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**Editor Name: Linda DiBella**

**Videographer name: Matt Lenz**

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**Authors and Affiliations:**

Christina N. Blaul1,2, Jenna Balestrini3, Hongyi Pan4, Julia Winkler4, Erica L. Herzog4, Huanxing Sun4

1 NSF Funded Biomedical MD-PhD REU in affiliation with Yale School of Medicine, Yale Pathology Department, Section of Pulmonary, Critical Care, and Sleep Medicine, New Haven, CT

2 University of California, Merced, School of Natural Sciences: Molecular and Cellular Biology, Merced, CA

3 Department of Anesthesiology, Yale School of Medicine, New Haven, CT

4 Department of Internal Medicine, Section of Pulmonary, Critical Care, and Sleep Medicine, Yale School of Medicine, New Haven, CT

**OTHER AUTHORS EMAIL ADDRESSES:**

Christina N. Blaul: [cblaul@ucmerced.edu](mailto:cblaul@ucmerced.edu)

Jenna Balestrini: [jbalestrini@draper.com](mailto:jbalestrini@draper.com)

Hongyi Pan: [Hongyi.pan@yale.edu](mailto:Hongyi.pan@yale.edu)

Julia Winkler: [Julia.winkler@yale.edu](mailto:Julia.winkler@yale.edu)

Erica L. Herzog: [Erica.herzog@yale.edu](mailto:Erica.herzog@yale.edu%20)

**Title: Use of Decellularized Mammalian Lung Tissues to Study Cell: Matrix Interactions**

**Corresponding Author:**

Huanxing Sun

Email: [huanxing.sun@yale.edu](mailto:huanxing.sun@yale.edu)

**A.** Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **(Y/N)\_\_\_\_\_No\_\_\_\_**

Can you record movies/images using your own microscope camera? **(Y/N)\_\_\_\_\_\_\_\_\_**

If no, JoVE will need to record the microscope images using our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope: **\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**B.** Software Usage: Does your protocol include detailed, step-by-step, descriptions of software usage? **(Y/N)\_\_\_No\_\_\_\_\_**

**C.** Which steps of your protocol will viewers benefit most from having filmed? Please list 4-6 individual steps using the step numbers listed in this document. (Please do not list entire sections.) \_\_\_\_\_\_\_\_\_\_\_\_\_\_**2.3; 3.1; 3.3; 5.1; 5.3; 6.6**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Authors, please answer this question with the steps listed here in the protocol section for use by the videographer.

**D.** What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1-2 individual steps using the step numbers listed in this document. (Please do not list entire sections.) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Authors, please answer this question with the steps listed here in the protocol section for use by the videographer.

**E.** Will the filming need to take place in multiple locations? (Y/N) \_\_Yes\_\_\_\_\_ If yes, how far apart are the locations? \_\_\_Very close, usually no more than 5 min by walking.

**1. Introduction (Experimental Goal and Author Interviews)**

**A. Experimental Goal: (read by voice talent at JoVE)**

The overall goal of this experiment is to investigate cell:matrix interactions in the lung. **(Intro)**

**B. Required Interview Statements: (Said by you on camera. Don’t forget to smile!)**

* 1. Erica L. Herzog: This method can help answer key questions in the lung fibrosis field, such as how the lung ECM affects differentiation in fibrosis.
  2. Huanxing Sun: The main advantage of this technology is that it can be adapted for use in many types of laboratories and with very small amounts of starting material.

**E. Ethics title card: (for human subjects or animal work, does not count toward word length total)**

* 1. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Yale University. The use of human tissues and cells has been approved by Yale’s Human Investigation Committee (HIC).

**Protocol: (read by voice talent at JoVE)**

Videographer notes: Overall the shoot went fairly well today but was also very frustrating at times and i had a few more general notes and they are listed below.

General Notes

- This protocol had a very large number of shots in it today and the researcher Huanxing Sun was the only one helping out on it. I as there for almost 6 hours and she was trying to perform a "real experiemt" rather than staging with stock and fake solutions most of the time. Besides the fact that she was very nervous in front of the camera you could tell she had been there already for many hours getting the lab set up for the shoot and we were on a time crunch to finish before 2:30pm as thats when she needed to be done by because the rest of the lab was waiting or us to finish using the facilities there.

Because of this we did not break for lunch and She was visibly exhausted/hungry the second half of the day and really not listening to my directions and suggestions well throughout the shoot. The stuff we obtained was good but it definitely could have been better. In addition, I also am aware there was a large language barrier and although she kept nodding her head yes I when i asked her to change things she really didnt listen to any of my directions and questions. This is really seen in the placement and arrangement of dishes and tubes. This resulted in me moving the camera to reframe shots and follow action and she rarely listened to what i was suggesting while we were filming.

-Therefore numerous shots start either than we needed to and some end abruptly because when i asked her she would just agree and say yes event though i wasn't sure she understood anything i was asking most of the time. But just so the editor is aware everything we shot goes in the order of the protocol so I hope that helps.

1. **Preparation of Mouse Lung Slices**

Video editor: I have no idea how accurate the edits to the shotlist are. According to the videographer, it was a rough shoot but these notes seem pretty tame.

* 1. After performing a right ventricular perfusion with PBS in a mouse according to the text protocol, in a cell culture hood, remove the lungs *en bloc* **[1-WIDE]** and separate them into single lobes. Place each lobe into a 60-mm sterile Petri dish **[2-CU].**
     1. Talent is in cell culture hood places dish with lungs
     2. Talent separates lungs into single lobes and transfers to separate Petri dishes
  2. Transfer each Petri dish into a 100-mm Petri dish **[1-CU].** Then, to snap freeze the tissue, float the nested Petri dishes with the lung on the surface of liquid nitrogen in its container, ensuring that liquid nitrogen does not get into the 60-mm dish containing the tissue **[2-CU].**
     1. Talent nests the petri dishes
     2. Talent floats the nested dishes in a liquid nitrogen container without getting the liquid nitrogen in the 60 mm dish on the tissue
  3. Using a multi-knife and sterile technique, slice each lobe into 1 mm slices **[1-ECU].** Then use PBS with 1 μg/mL of sodium nitroprusside to rinse the slices for 1 h **[2-CU-TXT].**
     1. Talent slices lobe with a multi-knife, then separate the slices by scalpel and forceps.
     2. Talent adds slices to a 50ml conical tune with 25 ml PBS/SNP to ~~slices and~~ rinse the slices (TEXT: SNP, Refer to text protocol for additional details), Editor, use the ‘SNP’ text for the sodium nitroprusside

1. **Decellularization, Sterilization and Storage**
   1. Transfer the slices to a 15-mL tube with 5 mL of decellularization, or decell solution **[1-CU-TXT].** Then roll the tube at 37 °C and 8 rpm overnight **[2-MED].**
      1. Talent transfers slices into tube with decell solution (TEXT: Wear mask when preparing decell solution)
      2. Talent places tube on shaker/roller in 37 °C ~~room~~ incubator.
   2. The following morning, transfer the slices to a 50 mL conical tube containing 35 mL of 1x PBS **[1-CU].** Shake the tube by hand or vortex for 30 s **[2-MED/CU].** Then transfer the slices to a new 50 mL conical tube containing 35 mL of 1x PBS and again shake by hand or vortex for 30 s **[3-MED/CU].**
      1. Talent transfers slices to conical tube with PBS
      2. Talent shakes and vortexes tube
      3. Talent finishes transferring slices to new tube with PBS, covers and shakes and vortexes tube
   3. Repeat this rinse 10 times or until there are few to no bubbles evident in the rinsate approximately 10 s after shaking **[1-CU].**
      1. Talent stops shaking tube and few to no bubbles remain after ~10 s
   4. Place the scaffold slices in 2 mL of benzonase buffer for a set of mouse lungs and incubate the tissue at RT for 10 min **[1-CU-TXT].** Then, transfer the slices to 5 mL of 50 U/mL benzonase warmed to 37 °C **[2-CU],** and incubate the samples, rolling at 37 °C for 1 h **[3-MED].**
      1. Talent places slices in benzonase buffer and sets on ~~bench~~ cell culture hood to incubate (TEXT: Refer to Table 1 in text protocol)
      2. Talent transfers slices to warm benzonase
      3. Talent places samples in shaker at 37 °C rolling
   5. With 25 mL of sterilization solution in a 50-mL conical tube, rinse the scaffold slices **[1-CU]** and place the tube on a shaker at 250 rpm and 37 °C for 1 h **[2-MED].** After repeating the sterilization rinse, store the prepared scaffold slices in sterilization solution at 4 °C **[3-WIDE].**
      1. Talent adds sterilization solution to tubes
      2. Talent places tube on shaker at 37 °C and starts shaking
      3. Talent places samples in fridge
2. **Preparation of Human Lung Tissue Slices**
   1. TEXT ON WHITE BACKGROUND:Fresh human tissue is potentially infectious biomaterial and should be handled with standard precautions **[1-TXT].**
      1. TEXT of the above statement
   2. Visually examine the lung tissue by eye **[1-MED]** to ensure that it is anatomically intact and does not appear to be grossly infected **[2-CU/ECU-TXT].**
      1. Talent examines lung tissue
      2. Lung tissue that appears anatomically intact and does not appear infected (TEXT: Refer to text protocol for tissue details)
   3. Then place the lung tissue in nested Petri dishes and snap freeze it as demonstrated earlier in this video **[1-MED/CU].**
      1. Talent places tissue in nested dishes and floats in liquid nitrogen
   4. Next, using a multi-knife and sterile technique, cut the tissue into 2 mm slices **[1-ECU].** Then with PBS containing 1 μg/mL of SNP, rinse the slices extensively for 1 h **[2-CU].**
      1. Talent cuts slices with multi-knife then separate the slices by scalpel and forceps.
      2. Talent adds PBS/SNP to slices to rinse
   5. Transfer the slices to a 50-mL tube containing 15 to 25 mL of decell solution **[1-CU],** and roll the tube at 8 rpm and 37 °C overnight **[2-MED].**
      1. Talent places slices in tube of 10 ml decell solution (2.5 X), then add PBS to make the final volume to 25 ml.
      2. Talent places tube into shaker to roll at 37 °C and 8 rpm
   6. Use PBS to extensively rinse the scaffold slices until there are no bubbles left **[1-CU].** Then transfer the scaffold slices into 10 mL of benzonase buffer and incubate the tissue at RT 10 min **[2-MED/CU].**
      1. Talent finishes shaking sample and bubbles disappear
      2. Talent transfers slices into benzonase buffer and sets to incubate in cell culture hood.
   7. Transfer the scaffold slices into a new 50 mL tube containing 15 – 25 mL of fresh, warm benzonase buffer nuclease **[1-CU-TXT]** and incubate the scaffold slices at 37 °C for 1 h **[2-WIDE].**

**4.7. Added Shot:** Talent transfers slices to tube of fresh warm benzonase buffer and sets on cell culture hood to incubate for 10 min. Video editor: I do not know how this added shot was actually slated.

* + 1. Talent transfers slices to tube of fresh warm benzonase buffer nuclease (TEXT; 90 U/mL, 37 °C)
    2. Talent places tube on shaker/roller in 37 °C incubator. ~~Talent places slices into incubator~~
  1. Place the scaffold slices in a 50 ml conical tube with 25 mL of sterilization solution **[1-CU].** Then shake the tube at 37 °C and 250 rpm for 1 h before repeating the sterilization rinse **[2-MED/CU].** Store the scaffold slices in sterilization solution at 4 °C until use **[3-WIDE].**
     1. Talent places scaffold slices in tube of sterilization buffer
     2. Talent places tube in shaking incubator and closes door to start shaking
     3. Talent places tubes in fridge

1. **Preparation of Scaffold and Seeding of Cells**
   1. Immediately prior to culturing, use PBS to rinse the slices two times for 5 min each **[1-CU].** Then transfer 3 slices per well into a tissue culture treated, Poly-L-Lysine coated 6-well plate, using forceps to unroll and evenly stretch slices **[2-CU].**
      1. Talent adds PBS to tube of slices and rinses
      2. Talent transfers slices into tissue culture treated, poly-L-lysine coated wells of 6-well plate
   2. Incubate the slices at 37 °C for at least 10 min to allow adherence to the bottom of the plate but do NOT dry the slices. This step is critical **[1-WIDE].**
      1. Talent removes plate of slices from incubator and checks that they are not dry
   3. Suspend 104, 105 or 106 fibroblasts in 50 μL of fibroblast medium **[1-MED/CU-TXT].** Then drizzle the cells over the slices **[2-CU].** Incubate the seeded slices in the 37 °C incubator with 5% CO2 for 30 - 60 min **[3-WIDE].**
      1. Talent suspends fibroblasts in medium (TEXT: Refer to Table 1 in text protocol)
      2. Talent drizzles cells over slices
      3. Talent places plate into incubator
   4. Then add 1.85 mL of additional medium to the wells **[1-CU]** and return the plate to the incubator for 24 h **[2-WIDE]**.Replace 500 μL of medium and repeat the medium change every 48 h for a maximum culture period of 14 d **[3-MED/CU].**
      1. Talent adds medium to wells
      2. Talent places plate into incubator
      3. Talent replaces 500 μL of medium in wells
2. **Digestion of the Scaffold**
   1. To lyophilize the decellularized scaffold, transfer the tissue into lysis buffer **[1-CU]** and incubate it at 55 °C overnight to obtain lung ECM solution **[2-WIDE-TXT].**
      1. Talent ~~transfers tissue~~ add lysis buffer to the tube with tissue
      2. Talent places sample in 55 °C incubator (TEXT: Refer to text protocol for additional details)
   2. Centrifuge the lung ECM solution at 3615 x g for 8 min **[1-MED].** Then remove the supernatant and add 0.5 mL of lysis buffer to the pellet **[2-CU]** and incubate it at 55 °C overnight to re-lyse it **[3-WIDE].**
      1. Talent places ECM solution into centrifuge which was already sets to 3615 x g speed and 8 minutes
      2. Talent finishes removing supernatant from tube and adds lysis buffer to pellet and resuspends
      3. Talent places tube in 55 °C incubator
   3. Combine the lysed ECM solutions, and dialyze the sample against 4 L of ddH2O to remove the lysis buffer. Repeat the dialysis 3 more times **[1-MED-TXT].**
      1. Talent finishes combining solutions into dialysis tubing and places tubing into ddH2O (TEXT: Perform 1st for at least 1 h, 2nd overnight, 3rd and 4th for at least 1 h)
   4. Using a 0.45 μm filter apparatus, filter sterilize the purified ECM solution **[1-CU].** Then after determining the protein concentration according to the text protocol, prepare ECM working solution by using cell culture medium to dilute the purified ECM solution to 20 μg/mL **[2-CU-TXT].**
      1. Talent filter sterilized ECM solution

Added shot: Talent show the protein assay reagent and a tube written with ECM solution concentration.

Added shot: To show all the reagents for ECM working solution preparation

Video editor: I am not sure of how these added shots were actually slated.

* + 1. Talent adds cell culture level water to an empty tube to start to prepare ECM working solution (TEXT: DMEM, 10% FBS, 1% pen/strep)
  1. To stimulate PBMCs with ECM solution, following their isolation, seed 1x106 PBMCs in a 12-well-plate in 500 μL of cell culture medium **[1-CU-TXT].**
     1. Talent seeds PBMCs in 12-well plate using cell culture medium (TEXT: Refer to text protocol for additional details)
  2. Treat the PBMCs by adding 50 μL of ECM working solution + 450 μL of cell culture medium for the final concentration of 1 μg/mL **[1-CU]**. Or add 500 μL of ECM working solution for a final concentration of 10 μg/mL, and culture the cells for up to 14 d **[2-CU-TXT].** 
     1. Talent adding 50 μL ECM working solution to wells then adds 450 μL of cell culture medium
     2. Talent adds 500 μL ECM working solution to wells (TEXT: Final medium volume is 1 mL/well)

1. **Results: Analysis of Decellularized Mammalian Lung Tissue**
   1. The initial steps of this protocol are designed to process intact lung tissue as shown here **[1-LM]** to yield an acellular culture substrate as seen in this image **[2-LM].**
      1. LAB MEDIA Figure 1A, Editor, add the text ‘Intact Lung Tissue’ at above or below the panel.
      2. LAB MEDIA Figure 1B, Editor, add in this panel next to panel 1A and add in the text ‘Acellular Culture Substrate’ above or below the panel when it is mentioned.
   2. Unlike native tissue, which stains abundantly with DAPI**,** the decellularized lung will display a complete absence of DAPI staining **[1-LM].** A quantitative fluorescence bead assay also reveals a nearly complete absence of DNA in the decellularized tissue **[2-LM].**
      1. LAB MEDIA Figure 2A, Editor, add in the top panel for the first part of the sentence and the bottom panel for the second part of the sentence.
      2. LAB MEDIA Figure 2B, Editor, for the ‘nearly complete absence of DNA,’ use an arrow from above to point to the ‘decellularized’ column.
   3. Removal of cellular proteins such as beta-actin and major histocompatibility class II can be confirmed by Western blot, whereas relative to intact lung, the decellularized tissue reveals a complete absence of signal **[1-LM].**
      1. LAB MEDIA Figure 2C, Editor, add in the left hand labels when each is mentioned. Then use an arrow from below to point to the ‘Dec’ column when ‘a complete absence of signal’ is mentioned.
   4. When examined histologically, H&E or trichrome staining as well as scanning electron microscopy reveal complete absence of cellular material but preserved alveolar architecture **[1-LM].** 
      1. LAB MEDIA Figure 3A and B, Editor, add in A and then for H&E staining,’ add in the left panel in B and for ‘scanning electron microscopy,’ add in the right side of B.
   5. In addition, appropriately decellularized tissue will retain collagen and proteoglycans, although glycosaminoglycans may be reduced relative to native tissue **[1-LM].**
      1. LAB MEDIA Figure 4, Editor, for ‘will retain collagen and proteoglycan,’ use arrows from below to point up at the bottom row of GVG/Elastin, Collagen I and Collagen IV. For’ glycosaminoglycans may be reduced,’ use an arrow to point up at the GAGs.
   6. As seen here, the slicing technique can be used to decellularize fibrotic tissue from animals or from fibrotic human lungs, and histologic evaluation reveals the disrupted architecture and ECM accumulation of fibrosis **[1-LM].**
      1. LAB MEDIA Figure 5A – C, Editor, for the first part of the sentence, add the panels in A and B. Add in ‘Normal Lung’ above the left side panels in B and ‘Fibrotic Lung’ over the right side panels in B. For the second part of the sentence, replace A and B with C and add ‘Normal Lung’ on top left as in A and ‘Fibrotic Lung’ on bottom left as in A.
   7. In addition, scaffolds prepared from fibrotic lungs produce tensile responses that are nearly an order of magnitude greater than scaffolds prepared from normal lung tissue **[1-LM].**
      1. LAB MEDIA Figure 5D, Editor, for ‘scaffolds prepared from fibrotic lungs,’ use an arrow to point to the blue and gray lines on the graph. For ‘scaffolds prepared from normal lung tissue,’ use an arrow to point to the red lines. When pointing out the blue and gray lines, also use an arrow pointing up to point out the SLS sample in the graph on the right and then move the arrow to the CLS sample when pointing to the red lines on the left graph.
2. **Conclusion (said by authors on camera)**
   1. Huanxing Sun: Once mastered, this technique can be done in 48 hours if it is performed properly.
   2. Huanxing Sun: While attempting this procedure, it’s important to remember to remove the decell solution completely after the decellularization.
   3. Erica Herzog: Following this procedure, other methods like preparing heart, kidney and liver scaffolds can be performed in order to answer additional questions regarding fibrosis diseases.
   4. Erica Herzog: After its development, this technique paved the way for researchers in the field of fibrosis to explore cell-matrix interactions in the lung.
   5. Erica Herzog: After watching this video, you should have a good understanding of how to use lung scaffolds to do cell culture and treat cells with ECM solution.
   6. Huanxing Sun: Don't forget that working with CHAPS can be extremely hazardous and precautions such as weighing and dissolving the reagent in a chemical hood should always be taken while performing this procedure.

**Provided Media**

Authors, Please list all images, movie files, or 3-D rendered animations that can be included in the video per editor’s request. The step in the script/video where these images will be inserted should be specified. For example:

6.2 – 0123\_PIname\_Figure1.tif - dual color imaging of tumor angiogenesis at 40X

6.2 – 0123\_PIname\_Figure2.tif - dual color imaging of tumor angiogenesis at 100X

Formats: For static images we prefer .tiff, .eps, Illustrator, Powerpoint or Photoshop files at dimensions of at least 720X480 pixels and 300 dpi. The higher resolution, the better. Likewise any exported movie files should have at minimum these dimensions and be rendered to .mov, .mp4, or .avi files.

Insert your media filenames here.

**General Preparation**

It’s critical for a smooth and organized shoot that all reagents are accounted for, in advance.

Any overnight or long incubation steps should be recognized and specimens/samples be prepared in advance so that prior steps can be recorded and shooting can continue with pre-prepared specimens/samples.

All tubes/flasks should be pre-labeled neatly before we arrive.

Ex. Luciferase assay done in 96 well plates should be labeled with negative/positive control wells and experimental samples are labeled accordingly.

You will receive more detailed preparation instructions are included in the email accompanying the finalized script.