM
20 1/2 inch gauge blunt end needle for trachea cannulation	Cml Supply	90120050D
30cc syringe	BD	309650
Anti Anti solution	Gibco	15240096
Automated vibrating blade microtome	Leica	VT1200S
Cell Viability Reagent (alamarBlue)	Thermofisher	DAL1025
Confocal	Zeiss	880
Dulbecco's Modified Eagle Medium and GLutaMAX, supplemented with 10% FBS, 1% Pen/Strep	Gibco	10569-010
Endothelin-1	Sigma	E7764
KCl	Sigma	7447-40-7
Mortar and Pestle	Amazon	
RIPA lysis and extraction buffer	Thermoscientific	89900
Surgical suture 6/0	FST	18020-60
TRIzol Reagent	Invitrogen, Thermofisher	15596026
UltraPure Low Melting Point Agarose	Invitrogen	16520050
Vibratome	Leica Biosystems	VT1200 S
Winged blood collection set (Butterfly needle) 25-30G	BD	25-30G

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues (Lines: 35-37, 330-332).
    - Thank you for taking the time to review our manuscript. We have read the manuscript and changed the above grammatical errors.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. We have removed all the commercial language from your manuscript and used generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
    - Thank you for taking the time to remove these symbols from the manuscript.

3. Line 96-121: Please remove step 1 and move the items listed to the Table of Materials. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.
    - Thank you for the suggestion. We have added the name, company and catalog number to all materials and created a Table.

4. Line 98: Please mention the strain of the mice used for the study.
    - Thank you very much for reviewing the manuscript. We have updated the materials table to include the strain of mice as well as the manuscript.

5. Line 137: Please mention how was the anesthetic induced? What was the dose used to euthanize the animal?
    - Thank you for the feedback. We have added details to expand how euthanize was performed.

6. Line 192-193: Please specify the volume of DMEM. Should the tissue be submerged in DMEM?
    - Thank you for the feedback. We have ensured the volume of DMEM is in the manuscript and elaborated that tissue should be submerged.

7. Line 249: Please specify the volume of the vasoconstrictors used. Please check if the units mentioned for the concentration are correct (i.e., 60 mM KCl or 1 µM Endothelin-1)
    - Thank you for the feedback. We have added the volume of 500 µL of either vasoconstrictors in the manuscript.

8. Line 252: Please elaborate on the imaging steps. How were the images captured? What was the time interval between each snap?

- Thank you for the feedback. We used an iPhone to capture the video by a Recording mode.

9. Line 274: Is the liquid nitrogen in the microcentrifuge tube discarded before adding the powder.
    - The liquid nitrogen should only chill the tubes, not be inside. WE agree that this is confusing the way it is worded and have addended the directions to make clearer. Thank you for bringing this to our attention.

10. Line 284-288: Please move the steps of the viability experiment to the protocol section and explain the results in the Representative Results section.
    - Thank you for this suggestion. We have separated viability experiment into the representative results section and protocol section.

11. Line 299- 303: Please include the detailed experimental steps for the preservation of cell labeling in the Protocol section.
    - Thank you, we have added a section on the steps used to demonstrate preservation of cell labeling in the protocol section.

12. Please discuss the results of vasoconstrictor experiment in the Representative results section.
    - Thank you for the feedback and reviewing our manuscript. We have added the vasoconstrictor experiment to the representative results.

13. Please remove the embedded table (Table 1) from the manuscript. Upload the table as a separate file to your Editorial Manager account in the form of an .xls or .xlsx file.
    - Thank you very much for the suggestion. The table has been uploaded as an excel file. Since there are inserted references in the table, if the table is removed, the references will be deleted automatically by the Endnote. Could we leave the table in the Word file to maintain references in order? Then can you remove the table later? Sorry about this Endnote technical issue.

14. Please include an Acknowledgements section, containing any acknowledgments and all funding sources for this work.
    - Thank you very much. We have added an acknowledgment section.

15. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

- Thank you very much. We have added a disclosure section for the authors.

16. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).

- Sorry, could you instruct how to do so.

17. As the video files are uploaded separately, please include description about each video file in the Figure Legends section (As an example "Video 1" description is included in the Figure and Legends section of the formatted manuscript file). Please use the abbreviation "µM"for the micromolar abbreviation instead of the letter u. Please include a scale bar.

- We uploaded each video separately.

18. Figure 3: Please include a title for the y-axis. Is the representation of the graph correct? Day 8 on the bar graph indicates that the viability is over 100%. Can the viability be more than 100%?

- Thank you for this feedback. We have added a title for the Y axis, which is showing the ratio of absorbance between the experimental (PCLS tissue) and control tissue (4% PFA fixed tissue) on different days after harvest. On Day 8, the value is greater than 100% because the absorbance of the experimental tissue is higher than the control.

19. Please highlight up to 3 pages 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 have highlighted 3 pages of the protocol in yellow that most convey the protocol and should be available to the reader.