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TITLE:

Engineered Lung Tissues Prepared from Decellularized Lung Slices

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SUMMARY:

This protocol describes a method to generate reproducible, small-scale engineered lung tissues, by repopulating decellularized precision-cut lung slices with alveolar epithelial type 2 cells, fibroblasts, and endothelial cells.

ABSTRACT:

There is a need for improved 3-dimensional (3D) lung models that recapitulate the architectural and cellular complexity of the native lung alveolus *ex vivo*. Recently developed organoid models have facilitated the expansion and study of lung epithelial progenitors *in vitro*, but these platforms typically rely on mouse tumor-derived matrix and/or serum, and incorporate just one or two cellular lineages. Here, we describe a protocol for generating engineered lung tissues (ELTs) based on the multi-lineage recellularization of decellularized precision-cut lung slices (PCLS). ELTs contain alveolar-like structures comprising alveolar epithelium, mesenchyme, and endothelium, within an extracellular matrix (ECM) substrate closely resembling that of native lung. To generate the tissues, rat lungs are inflated with agarose, sliced into 450 µm-thick slices, cut into strips, and decellularized. The resulting acellular ECM scaffolds are then reseeded with primary endothelial cells, fibroblasts, and alveolar epithelial type 2 cells (AEC2s). AEC2s can be maintained in ELT culture for at least 7 days with a serum-free, chemically-defined growth medium. Throughout the tissue preparation and culture process, the slices are clipped into a cassette system that facilitates handling and standardized cell seeding of multiple ELTs in parallel. These ELTs represent an organotypic culture platform that should facilitate investigations of cell-cell and cell-matrix interactions within the alveolus as well as biochemical signals regulating AEC2s and their niche.

INTRODUCTION:

Alveoli are the functional units of the distal lung, comprising a meshwork of gas-exchanging airspaces lined by alveolar epithelial type 1 cells (AEC1s) and type 2 cells (AEC2s). Underlying the

epithelium is a dense network of capillaries as well as supporting mesenchyme, all buttressed by an extracellular matrix (ECM) scaffold that provides both strength and flexibility to these delicate air sacs[1]. The alveoli are also the site of injury in numerous lung pathologies, including idiopathic pulmonary fibrosis[2], acute respiratory distress syndrome[3], and severe coronavirus disease-19 (COVID-19)[4]. Although work over the past decade has uncovered a remarkable plasticity within the lung epithelium, the mechanisms that enable distal lung repair in some settings – and that preclude repair in others – remain an area of intense investigation[5]. The development of improved *in vitro* platforms to model the alveolus would facilitate studies of alveolar biology, regeneration, and therapeutics.

AEC2s self-renew and differentiate into AEC1s, and thus are considered the primary stem cell of the distal lung[6-8]. However, these cells pose a particular challenge to *in vitro* study given the difficulties associated with culturing primary AEC2s without a loss of phenotype[9]. In conventional 2-dimensional (2D) culture, AEC2s flatten and adopt some features of AEC1-like cells[10]. In contrast, 3D culture strategies, most commonly organoids, support the maintenance of differentiated features in primary AEC2s[6,11,12] and permit long-term culture of pluripotent stem cell (PSC)-derived AEC2s[13,14]. Organoids have been used to model distal lung development[15], viral infection[11,15], and AEC2-related genetic disease[13,16,17], enabling important insights into AEC2 biology and regeneration. However, these culture models typically comprise just one or two cellular lineages, and embed the cells in gel-type matrices that fail to recapitulate either the architecture or the ECM substrate of the native lung alveolus.

The ECM is a critical regulator of cell phenotype and behavior via molecular, topological, and mechanical cues; comprises a key component of tissue-specific niches regulating stem cell fate; and serves as a reservoir that modulates the availability of locally-secreted growth factors[18-21]. Culturing cells on native ECM may thus increase the predictive capacity of *in vitro* systems to model the biology of *in vivo* tissues. Decellularization, a process that removes cellular material from tissues via detergents, enzymes, or physical or other methods, can preserve in large part the ECM scaffolding of a native organ, when carefully performed[22,23]. Such scaffolds can be repopulated with cells for 3D biomimetic culture. However, while decellularized scaffolds are widely used for tissue engineering applications, their use for routine cell culture has been limited. Several previous studies have reported the decellularization and recellularization of lung slices or small lung tissue segments. In addition to proof-of-concept studies[24-26], repopulated lung slices have been used to study fibroblast-matrix adhesion[27,28] and to investigate the effect of diseased lung matrices on fibroblast phenotype[27,29]. With improved technologies available for generating precision-cut tissue slices, decellularized lung slices could offer a convenient and small-scale platform with which to culture cells, while preserving alveolar, airway, and vascular substructures. Incorporating multiple cell types would enable studies of cell-cell interactions within a physiologically relevant 3D environment. However, improved strategies are needed to facilitate the handling of tissues throughout the culture process, and to ensure controlled and reproducible seeding of tissues with known numbers of cells.

Here, we present a protocol to generate engineered lung tissues (ELTs) by repopulating decellularized precision-cut lung slices (PCLS) with primary endothelial cells, AEC2s, and

fibroblasts. In an adaptation of our previously described engineered heart tissue system[30] and whole lung decellularization-recellularization strategies[22,31], we describe procedures to cut PCLS from rat lungs and to clip the slices into reusable tissue culture cassettes that simplify and standardize downstream manipulations. Clipped slices are decellularized to form acellular ECM scaffolds, which are repopulated in customized seeding baths. Lung slice scaffolds preserve critical ECM components and architecture, and support the growth of AEC2s within multi-lineage alveolar-like structures for at least 7 days. ELTs represent a novel alveolar co-culture system within a physiologically relevant 3D matrix, which should support the development of lung tissue engineering strategies, while facilitating basic biological studies of AEC2s and the alveolus.

PROTOCOL:

All animal experimental procedures described in this paper were approved by the Yale Institutional Animal Care and Use Committee.

1. Creation of Tissue Culture Cassettes and Seeding Baths

NOTE: Once made, tissue culture cassettes and seeding baths may be autoclaved and reused for repeated rounds of ELT culture.

1.1. Tissue Culture Cassettes

1.1.1. Use a laser cutter to cut tissue culture cassette frames and clips out of 3/32 inch thick polytetrafluoroethylene (PTFE) according to the designs provided in **Supplementary File 1** and **Supplementary File 2**, respectively. Use a laser cutter to cut tissue culture cassette tabs out of 1/16 inch thick PTFE according to **Supplementary File 3**. Cut outlines 3x using 80% power and 15% speed (for a 30 W laser cutter).

1.2. Seeding Baths

1.2.1. Use the seeding bath CAD files (**Supplementary File 4** and **Supplementary File 5**) to 3D print the base and ring of the seeding bath mold, respectively, using clear resin.

1.2.2. Soak the molds in a solution of 10% poloxamer 407 in distilled water overnight before use to aid in PDMS release. Let air dry, then fit the ring over the base of the mold and wrap in flexible plastic film to help prevent leaking.

1.2.3. Prepare at least 60 g per mold of polydimethylsiloxane (PDMS) by mixing PDMS elastomer in a 10:1 ratio with curing agent, and pour into the 3D printed mold. Degas the PDMS in a vacuum desiccator for 30 min to remove any air bubbles.

1.2.4. Bake seeding baths at 60 °C for 8 h.

2. Preparation of Precision-Cut Lung Slices from Rat Lungs

2.1. Organ Harvest

2.1.1. Prepare a split perfusion system comprising gravity- and pump-driven limbs, as pictured in **Figure 1**. Connect a pulmonary artery (PA) cannula to the end of the tubing, comprising a 1/16 inch barbed Y-connector attached to a ½ inch length of LS 14 silicone tubing and a 3/32 inch female luer-lock connector (see **Figure 1**). Do not attach a check valve to the cannula at this time.

2.1.2. Prime the lines with PBS containing 100 U/mL heparin and 0.01 mg/mL sodium nitroprusside (SNP) for anticoagulation and vasodilation, respectively. Pre-set the perfusion pump to 30 mL/min.

NOTE: Add SNP fresh to the heparin solution and keep protected from light.

2.1.3. Dose an adult (8-12 week-old, approximately 300-350 g) Sprague-Dawley rat with an intraperitoneal (IP) injection of 400 U/kg heparin for anti-coagulation, followed by an IP injection of ketamine (75 mg/kg) and xylazine (5 mg/kg) for anesthesia. Confirm a surgical plane of anesthesia via lack of response to noxious stimulus (toe pinch).

2.1.4. Trim the chest and abdomen of fur using hair clippers. Then spray with 70% ethanol and wipe 3x with 10% povidone-iodine.

2.1.5. Grasp the skin below the level of the diaphragm with rat tooth forceps. Then make a ½ inch transverse incision in the skin with fine-pointed scissors. Grasp the exposed abdominal fascia with the forceps, make a ½ inch transverse incision in the fascia, and then extend the incision through the skin and fascia across the width of the upper abdomen.

2.1.6. Use the tip of the fine scissors to make a small incision (not more than 1/8 inch) in the center of the anterior diaphragm, causing the lungs to retract in the thorax. Extend the incision in the diaphragm across the full width of the chest.

2.1.7. Make two vertical incisions through the full height of the ribcage toward the neck, being careful not to damage the lungs. Extend the incision through the left ribs to cut through the collarbone and along the side of the neck to the level of the larynx, exposing the trachea.

2.1.8. Dissect the trachea free from surrounding connective tissue and from the esophagus. Make a transverse incision across the anterior half of the trachea between two cartilage rings, close to the larynx. Thread a 4-0 polypropylene suture behind the trachea, below the level of the incision, and loosely pre-tie the first half of a surgeon's knot with two twists.

2.1.9. Place a cannula comprising a 1/16 inch barbed Y-connecter connected to a one-way check valve and a ½ inch length of LS 14 silicone tubing with a 3/32 inch female luer-lock connector (see **Figure 1**) into the trachea by inserting one limb of the Y-connector into the tracheal incision toward the direction of the lungs.

2.1.9.1. Position the pre-tied suture loop around the trachea at the level of the inserted cannula and tighten around the inserted Y-connector to secure the cannula in place. Add two single-twist throws of the suture to complete the knot.

2.1.10. Fill a 10 mL syringe with air and connect to the Luer-lock of the tracheal cannula.

2.1.11. Clamp the inferior vena cava close to the diaphragm using a curved hemostat, then inject the heart with 150 U heparin (1000 U/mL) via the right ventricle (RV).

2.1.12. Partially open the gravity line stopcock, to produce a slow but steady dripping of PBS/heparin/SNP from the PA cannula prepared in step 2.1.1.

2.1.13. Thread the needle of a 4-0 polypropylene suture behind the base of the PA where it exits the RV. Use the first half of a surgeon's knot to pre-tie a loose loop of suture around the base of the PA.

2.1.14. Make a small incision (not more than 1/8 inch) in the RV just below and perpendicular to the PA using fine scissors, then insert one limb of the PA cannula Y-connector into the base of the PA. Secure the suture around the PA and the inserted connector and add a single-twist throw to complete the surgeon's knot.

NOTE: Cannulating the PA under flow prevents the introduction of air bubbles into the vasculature that can preclude adequate clearing of the lungs.

2.1.15. Attach a one-way valve to the other end of the PA catheter Y-connector, then cut off the apex of the heart to allow blood flow efflux via the left ventricle.

NOTE: Failure to cut off the apex of the heart prior to perfusing via the pump can cause damage to the blood-gas barrier, leading to leakage of fluid into the airspaces.

2.1.16. Switch the perfusion line to the pump side using the stopcock connecting the two lines, then turn on the pump at 30 mL/min. While perfusing the lungs via the PA, manually ventilate the lungs via the 10 mL tracheal syringe at approximately 10-15 breaths/min, to facilitate clearing the lungs of blood. Perfuse the lungs until they turn mostly white, usually requiring 40 mL of PBS/heparin/SNP or less.

NOTE: Inadequate clearing of the lungs of blood may impair downstream decellularization.

2.1.17. Cut the posterior trachea just above the level of the tracheal cannula, and then dissect the lungs and heart free of all remaining connective tissue and extract the lungs and heart *en bloc*.

2.1.18. Fill a 10 mL syringe with 2% low melting point agarose in Hank's balanced salt solution

(HBSS) without phenol red, prewarmed to 42 °C.

NOTE: The exact volume of agarose required will vary by lung size. Larger lungs (i.e., from rats larger than 400 g) will require more than 10 mL agarose.

2.1.19. Manually inflate the extracted lungs 3x with 10 mL of air (i.e., to approximately total lung capacity) via the trachea cannula to help recruit collapsed parenchyma.

2.1.20. Immediately inflate the lungs with the prepared syringe of agarose by manually injecting the agarose via the trachea cannula at a rate of approximately 40 mL/min, just until the most distal tips of the lung lobes are inflated. If distal regions of lung remain collapsed, inject an additional 1-2 mL of agarose.

2.1.20.1. Cap the trachea by attaching the white cap from a 4-way stopcock to the female luer-lock of the tracheal cannula. Place the lung in a 150 mm Petri dish on ice to allow the agarose to solidify.

NOTE: Inflation of the lung shortly after extraction is critical to ensure uniform filling of the lung parenchyma, and subsequent successful tissue slicing. If the lung inflates very unevenly, do not proceed with lung slicing as slice quality will be poor.

2.2. Lung Slicing

NOTE: Exact slicing procedure may need to be adapted based on the vibratory microtome (vibratome) being used; additional examples of PCLS preparation with various tissue slicers have been published previously[32-34].

2.2.1. Pre-chill the metal chilling block at -20 °C and keep on ice when not using throughout the slicing procedure.

2.2.2. Use a small drop of cyanoacrylate glue to attach a blade to the blade holder. Carefully attach the blade holder to the vibratome using an Allen wrench so that it just lines up with the end of a specimen tube inserted into the buffer tray.

2.2.3. Prepare 6-well plates with 3 mL per well sterile ice-cold HBSS without phenol red to collect the slices.

2.2.4. Using a scalpel, cut a piece of lung tissue approximately 1-1.5 cm^3.

NOTE: Lung tissue from the lower and middle portions of the left lobe, as well as from the right middle and bottom lobes, most readily yields larger tissue slices that maximize alveolar area. If uninflated tissue regions or areas of connective tissue are present, either trim off this tissue with scissors or orient downward toward the plunger; such tissue tends not to cut cleanly.

2.2.5. Place a small drop of cyanoacrylate glue onto the plunger of the specimen tube. Dab lung tissue on a paper wipe to remove excess moisture, and then immediately place the lung tissue on top of the plunger using a pair of forceps.

2.2.6. Slide the metal tube of the specimen tube up to the level of the top of the tissue and hold in place, with the plunger receded. Pipette pre-warmed 2% agarose in HBSS into the top of the tube to completely surround the tissue.

2.2.7. Place the ice-cold chilling block around the tissue for about 1 min to allow the agarose to solidify.

2.2.8. Insert the specimen tube into the buffer tray. Fill the tray with ice-cold PBS to mid-way up the tissue block. Turn the motor box switch to fast forward (FF) to advance the motor box plunger so that it is just touching the base of the specimen tube.

2.2.9. Set the desired settings for slice thickness, cutting speed, and oscillation frequency, for example, 450 µm thickness, speed 4 and oscillation frequency 5. Select **Continuous mode**, and then flip the switch to **On** to begin slicing.

2.2.10. As tissue slices fall into the buffer tray, transfer them to the prepared 6-well plates using an inoculating loop or spatula.

2.2.11. Stop slicing when about ~2 mm thickness of tissue remains in the specimen tube, to avoid damaging the blade or cutting tissue containing glue.

2.2.12. Repeat the above steps to slice additional lung tissue, as desired.

2.2.13. Decellularize slices immediately for scaffold preparation, or snap-freeze and store at -80 °C for up to 2 months. To freeze, transfer 4-6 slices to a 35 mm Petri dish and carefully aspirate any excess fluid from around the slices. Place the dishes in a bath of dry ice and 100% ethanol to snap-freeze, then wrap in foil, seal in a plastic bag, and transfer to -80 °C.

NOTE: Do not place fresh slices directly into a -80 °C freezer as the relatively slow rate of freezing can cause ice crystals to form that can damage the tissue.

3. Preparation of Lung Tissue Scaffolds

3.1. Preparation of Materials and Decellularization Solutions

3.1.1. Autoclave frames, clips, and tabs.

3.1.2. Prepare decellularization solutions as outlined in **Table 1**.

NOTE: Add benzonase nuclease to pre-warmed buffer immediately before use and sterile filter.

Prepare Triton X-100 and sodium deoxycholate (SDC) solutions within 24-48 h of the decellularization procedure. Prepare antibiotic/antimycotic solutions and benzonase buffer up to 30 d in advance and store at 4 °C.

3.2. Cutting and Clipping Lung Slices

NOTE: While cutting and clipping may be done non-sterilely on the benchtop, the decellularization steps in section 3.3 and all subsequent handling of the tissue scaffolds must be performed in a laminar flow hood.

3.2.1. Fill a 100 mm Petri dish about one-third full with PBS. Transfer cassettes and tabs to the dish using forceps.

3.2.2. If using frozen slices, thaw one dish at a time by pouring room temperature PBS into the dish to cover the slices. Keep remaining dishes on dry ice.

3.2.3. Transfer a thawed slice to a 150 mm Petri dish. Gently unfold the slice using fine forceps, if necessary, so that it lies flat, then carefully aspirate excess PBS from around the tissue.

3.2.4. Use a razor blade, with a ruler as a guide, to cut a 3 mm wide strip from the slice by pressing the full length of the blade firmly against the dish and rocking it slightly side to side with the blade edge held in place. Alternatively, use a rotary cutter retrofitted with 2 parallel blades separated by a 3 mm custom-made spacer (e.g., made of acetal [polyoxymethylene]) to cut tissue strips. Avoid any tears, holes, large airways or vessels, or thick connective tissue.

NOTE: For successful clipping, the strip must be at least 9 mm long.

3.2.5. Using forceps, transfer the tissue strip to the prepared 100 mm Petri dish.

3.2.6. Clip the tissue strip into the cassette: float the tissue above the cassette, centering the tissue to overhang the holes in the clips at either end. With fine forceps, place a tab partly into the hole at one end, gently straighten the tissue, and then place the second tab. Using forceps in each hand, press each tab in completely to secure the tissue.

NOTE: If having difficulty keeping the tissue in place prior to clipping, aspirate some PBS from the dish to lower the fluid level. Be careful not to stretch the tissue in placing the second clip, as this may lead to tearing.

3.2.7. Repeat the thawing, cutting, and clipping procedure in steps 3.2.2-3.2.6 for as many tissues as desired.

3.3. Slice Decellularization

3.3.1. Once all slices are clipped, transfer the 100 mm dish containing the cassettes to a laminar

flow hood.

3.3.2. Begin step 1 of the decellularization protocol (see **Table 2**): using a curved hemostat to grasp the notched sides of each cassette, transfer cassettes to 6-well plates (2 tissues/well) filled with 3 mL of PBS + ions + antibiotics/antimycotics per well (see solution recipe in **Table 1**).

3.3.3. Place well plates on an orbital shaker at 30 rpm for 10 min.

3.3.4. Continue with step 2 of the decellularization protocol (see **Table 2**): aspirate the fluid from each well, then replace with 3 mL/well PBS + ions, place plate on orbital shaker at 30 rpm, and incubate for 5 min.

3.3.5. Repeat step 3.3.4 for each of the solutions and corresponding durations as outlined in the decellularization protocol in **Table 2**.

3.3.6. After the final rinse step with PBS + antibiotics/antimycotics (step 20 of **Table 2**), transfer tissues to sterile 6-well plates with fresh PBS + antibiotics/antimycotics, and incubate at 37 °C for 48 h.

NOTE: After sterilization with antibiotics/antimycotics, lung tissue scaffolds can be seeded immediately, or stored at 4 °C for up to 30 d.

4. Slice Recellularization and Culture

NOTE: **Figure 2** shows a proposed timeline for tissue seeding and culture, in which slices are seeded first with rat lung microvascular endothelial cells and cultured in low-serum endothelial medium; then seeded with rat AEC2s and rat lung fibroblasts with a serum-free AEC2 growth medium (adapted from Jacob et al.[13] and You et al.[35]); see additional notes on cell sources used in Results and culture media details in **Table 3**. This strategy yields alveolar-like structures containing AEC2 monolayers.

4.1. Preparing Tissue Scaffolds for Seeding (Day -4 or -3)

4.1.1. If using tissue scaffolds stored at 4 °C, incubate scaffolds overnight at 37 °C with fresh PBS + antibiotics/antimycotics (10% penicillin/streptomycin, 4% amphotericin B, 0.4% gentamicin in PBS) prior to seeding.

4.1.2. Rinse scaffolds 3x with sterile PBS (5 mL/well), 5 min each.

4.1.3. Examine scaffolds under a phase contrast microscope at 5x magnification to select tissues for seeding.

NOTE: The best scaffolds for seeding have no tears or holes and do not contain large airways or vessels. While scaffolds with the features may be seeded successfully, repopulation patterns may

differ from those observed in alveolar areas.

4.2. Endothelial Cell Seeding (Day -3)

4.2.1. Count endothelial cells using a hemocytometer and prepare the endothelial cell suspension in endothelial medium (see **Table 3**) at 5 x 10^6 cells/mL, with sufficient cells to seed 500,000 endothelial cells per slice (e.g., for 12 slices, resuspend 6 x 10^6 cells in 1.2 mL medium).

4.2.2. Place autoclaved seeding baths in 100 mm Petri dishes. Transfer rinsed scaffolds upside down to seeding baths: use a fine curved hemostat to grasp a cassette by the notched sides, use a straight hemostat or forceps to grasp one end of the cassette (being careful not to touch the tissue itself) and flip, and then grasp the cassette again with the tips of the fine curved hemostat via the holes along the notched sides, and place in a seeding bath well. Repeat for remaining cassettes.

NOTE: When correctly placed, scaffolds will be centered, upside down, in the bottom of each well. If needed, press down gently on the corner of the cassette with the tips of a hemostat to ensure that the cassette is seated flat in the well. Improper seating of the cassette can lead to poor tissue seeding. It is acceptable if the well contains a small amount of PBS.

4.2.3. Swirl the prepared cell suspension gently to mix, then use a manual pipette to pipette 100 µL cells directly on top of each tissue at the base of the well, being careful not to damage the tissue with the pipette tip.

4.2.4. Transfer seeded tissues to the cell culture incubator at 37 °C/5% CO_2.

4.2.5. After 2 h, add 900 µL pre-warmed culture medium to each well using a manual pipette, then return to incubator. If a cassette becomes unseated (floats) upon adding medium, gently press down on the corner of the cassette with the pipette tip so that it lies flat in the well.

4.2.6. Change medium on day -2. Remove medium by tilting the Petri dish and manually pipetting with a pipette tip lightly placed in the corner of the well, so as not to disturb the cassette. Replace with 1 mL of fresh endothelial medium per well.

4.3. AEC2 and Fibroblast Seeding and Tissue Culture (Day 0)

4.3.1. Count AEC2s and fibroblasts using a hemocytometer. Prepare a 1:1 cell suspension of AEC2s and fibroblasts in AEC2 growth medium (epithelial base medium + AEC2 supplements; see **Table 3**) at 5 x 10^6 total cells/mL, with sufficient cells to seed 500,000 cells (250,000 AEC2s and 250,000 fibroblasts) per slice (e.g., for 12 slices, resuspend 3 x 10^6 AEC2s + 3 x 10^6 fibroblasts together in 1.2 mL medium).

4.3.2. Pipette out the medium from each well of the seeding bath as described in step 4.2.6. Swirl the prepared cell suspension gently to mix, then pipette 100 µL cells directly on top of each tissue

at the base of the well.

NOTE: It is acceptable if a small amount of endothelial medium remains in the well prior to AEC2/fibroblast seeding.

4.3.3. Transfer seeded tissues to the cell culture incubator at 37 °C/5% CO_2.

4.3.4. After 2 h, add 900 µL pre-warmed AEC2 growth medium to each well, then return to incubator.

4.3.5. After 24 h of culture (day 1), prepare a 12-well plate with 1 mL of pre-warmed AEC2 growth medium per well per cassette.

4.3.6. Pipette 800 µL of medium from each well of the seeding bath. Remove cassettes from the seeding bath: grasp each with a fine curved hemostat via the holes along the notched sides, transfer to a straight hemostat or forceps to grasp the cassette at one end and flip, then use the curved hemostat to grasp the cassette via the notched sides and transfer right-side-up, one per well, to the prepared 12-well plate.

4.3.7. Change culture medium in the 12-well plate every other day until day 7 or for the desired length of culture: use a glass Pasteur pipette to aspirate the medium from each well, being careful not to touch the tissue; pipette in 1 mL fresh AEC2 growth medium per well.

NOTE: The degree of tissue repopulation may be monitored via phase contrast microscopy at 5x magnification throughout the duration of culture.

5. Tissue Harvest and Sample Analysis

5.1. To fix ELTs for histology and immunofluorescent staining, transfer tissue culture cassettes to 10% neutral-buffered formalin and incubate for 3-4 h at room temperature on a rocker. Remove tissues from cassettes by using the tip of a fine-pointed forceps to cut the tissue where it meets the tabs. Process tissue according to routine methods for paraffin embedding and histology; no specialized techniques are required.

5.2. To process ELTs for qRT-PCR, rinse tissues in cassettes in PBS 2x, then remove tissues and snap freeze or proceed with lysis for RNA extraction.

NOTE: Pooling at least 2 slices seeded with 1 x 10^6 cells and cultured for 7 days should yield ample RNA for downstream PCR analysis.

REPRESENTATIVE RESULTS:

An overview of the process to generate ELTs – comprising lung slicing, slice clipping and decellularization, and scaffold repopulation – is presented in **Figure 3**. The ELTs presented here were cultured using primary rat lung microvascular endothelial cells (see **Table of Materials**),

neonatal rat AEC2s, and lipofibroblast-enriched neonatal rat lung fibroblasts[36]. AEC2s were freshly isolated via magnetic bead-based sorting as previously described[37]; alternative isolation protocols have been detailed and discussed elsewhere[38-40]. Purity of isolated rat AEC2s can be assessed via flow cytometry for rat-specific AEC2 surface marker RTII-70[41], or via staining of a cytocentrifuged cell sample for RTII-70 or pro-surfactant protein C (pSPC). Rat lung fibroblasts were isolated from postnatal day 7-9 rat pups according to an adaptation of a published protocol[42] and used at passage 1-2; alternative isolation protocols have been described elsewhere[43,44]. Purity of isolated fibroblasts can be assessed via staining of cultured or cytocentrifuged cells for mesenchymal marker vimentin, and lipofibroblast enrichment can be assessed via staining for Oil Red O[45].

When the lung tissue is uniformly inflated with agarose, and tissue pieces strategically selected and oriented for slicing so as to maximize total and parenchymal tissue area, one rat lung may yield tissue for >100 alveolar ELTs. Strips of PCLS exhibit sufficient mechanical integrity to be clipped into tissue cassettes with few (<5%) instances of tearing (**Figure 3B**).

The protocol for decellularizing lung slices is closely based on our previously published whole lung decellularization protocol, which by quantitative proteomics was demonstrated to preserve many ECM components at levels not significantly different from those in native lung[22]. Decellularized slice scaffolds preserve the native architecture of the alveoli, as viewed by hematoxylin and eosin (H&E) staining (**Figure 4A,B**) and by phase contrast microscopy (**Figure 4C**). We typically exclude scaffolds containing large airways or vessels (**Figure 4D**) or tears, although the former can be included if they are of interest to the researcher. Decellularization leads to a 96% reduction in tissue DNA content as measured by an assay for double-stranded DNA (see **Table of Materials**; 0.50 µg/mg ± 0.073 µg/mg vs 0.018 µg/mg ± 0.0035 µg/mg in native vs decellularized tissue, respectively, mean ± SEM) (**Figure 5A**), with no DNA visible by hematoxylin staining (**Figure 4B**). Histological and immunofluorescent staining of decellularized scaffolds reveals maintenance of ECM proteins collagen, elastin, collagen IV, and laminin with architecture and quantity similar to that in native lung slices (**Figure 5B-E**). Note that the nuclei of native tissues stain blue/black with trichome (for collagen) and EVG (for elastin) stains. Immunofluorescent staining was performed as described previously, using standard methods for staining tissues[37]. The antibodies used and their respective concentrations are listed in **Table 4**.

Successful scaffold repopulation leads to highly cellular ELTs after 7 days, with an alveolar-like repopulation pattern visible by light microscopy (**Figure 6A-C**). In some cases, with very high cellularity, organoid-type structures may be visible (**Figure 6A,B**). Unsuccessful tissue seeding can be visualized by phase-contrast microscopy during culture (**Figure 6C**). After culturing tissue scaffolds with AEC2s, fibroblasts, and endothelial cells, ELTs are densely repopulated with alveolar-like structures comprising all three cell lineages (**Figure 6D,E**). At day 7 or 8, AEC2s maintain cuboidal morphology and express surfactant protein-B (SPB) and lamellar body protein ABCA3, without evidence of significant differentiation to AEC1s (**Figure 6E,F**). AEC2s are highly proliferative in ELTs, as demonstrated by 5-ethynyl-2'-deoxyuridine (EdU) incorporation following a 2 h pulse at 10 µM (**Figure 6G**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of perfusion system for lung extraction and clearing. (**A**) The perfusion system comprises a gravity-driven limb, used for initial cannulation of the pulmonary artery under flow; and a pump-driven limb, used to clear the lungs efficiently after initial cannulation. The pump line includes a "pulse dampener" that dampens the spikes in pressure caused by the pump. The design of the tracheal and pulmonary arterial cannulas is detailed at left. SNP = sodium nitroprusside. (**B**) Details of pulse dampener assembly. BPT and silicone refer to types of tubing. (**C**) Positions of tracheal and pulmonary arterial cannulas placed during lung extraction.

Figure 2: Culture timeline for tri-lineage recellularization. Proposed timeline for tri-lineage ELT seeding and culture, including timing of two-phase seeding. Cell numbers for seeding and culture medium for each phase are indicated. See culture media details in **Table 3**. AEC2 = alveolar epithelial type 2 cell. EC = endothelial cell. FB = fibroblast.

Figure 3: Schematic of engineered lung tissue preparation. (**A**) Native lung tissue is cut into slices using a vibratome. (**B**) Precision-cut lung slices are cut into standardized 3 mm wide strips, clipped into polytetrafluorethylene (PTFE) tissue-culture cassettes, and detergent-decellularized to yield acellular extracellular matrix scaffolds. (**C**) Scaffolds are reseeded in specialized seeding baths that confine the seeding area to the area of the tissue, and then cultured in a standard well plate.

Figure 4: Structure of decellularized lung scaffolds. H&E staining of native (**A**) and decellularized (**B**) lung slices showing preservation of alveolar architecture after decellularization. (**C,D**) Examples of decellularized ECM scaffolds viewed at 5x magnification by phase contrast microscopy, comprising predominately alveolar tissue (**C**) or containing large branching airways and vessels (**D**, black and red arrowheads). Scale bars, 50 μm (**A,B**); 500 μm (**C,D**).

Figure 5: DNA removal and matrix preservation in decellularized lung scaffolds. (**A**) Quantification of DNA in native and decellularized lung slices (mean ± SEM, n = 5). Welch's *t* test, $^{**}P < 0.01$. Decell = decellularized. (**B,C**) Histological staining of native and decellularized lung slices for collagen (**B**) and elastin (**C**). Arrowheads, elastin preserved in alveolar entrance rings of decellularized tissue. (**D,E**) Immunofluorescent staining of native and decellularized lung slices for collagen IV (**D**) and laminin (**E**). Scale bars, 50 μm. In all panels, dotted boxes outline the image region that is magnified to the right in each respective panel.

Figure 6: Cellular repopulation of engineered lung tissues. (**A-C**) Examples of recellularized ELTs on day 7 of culture, as visualized during culture by phase contrast microscopy. The recellularization pattern mirrors the alveolar structure of the tissue. In some areas of high cellularity, organoid-like structures may form (arrowheads). (**A**) and (**B**) represent successful cell repopulation, whereas (**C**) represents a poor level of recellularization after 7 days of culture. (**D-G**) Staining of recellularized ELTs on day 7 or 8 of culture. (**D**) H&E staining showing cellular repopulation of the alveolar septa. (**E**) Immunofluorescent staining labels engrafted proCollagenIα1^{+} fibroblasts, ABCA3^{+} AEC2s, and CD31^{+} endothelial cells. (**F**) Tissues contain abundant SPB^{+} AEC2s but few RTI-40 (podoplanin)$^{+}$ AEC1s under these conditions. (**G**) Many

AEC2s are proliferating in ELTs, as measured by EdU incorporation. Scale bars, 500 µm (**A-C**); 25 µm (**D-G**).

Table 1: Decellularization solutions. Preparation details for decellularization solutions.

Table 2: Decellularization protocol. Details of protocol for decellularizing lung slices.

Table 3: Culture media. Preparation details for endothelial and AEC2 growth media.

Table 4: Antibodies used for immunostaining. Details of antibodies and their concentrations used for immunostaining.

Supplementary File 1: Design for laser cutting tissue culture cassette frames.

Supplementary File 2: Design for laser cutting tissue culture cassette clips.

Supplementary File 3: Design for laser cutting tissue culture cassette tabs.

Supplementary File 4: CAD file for seeding bath mold base.

Supplementary File 5: CAD file for seeding bath mold ring.

DISCUSSION:

This paper describes the use of decellularized precision-cut lung slices as a platform to generate engineered lung tissues *in vitro*, which contain multi-lineage alveolar-like structures. By combining strategies that we previously developed to repopulate high-fidelity acellular ECM lung scaffolds for whole lung engineering[22,31], with our robust system for culturing small-scale engineered heart tissues[30], this protocol enables the use of physiologically relevant lung ECM as a tissue culture substrate, in a repeatable and moderate-throughput manner.

The methods presented here detail ELT scaffold preparation from rat lungs, which are readily attainable, may be extracted *en bloc* with direct access to intact airways for agarose inflation, and are of larger size than mouse lung. However, any lung tissue that can be inflated with agarose and yield slices at least 9 mm in length may be used within this system. Regardless of tissue source, uniform inflation of the lung tissue with agarose is the most critical step for ensuring success of downstream tissue slicing, clipping, and tissue handling. Underinflated lung tissue tends not to slice cleanly, while overinflated tissue may tear during clipping. Following agarose gelation, appropriately inflated tissue regions are firm but provide a little give when gently pressed with forceps. For intact rat lungs, we found that pre-inflating the extracted lungs with air several times, followed by agarose inflation as soon as possible after extraction, results in the best slicing outcomes and best quality of resulting tissue scaffolds. The appropriate volume of agarose needs to be optimized empirically; for a rat lung the volume required to inflate the lung to total lung capacity is approximately 30 mL/kg animal mass (e.g., 10.5 mL agarose for lungs from a 350 g rat). For larger resected lung tissues with less straightforward airway access (such

as those from human donors), some additional troubleshooting may be necessary to inflate the tissue via a bronchus[32]. During subsequent lung slicing, the selection and orientation of the tissue on the plunger is another important step to 1) ensure that the slices are large enough to generate tissue strips that can be clipped into tissue culture cassettes and 2) maximize parenchymal (alveolar) tissue area, excluding large airways or vessels.

Clipping the PCLS into tissue culture cassettes can be a challenging step initially, but the cassettes greatly simplify tissue handling during decellularization and seeding. Two potential problems that may arise are tissue tearing (either during the process of clipping, or during decellularization), or tissue positioning in the clips that results in poor downstream seeding (e.g., no seeding, or seeding just at the ends). Tearing may be the result of agarose overinflation, overstretching of the tissue during tab insertion, or leaving too little overhang to provide adequate tissue grip when inserting the tabs. Note that slices that tear at one clip end may be successfully seeded, however, they are difficult to visualize under the microscope during culture as the tissue is not flat. Poor tissue seeding (such as that in **Figure 6C**) is likely the result of the slice not lying flat between the two clips, and thus making poor contact with the base of the seeding bath well when flipped upside-down. Another possible cause is improper seating of the cassette in the bottom of the seeding bath well. In terms of clipping, apply slightly more tension in the tissue when placing the second clip to help it lie flat. Some slices have a slight concavity; in these cases clip the slice with the convex side up. With practice, we typically experience failed seeding with fewer than 2% of slices.

One limitation of this protocol is the requirement for some specialized equipment – a laser cutter and a 3D printer – to generate the initial materials for ELT preparation. However, once the tissue culture cassettes and seeding baths are created, no additional special materials are required. The lung slicing and decellularization steps of ELT scaffold preparation are moderately time-consuming; however, these steps may be performed in advance, or in numbers sufficient to prepare for multiple experiments at the same time. Many PCLS (>100 if optimizing for parenchymal regions) can be cut from a single lung and snap-frozen for later use. While a single freeze-thaw cycle may cause minor ultrastructural damage to the ECM[46], even multiple freeze-thaw cycles have been demonstrated not to cause a significant loss in ECM[23,47]. PCLS may also be clipped and decellularized in advance of an experiment, to be used within one month. (Notably, the described decellularization protocol can be accomplished in approximately 6 hours, which represents a significant advantage over previously described methods that require a day or more[27,28].) Once the scaffolds are prepared, the cell seeding process is simple and fast, and the culture of ELTs does not require specialized techniques.

A caveat of the described ELT method is the lack of region-specific seeding, i.e., the delivery of AEC2s specifically to the alveolar space, or endothelial cells specifically to the vascular space. Nevertheless, although cells are simply seeded on top of the tissue scaffolds, the pattern of recellularization is non-random, with some semblance of alveolar-like organization, including epithelial rings. We suspect that cell-cell interactions, as well as local differences in ECM composition and geometry[20,21], likely contribute to the observed recellularization patterns. In support of this hypothesis, a previously published study, in which fibroblasts were seeded non-

specifically onto decellularized lung slices, demonstrated that the pattern of tissue repopulation and associated cellular phenotypes varied significantly by microscopic tissue region and ECM scaffold source (e.g., healthy versus diseased)[27]. Fibroblasts were also observed to invade into the interstitium – the location in which they reside in native lung tissue[1,27]. The primary alternative method that we can imagine to culture cells on lung slices in a truly region-specific manner would entail seeding intact decellularized lungs via the airway[31,48] and vascular compartments[49,50], and then slicing the recellularized tissue. However, this alternative 1) is significantly more cost-, time-, and resource-intensive; 2) is lower throughput; 3) requires increased numbers of animals; and 4) is associated with an increased risk of contamination due to the challenges of whole lung culture and subsequent slicing of the seeded lung. While not recapitulating all aspects of native cellular organization, the ELT platform enables lung cell culture on a physiologically relevant ECM substrate, in a manner that is accessible to many more labs.

The flexibility of the ELT system is a major advantage of this platform, and should allow small-scale lung tissue culture with any number of tissue scaffolds, cells, or culture media of interest. The use of scaffolds derived from diseased tissue or from injury models may permit the study of cell-cell or cell-matrix interactions in the setting of disease-altered ECM[27,29,51]. However, note that the decellularization protocol may need to be adapted to account for matrix differences among species[52]. The described seeding strategy can be used for any cell type, and the culture timeline adapted to fit the needs of the researcher. As a starting point, 1 x 10^6 cells per scaffold should yield a highly cellular tissue within 7 days of culture, whereas 1 x 10^5 total cells result in poor cellularity. In any adaptation of the timeline, the tissue culture cassettes should be removed from the seeding bath 24 h following the last tissue seeding. Here, with the goal of modeling some of the cellular complexity of the lung alveolus, we describe a tri-culture recellularization strategy that supports the maintenance of well-differentiated neonatal AEC2s in alveolar-like structures for at least 7 days. Our results also demonstrate the successful engraftment of both fibroblasts and endothelial cells within ELTs, emphasizing the broad applicability of the culture substrate and its suitability for co-culture studies. Seeding adult cells in ELTs may facilitate the modeling of more quiescent alveolar structures, while seeding human PSC-derived AEC2s, including those with genetic modifications, could facilitate translational studies of human disease[13,53]. In general, the bottom-up approach enabled by the ELT platform presents the opportunity to investigate the contributions of particular cell types to readouts of interest – such as AEC2 proliferation or differentiation state.

In summary, this protocol outlines a robust system for generating engineered lung tissues for co-culture studies of AEC2s, fibroblasts, and endothelial cells within acellular ECM lung slice scaffolds. ELTs represent a novel 3D culture strategy for primary AEC2s, which to date have typically relied upon less-physiologic gel-type matrices to maintain a well-differentiated phenotype[6,11,12]. The current platform builds upon previous work in the repopulation of decellularized lung slices[24-29], but offers several advantages: 1) a tissue culture cassette system to facilitate ELT handling during decellularization, seeding, and culture; 2) a customized seeding bath to precisely seed a known number of cells on each slice scaffold; and 3) a tri-culture reseeding strategy that enables alveolar tissue repopulation with epithelial, mesenchymal, and endothelial cells. Thus, ELTs represent an important step forward toward creating reproducible

in vitro models that capture the cellular and substrate complexity of the native alveolus and the AEC2 stem cell niche.

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DISCLOSURES:

L.E.N. is a founder and shareholder in Humacyte, Inc, which is a regenerative medicine company. Humacyte produces engineered blood vessels from allogeneic smooth muscle cells for vascular surgery. L.E.N.'s spouse has equity in Humacyte, and L.E.N. serves on Humacyte's Board of Directors. L.E.N. is an inventor on patents that are licensed to Humacyte and that produce royalties for L.E.N. L.E.N. has received an unrestricted research gift to support research in her laboratory at Yale. Humacyte did not influence the conduct, description or interpretation of the findings in this report.

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Figure 1

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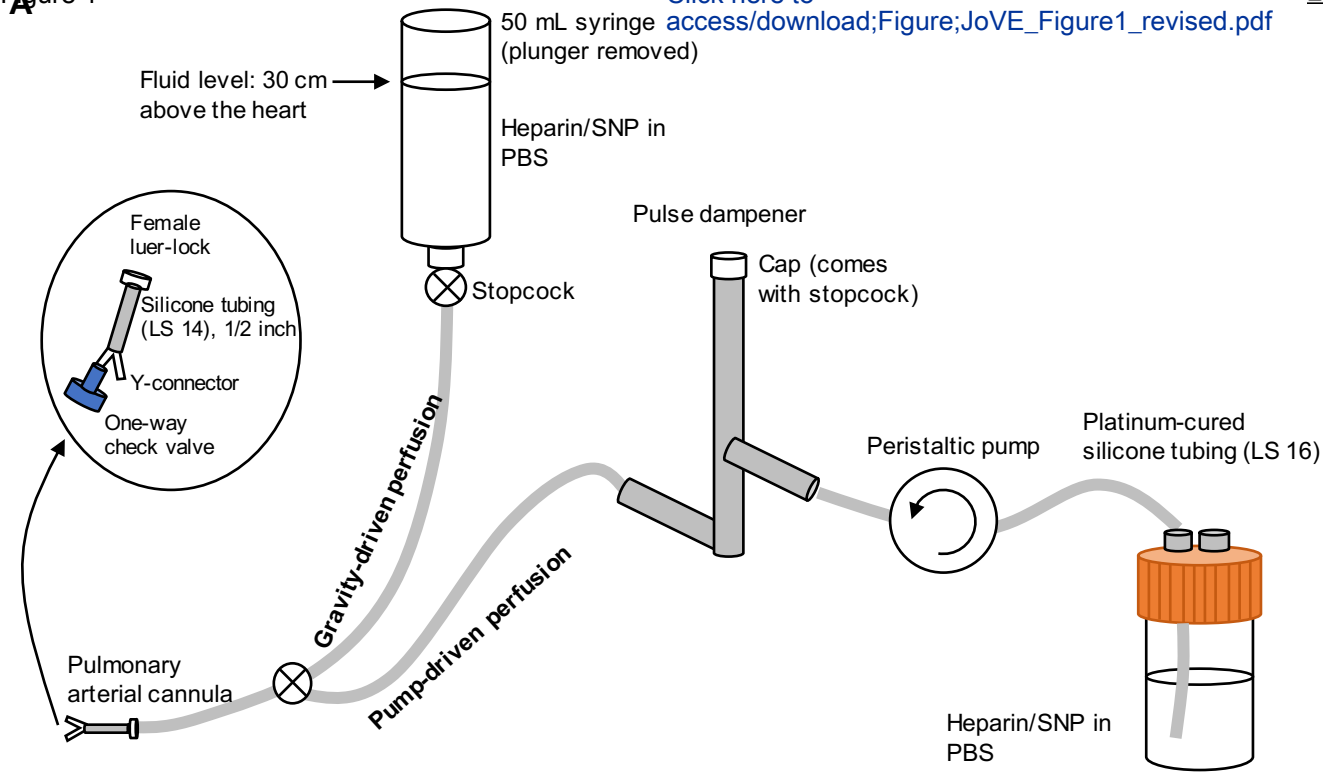

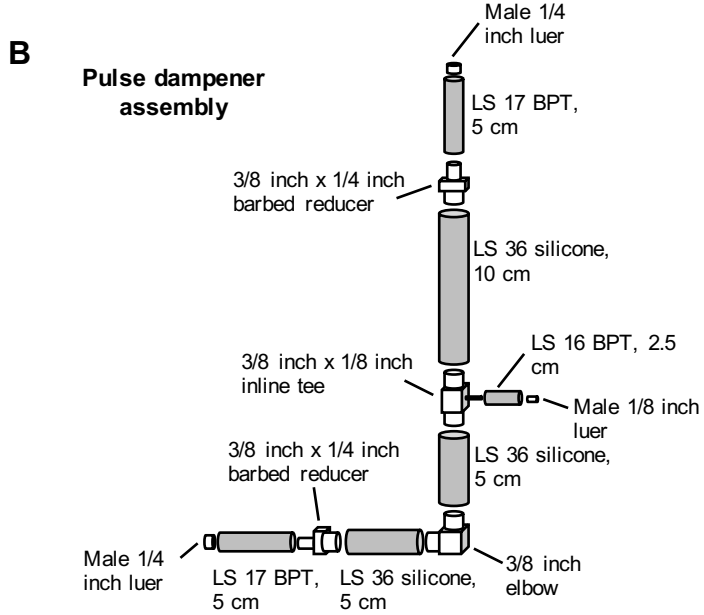

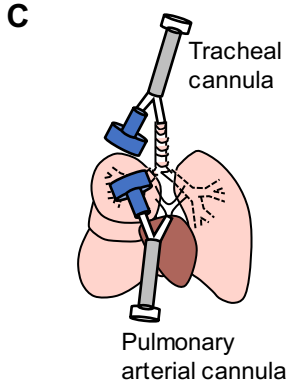

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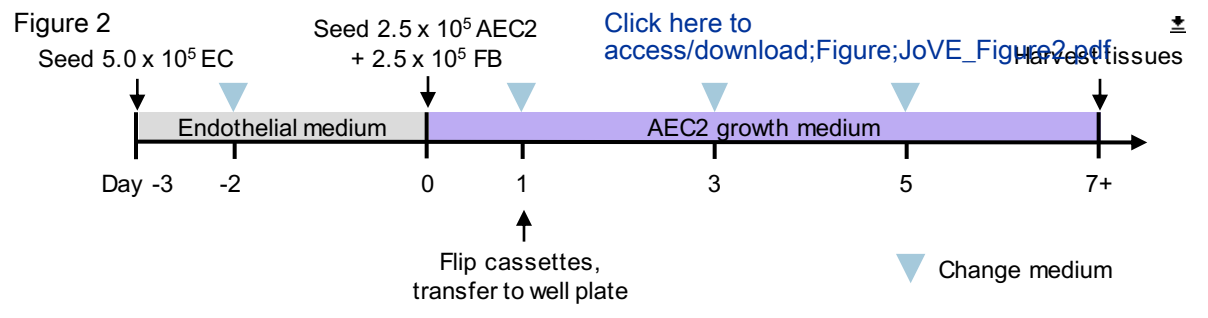
Seed 5.0 x 10^5 EC
Seed 2.5 x 10^5 AEC2
+ 2.5 x 10^5 FB
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Harvest tissues
Endothelial medium
AEC2 growth medium
Day -3
-2
0
1
3
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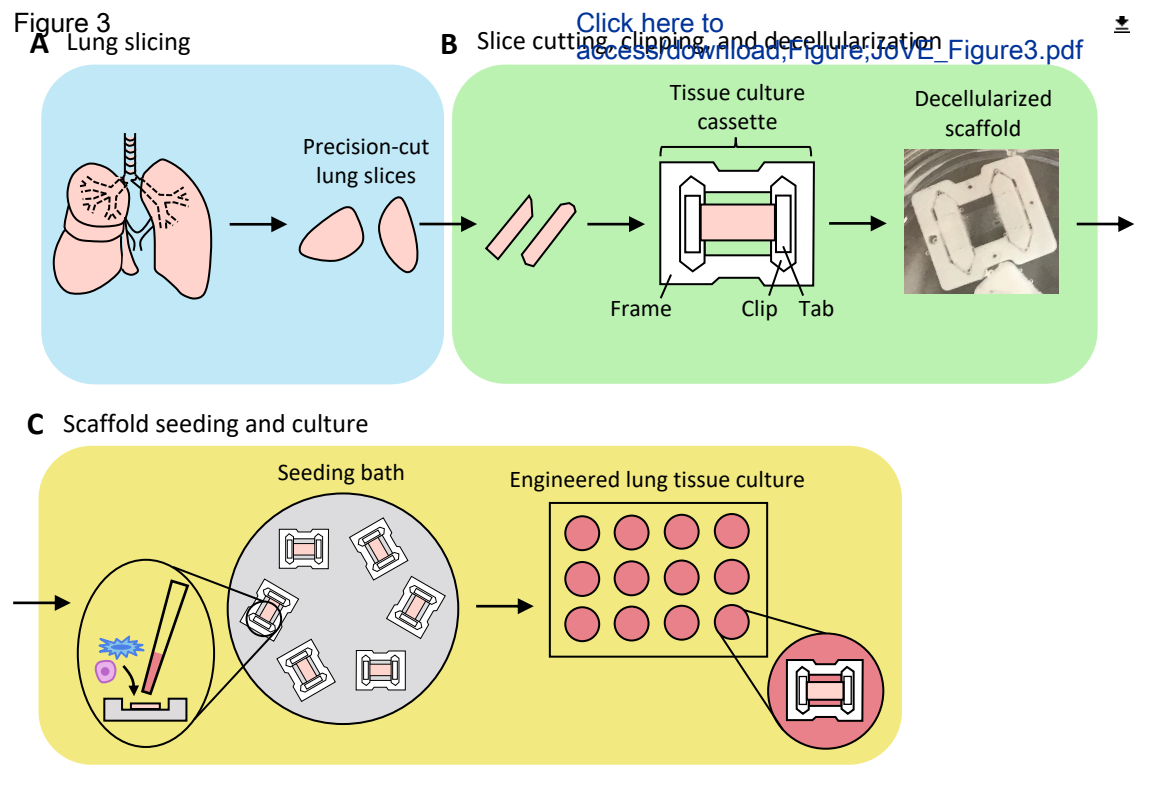
7+
Flip cassettes,
transfer to well plate
Change medium

Figure 3



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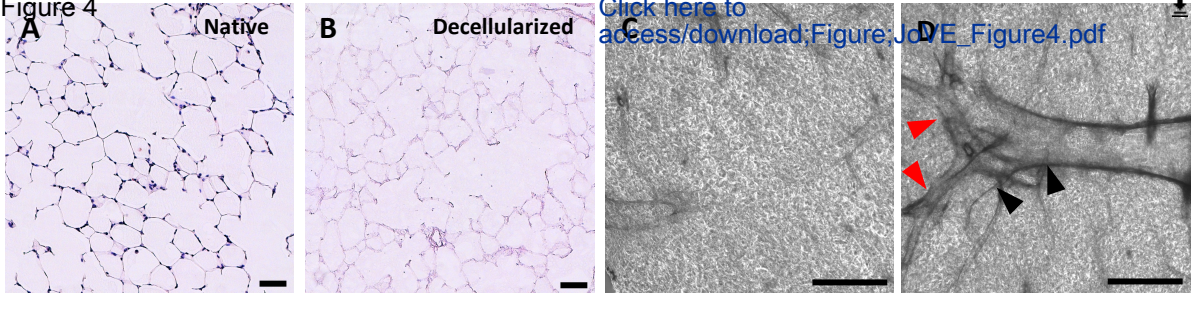

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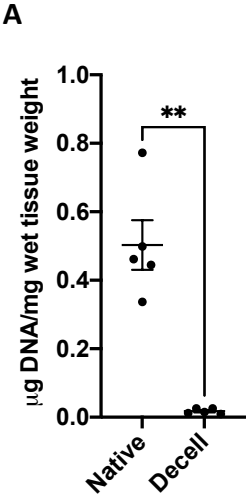

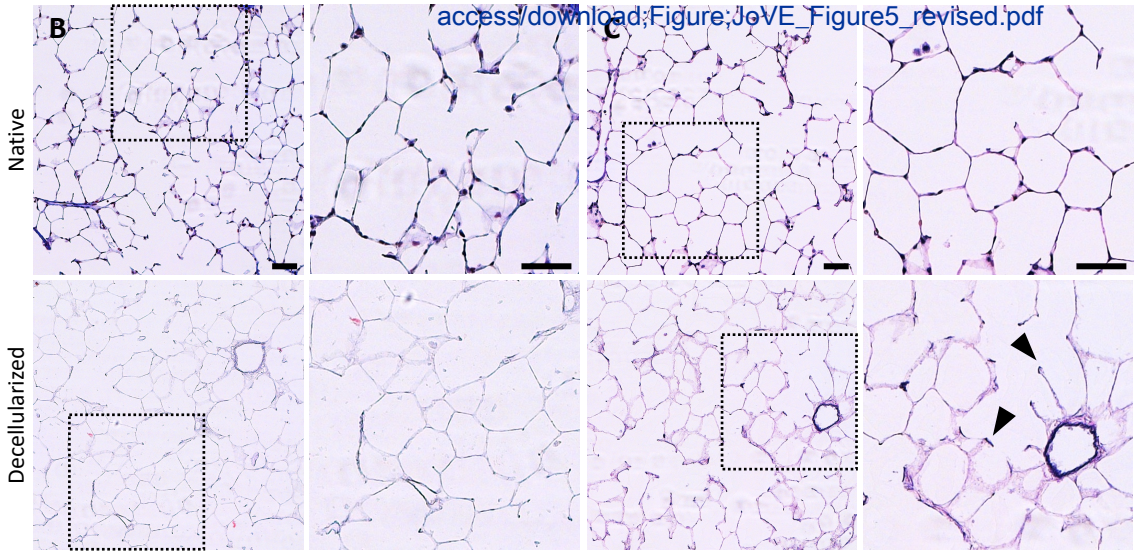

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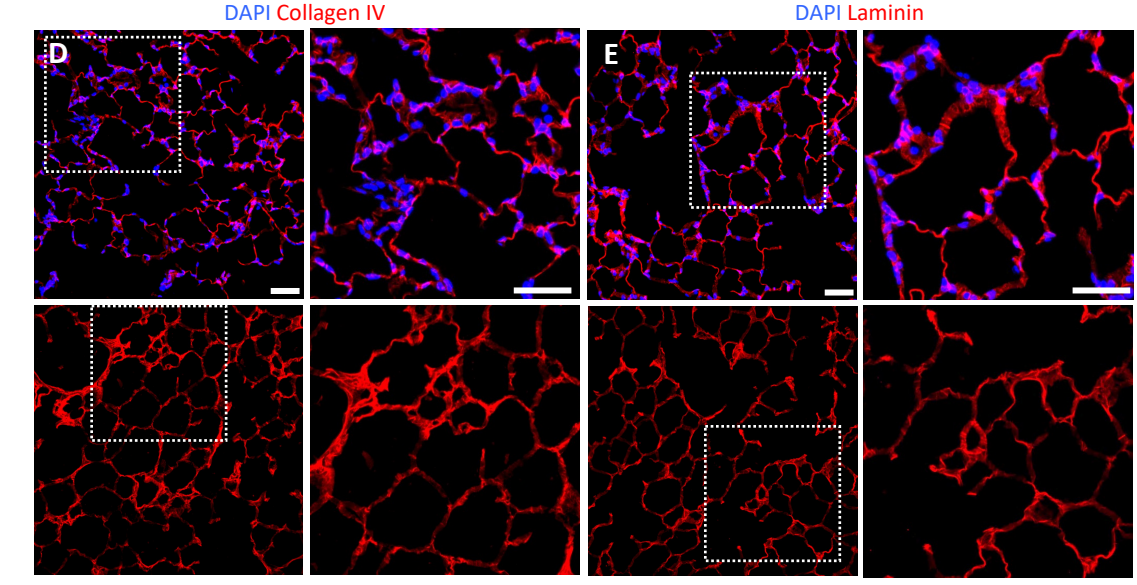

Figure 6

Click here to access/download;Figure;JoVE_Figure6.pdf

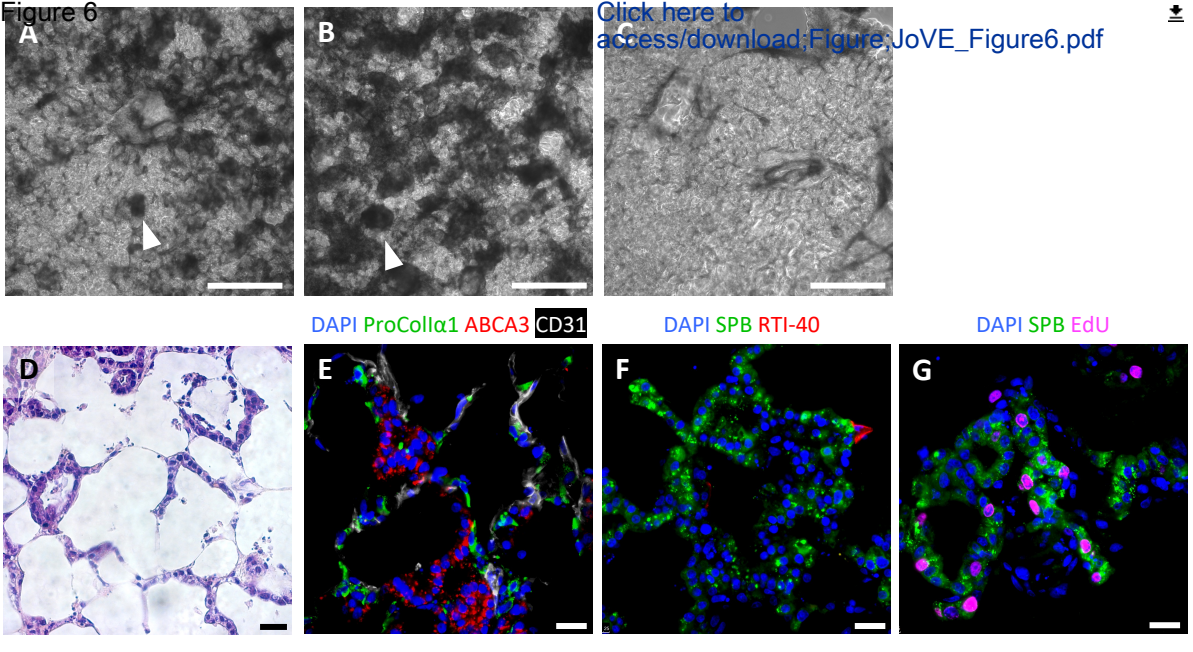

Table 1

Solution	Solution details	pH	Volume required (per 6-well plate)
PBS	PBS without Ca2+/Mg2+	7.4	126 mL
PBS + ions	PBS with Ca2+/Mg2+	7.4	18 mL
PBS + Abx (antibiotics/antimycotics)	PBS + 10% penicillin/streptomycin + 4% amphotericin B + 0.4% gentamicin	7.4	36 mL
PBS + ions + Abx (antibiotics/antimycotics)	PBS + ions + 10% penicillin/streptomycin + 4% amphotericin B + 0.4% gentamicin	7.4	18 mL
Benzonase buffer	diH2O + 50 mM Tris-HCl + 1 mM MgCl2 + 0.1 mg/mL BSA	8	36 mL
Benzonase	Benzonase buffer + 20 U/mL benzonase	8	36 mL
NaCl	PBS + 1M NaCl	7.4	18 mL
Triton 0.0035%	PBS + ions + 0.0035% Triton X-100	7.4	18 mL
Triton 0.5%	PBS + 0.5% Triton X-100 + 5 mM EDTA	7.4	18 mL
SDC 0.01%	PBS + 0.01% SDC + 5 mM EDTA	8	18 mL
SDC 0.05%	PBS + 0.05% SDC + 5 mM EDTA	8	18 mL
SDC 0.1%	PBS + 0.1% SDC + 5 mM EDTA	8	18 mL

NOTE: All solutions must be sterile filtered.

Notes

Use to prepare PBS + Abx, NaCl, Triton 0.5%, and 3x SDC solutions
Use to prepare PBS + ions + Abx and Triton 0.0035% solutions

Antibiotic/antimycotic stock solutions: 10,000 U/mL penicillin/10,000 µg/mL streptomycin; 250 µg/mL amphotericin B; 50 mg/mL gentamicin

Pre-warm to 37 °C

Pre-warm to 37 °C; add benzonase right before use and sterile filter.

Prepare fresh, within 24 h of using

Prepare fresh, within 24 h of using

Prepare fresh, within 24 h of using

Prepare fresh, within 24 h of using

Prepare fresh, within 24 h of using

Table 2

Click here to access/download;Table;Table 2 - Decellularization protocol.xlsx

Step	Solution	Time (min)
1	PBS + ions + Abx	10
2	PBS + ions	5
3	Triton 0.0035%	20
4	Benzonase buffer	10
5	Benzonase	30
6	NaCl	5
7	PBS	8
8	SDC 0.01%	15
9	SDC 0.05%	15
10	SDC 0.1%	15
11	PBS	8
12	Benzonase buffer	8
13	Benzonase	60
14	PBS	12
15	Triton 0.5%	3
16	PBS	15
17	PBS	15
18	PBS	15
19	PBS	15
20	PBS + Abx	10
	Store in fresh PBS + Abx	

Table 3

Medium	Medium details	Volume required (per 12 ELTs)
Endothelial medium	MCDB-131 Complete without serum + 2% FBS + 0.1% gentamicin	24 mL
Epithelial base medium	50% DMEM / 50% F12 + 15 mM HEPES + 1.5 mM L-glutamine + 1% penicillin/streptomycin + 0.1% gentamicin + 10 µg/mL insulin + 5 µg/mL transferrin + 0.1% BSA Fraction V	48 mL
AEC2 supplements	3 µM CHIR99021 10 ng/mL KGF 50 nM dexamethasone 0.1mM 8-Bromo cAMP 0.1 mM IBMX 0.01 µM retinoic acid	

Notes

MCDB complete medium without serum contains 1% penicillin/streptomycin

We use a combination of low- and high-glucose DMEM to achieve a glucose concentration of 150 mg/dL in the complete medium

With L-glutamine in basal medium, final concentration = 4 mM

Stock = 10,000 U/mL penicillin/10,000 µg/mL streptomycin
Stock = 50 mg/mL gentamicin

Add supplements fresh to sterile-filtered epithelial base medium to make AEC2 growth medium; protect from light.

Table 4

Click here to access/download;Table;Table 4 - Antibodies used for immunostaining.xlsx

Primary Antibody	**Source**	**Species**	**Concentration**
anti-ABCA3	Abcam #ab24751	Mouse	1:50
anti-CD31	R&D Systems #AF3628	Goat	5 µg/mL
anti-Collagen IV	Abcam #ab6586	Rabbit	1:500
anti-Laminin	Abcam #ab11575	Rabbit	1:200
anti-ProCollagenIα1	Rockland #600-401-D19	Rabbit	1:50
anti-RTI-40 (podoplanin)	Terrace Biotech #TB-11ART1-40	Mouse	1:200
anti-SPB	Santa Cruz #sc-13978	Rabbit	1:50
Secondary Antibody	**Source**	**Species**	**Concentration**
anti-goat IgG, Alexa Fluor 647	ThermoFisher #A-21447	Donkey	1:500
anti-mouse IgG, Alexa Fluor 555	Invitrogen #A21424	Goat	1:500
anti-mouse IgG, Alexa Fluor 568	Invitrogen #A10037	Donkey	1:500
anti-rabbit IgG, AlexaFluor 488	Invitrogen #A11034	Goat	1:500
anti-rabbit IgG, Alexa Fluor 488	Invitrogen #A21441	Chicken	1:500
anti-rabbit IgG, Alexa Fluor 555	Invitrogen #A21429	Goat	1:500

Dear Dr. Saha,

Thank you for the opportunity to revise our manuscript entitled, “Engineered Lung Tissues Prepared from Decellularized Lung Slices” (Manuscript ID JoVE63151). We thank the editors and reviewers for their careful reading and constructive comments. We have made substantial changes based on these comments, particularly in the form of additional details throughout the Protocol and a revised Discussion of the method. We believe the revised manuscript to be significantly improved in terms of content and clarity. A point-by-point response to the editorial and reviewers’ critiques is below.

Thank you again for your consideration of this manuscript.

Sincerely,
Laura Niklason

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proofread the manuscript and corrected spelling and grammar issues where noted.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have removed all instances of personal pronouns from the Protocol.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: parafilm
All commercial language has been removed from the manuscript.

4. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
Text discussing the protocol has been removed from the Protocol portion of the manuscript.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). 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.” However, notes should be concise and used sparingly.
We have removed all instances of personal pronouns and phrases such as “could be” and “should be” from the Protocol, and we have revised all Protocol steps to use the imperative tense.

6. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
Additional details have been added to numerous steps in the Protocol.

7. Please provide incision size and the suture size throughout the protocol.
These details have been added.

Step 1.2.3: How long was the degassing performed? Please specify.
This detail has been added.

Step 2.1.1: Please specify the cannula size.
Additional details of the cannula components have been added.

Step 2.1.4: How was fur trimmed? How much was the concentration of povidone-iodine?
These details have been updated in the manuscript and the Table of Materials.

Step 2.1.5: How was the diaphragm punctured? Please specify all the incision sizes.
Additional details have been added for this step (including details of the diaphragm puncture and incision sizes), which now encompasses steps 2.1.5, 2.1.6, and 2.1.7.

Step 2.1.6: How were the cannula and connecter placed and secured?
Additional details have been added to clarify this step, now encompassing steps 2.1.8-2.1.10.

Step 2.1.9: Please specify the cannula and suture size.
The cannula is prepared and described in step 2.1.1. during the set-up of the perfusion system – a reference to that step has been added in step 2.1.12 for clarity. The suture is 4-0 polypropylene as specified. This step now encompasses steps 2.1.13 and 2.1.14.

Step 2.1.11: What was the perfusion rate?
The perfusion rate was specified during initial perfusion system set-up in step 2.1.2. This detail has been repeated in this step for additional clarity (now step 2.1.16).

Step 2.1.14: How were the agarose inflation and trachea capping done?
Additional details for this step have been added (now steps 2.1.19 and 2.1.20).

Step 2.2 NOTE: Please include a citation regarding the slicing procedure

The described slicing procedure was learned through training at our institution, not through any published work. However, we have added references to other articles demonstrating PCLS generation by means of various tissue slicers.

Step 2.2.2: What is the volume of HBSS?
This detail has been added (now step 2.2.3).

Step 3.3.2: Which ions, antibiotics, antimycotics were used in this study and at what concentration?
All decellularization solution recipes are detailed in Table 1 – Decellularization solutions. An additional reference to this table has been added in this step for clarity.

Step 4.1.3, 4.3.7: Please provide all microscope settings and parameters along with all the buttons click, software steps associated.
We have added a recommended magnification and further specified the use of a phase contrast microscope in these steps. Examining the tissue scaffolds and repopulated ELTs during culture is as simple as looking through the eyepieces of the microscope; no additional settings or software are required.

Step 4.2.1, 4.3.1: How were the cells counted? Please describe
These details have been added.

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The Discussion has been substantially revised to more clearly cover the requested topics.

9. Please do not abbreviate journal names in References.
The references have been revised to include full journal names.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this work, the authors present a protocol to generate engineered lung tissues (ELTs) by repopulating decellularized, precision-cut lung slices (PCLS) with primary endothelial cells, type II alveolar epithelial cells (AECs), and fibroblasts. Specifically, this work describes procedures to mount rat PCLS into reusable tissue culture cassettes and to decellularize such clipped slices generating acellular ECM scaffolds, which are then repopulated in customized seeding baths.

The involved protocol is quite well described, visual demonstration via JoVE video will be very helpful in guiding the user, especially a novice, through the intricate steps of reproducibly generating the tissue slices

We thank this reviewer for their thoughtful critiques; detailed responses are provided below.

Major Concerns:

Lines 337- 405: The concept of region-specific seeding is my main conceptual problem with this protocol: Simply seeding the various cell types, even, as suggested, sequentially, will not ascertain the appropriate zip code effect, i.e. that the various cell types will land on/migrate to the appropriate structures, e.g. AE2 in the alveolar space and EC in the vascular/capillary space. I am not convinced about the validity of the optimistic statement in lines 551/552. My skepticism is confirmed, in part ,by close inspection of Figure 6 panels E-G.

We agree with this reviewer that the seeding method for ELTs does not ascertain region-specific seeding. We also agree that we are not precisely recapitulating all features of alveolar cellular organization by this method. However, we stand by our observation that *despite this limitation*, the cells are repopulating the tissue in a non-random fashion, which we believe is likely influenced by cell-cell interactions as well as by differences in ECM composition and geometry in different microscopic regions of the decellularized scaffold. This speculation is based on our reading of other literature. One related study of particular mention that supports this hypothesis is that by Burgstaller *et al.* 2018 (Am J Physiol Lung Cell Mol Physiol, 2018 May 1;314(5):L708-L723), in which fibroblasts were seeded non-specifically onto decellularized lung slices. The cellular repopulation pattern and cellular phenotype were found to be significantly influenced by tissue type (i.e. healthy vs diseased) and by microscopic tissue region. Fibroblasts were additionally observed to invade into the interstitium – the location in which they reside in native tissue.

We have revised the Discussion to more clearly discuss this limitation (see lines 832-854), and have revised some of our language. However, we note that this limitation does not diminish what we believe to be the critical advantage of ELT culture: the ability to perform cell culture studies using a physiologically relevant 3D ECM substrate, in a manner accessible to many labs. The primary alternative that we imagine to culture cells on lung slices in a truly region-specific manner would entail seeding intact decellularized lungs via the airway and vascular compartments, and then slicing the tissue. However, this alternative method 1) is significantly more cost-, time-, and resource-intensive; 2) is lower throughput; 3) requires increased numbers of animals; and 4) is associated with an increased risk of contamination due to the challenges of whole lung culture and subsequent slicing of the seeded lung.

Minor Concerns:

Lines 35/36: For the sake of scientific rigor, the authors should cite the source of their 3 primary cells/cell lines (EC, AEC, and FB) and inform the audience how they validated their purity.

These details have been added in the beginning of the Representative Results section (lines 630-654).

Line 144: provenance /diameter of pulmonary artery cannula is not described
The pulmonary artery cannula is hand assembled. Additional details have been added to this step (step 2.1.1) describing the component parts, and an additional reference has been made to a drawing of the cannula in Figure 1.

Lines 198 pp: why are so may sections highlighted? he alveolar space and EC in the vascular/capillary space. \
The journal requests that "For a protocol section that exceeds 3 pages, highlight in yellow up to 3 pages to be featured in the video." As there are many steps to this protocol, we have proposed to feature in the video those portions of the protocol that are critical and/or unique to ELT generation and cannot be easily learned from another source.

Lines 427-445… the provenance /concentrations of the antibodies used for IHC staining are not, listed the IHC procedures are not describe in detail (references to prior publications?)
We apologize for this omission. ELT staining does not require specialized procedures, so we have added a reference to our standard immunostaining method from a prior publication in Results (see lines 675-676). The new Table 4 includes all antibodies and their respective concentrations used for the immunostaining images presented in this manuscript.

Lines 427 and 535: The statement that the trichrome and elastin show maintenance of these ECM proteins in the decell slices with the same quantity as the native lung is not tenable.
We would appreciate clarification on your concern with this point. If you are referring to the intensity of the histological staining in Figure 5B,C, we note that it is not an entirely straightforward comparison, as the cellular material in native tissues is also stained darkly by trichrome (nuclei are dark red to blue) and EVG (nuclei are blue/black). Thus, native tissues will necessarily stain more intensely than their decellularized counterparts. On close inspection we believe the referenced statement that ECM proteins in decellularized tissues are maintained at a quantity "similar" to that of native lung is reasonable, and that there is high preservation of ECM in both larger airways and vessels and in the alveoli. For example, note the preservation of elastin in the alveolar entrance rings in the decellularized tissue (arrowheads, revised Figure 5C). We have added images at higher magnification for all image panels in Figure 5 to allow better visualization.

Of note, the decellularization protocol used for ELT preparation is closely based on our lab's whole lung decellularization protocol, which by quantitative proteomics was demonstrated to preserve many ECM components at levels not significantly different from those in native lung (Calle 2016, Acta Biomater, 2016 Dec;46:91-100). While the protocol used for lung slices in this manuscript is not exactly the same – the whole lung protocol is based on *volume*; the lung slice protocol used in this manuscript is based on the *duration* for each of those volume-based steps – both expose the tissue to very low concentrations of detergent (not more than 0.5%) for very short periods of time (only 3-20 min for any individual detergent step). A reference to this

paper has been added (see lines 661-663) to help clarify the provenance of the ELT decellularization method.

Line 471: what kind of light microscopy?
Phase contrast microscopy was used to take the images in Figure 4C,D and Figure 6A-C. This detail has been added/corrected in the figure legends and related protocol steps.

Lines 483/484: Figure 6: "organoid formation" in Panels A and B (similar to the aggregation of fibroblasts seen in panel E) can be taken as euphemism for unsuccessful recellularization of alveolar structures, Panel A shows unevenness of reseeding; how frequent are images like panel C ? (Reproducibility of successful seeding?)
We used the phrase “organoid-like structures” to describe the formation of epithelial spheroid-type clusters on slices, which have the appearance of organoids or alveolospheres on histology (not shown in the images in Figure 6). We agree that these structures suggest that not all of the cells are repopulating alveolar structures.

In most cases, we find that across any single experiment (e.g. 12 or more ELTs) there is a high level of consistency of seeding density. In addition, the level of consistency has improved over time, which we suspect is related to improved technique in clipping slices. With experience, slices such as those in panel C account for less than 2% of our recellularized ELTs. We have added a discussion of this point and related troubleshooting to the Discussion (see Lines 802-815).

Reviewer #2:
The manuscript is clearly written and the methodology described to develop ELTs is quite well explained. There are some minor aspects that should be addressed in the final version of the manuscript:
Thank you for the careful reading and constructive comments; please see responses to specific comments below.

- 1.1.1 Could be of interest to include an alternative method in case that laser cutter is not available.
Unfortunately, a laser cutter (either the specified one or another) is required to cut the tissue culture cassettes. This point has been added as a limitation in the Discussion (see lines 817-818).

- 1.2.4 PDMS could be cured at RT (24h-48h) if needed?
In theory the PDMS could be cured at RT, however, we have not tested this ourselves. We are inclined to leave the curing instructions as currently described, as this is our standard protocol.

- 2. Could be interesting to add in the NOTE some hints to use lung tissue from other sources in function of the weight/volume of the organ.
While we leave the optimization related to specific alternative lung tissue sources to those researchers using them, we have added some additional details in the Discussion about the

critical step of lung inflation with agarose (see lines 774-797).

- 2.2 I suggest giving some extra tips/hints in the NOTE for using different microtomes.
As further information on cutting PCLS by different methods, we have added several references to other JoVE articles that demonstrate the use of different tissue slicers for preparing PCLS.

- 2.2.12 Some typo errors. Could it be done in liquid nitrogen? What if slices are just placed in the -80ºC freezer (not snap frozen)?
We apologize but we do not see any typos here – could you please clarify (now step 2.2.13)? In theory, the slices could be frozen in liquid nitrogen. However, as above, we have described the method that we have optimized for this protocol. We do not recommend placing slices directly in the freezer due to the higher risk of ice crystal formation and have added a related NOTE on that point to this step.

- There are no sterilization steps between 3.2 and 3.3?
This observation is correct. The decellularization protocol begins by incubating the clipped, native tissue slices in a high concentration of antibiotics/antimycotics (see step 3.3.2), but there is no initial, definitive sterilization step. Rather, the tissues are rendered sterile through the course of decellularization and final antibiotics/antimycotics incubation (see step 3.3.6). To date, we have decellularized hundreds of lung slices by the described method, and only one slice has developed contamination during culture. We highly suspect this case was due to an inadequate duration of antibiotics/antimycotics incubation at the end of the protocol (<15 hrs, a significant departure from the specified 48 hrs).

- 4. It would be interesting to include a table with references to protocols to isolate the different cell types from rats.
References to the methods used to isolate AEC2s and fibroblasts for the ELTs presented here, as well as to a few other published protocols, have been added at the beginning of the Representative Results section (see lines 630-654).

- There is some slight agitation in step 4.2.3 as used to do in standard cell passage? Same for AEC2s and FBs.
We included these steps directing the reader to “swirl the prepared cell suspension” prior to seeding not as an agitation step as done in cell passaging after trypsinization, but rather to help resuspend cells that tend to sink as the cell suspension sits (e.g. during the time taken to transfer scaffolds to the seeding baths in step 4.2.2 prior to seeding). This is of particular concern for cells of larger size such as fibroblasts. Gently swirling the cell suspension helps to redistribute cells, with less risk of shear stress-associated damage that can result from repeated pipetting.

- 5. Could you include some hit for confocal immunohistochemistry of the ELTs?
We have added a reference in the Results for our staining methods (see lines 675-676). ELTs do not require any specialized techniques for immunostaining, and can be treated as lung tissue.

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Supplemental Coding Files

Supplementary File 4 - Seeding bath mold base.SLDPRT

Supplemental File 3

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Supplemental Coding Files

Supplementary File 3 - Tissue culture cassette tabs.png

Supplemental File 5

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Supplemental Coding Files

Supplementary File 5 - Seeding bath mold ring_3D drawing.tif

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Author(s): Katherine L. Leiby, Ronald Ng, Stuart G. Campbell, Laura E. Niklason

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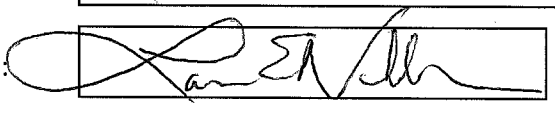
Signature:	
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