Journal of Visualized Experiments

Decellularization of the murine cardiopulmonary complex

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61854R1
Full Title:	Decellularization of the murine cardiopulmonary complex
Corresponding Author:	Janine T Erler University of Copenhagen Biotech Research and Innovation Centre Copenhagen, DENMARK
Corresponding Author's Institution:	University of Copenhagen Biotech Research and Innovation Centre
Corresponding Author E-Mail:	janine.erler@bric.ku.dk
Order of Authors:	Alejandro Mayorca
	Maria Rafaeva
	Oliver Willacy
	Chris Madsen
	Raphael Reuten
	Janine T Erler
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Copenhagen, Denmark
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Biochemistry
Please provide any comments to the journal here.	

TITLE:

Decellularization of the Murine Cardiopulmonary Complex

AUTHORS AND AFFILIATIONS:

Alejandro E Mayorca-Guiliani[1]#, Maria Rafaeva[1], Oliver Willacy, Chris D Madsen[2], Raphael Reuten[1], Janine T Erler[1]#

1. Biotech Research and Innovation Centre, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark
2. Department of Laboratory Medicine, Division of Translational Cancer Research, Lund University, Lund, Sweden

Email addresses of co-authors:
Alejandro E Mayorca-Guiliani (alejandro.mayorca@bric.ku.dk)
Maria Rafaeva (maria.rafaeva@bric.ku.dk)
Oliver Willacy (oliver.willacy@bric.ku.dk)
Chris D Madsen (chris.madsen@med.lu.se)
Raphael Reuten (raphael.reuten@bric.ku.dk)
Janine T Erler (janine.erler@bric.ku.dk)

Correspondence to:
Alejandro E Mayorca-Guiliani (alejandro.mayorca@bric.ku.dk)
Janine T Erler (janine.erler@bric.ku.dk)

KEYWORDS:

Decellularization, Lungs, Heart, Extracellular Matrix

SUMMARY:

This protocol aims to decellularize the heart and lungs of mice. The resulting extracellular matrix (ECM) scaffolds can be immunostained and imaged to map the location and topology of their components.

ABSTRACT:

We present here a decellularization protocol for mouse heart and lungs. It produces structural ECM scaffolds that can be used to analyze ECM topology and composition. It is based on a microsurgical procedure designed to catheterize the trachea and aorta of a euthanized mouse to perfuse the heart and lungs with decellularizing agents. The decellularized cardiopulmonary complex can subsequently be immunostained to reveal the location of structural ECM proteins. The whole procedure can be completed in 4 days.

The ECM scaffolds resulting from this protocol are free of dimensional distortions. The absence of cells enables structural examination of ECM structures down to submicron resolution in 3D. This protocol can be applied to healthy and diseased tissue from mice as young as 4-weeks old, including mouse models of fibrosis and cancer, opening the way to determine ECM remodeling

associated with cardiopulmonary disease.

INTRODUCTION:

The ECM is a three-dimensional network made of proteins and glycans that accommodates all cells in a multicellular organism, giving organs their shape and regulating cell behavior throughout life[1]. From egg fertilization onwards, cells build and remodel the ECM, and are in turn strictly controlled by it. The purpose of this protocol is to open a way to analyze and map mouse ECM, as mice are the most used model organism in mammalian pathophysiology.

The development of this method was driven by the need to characterize and isolate metastasis-associated native ECM[2]. As tumors lack proper anatomical vascularization and mice are relatively small organisms, microsurgical procedures were designed to retrogradely catheterize the aorta, while isolating the circulation of the major vessel leading to a tumor (e.g., the pulmonary veins), thus focusing reagent flow and allowing tumor decellularization. This method produces ECM scaffolds with a conserved structure[2] that can be immunostained and imaged, allowing ECM structure mapping in submicron detail. To carry out this protocol, it is necessary to acquire surgical and microsurgical skills (dissection, microsuturing and catheterization) that may represent a potential limitation to its use. To our knowledge, this method represents the state-of-the-art for native ECM structure imaging analysis[2,3].

PROTOCOL:

All procedures included here have been reviewed and approved by the ethical committee regulating experimental medicine in the University of Copenhagen and agree with Danish and European legislation. To demonstrate this protocol, we have used female BALB/cJ mice of 8-12 weeks of age and an MMTV-PyMT female mouse of 11 weeks of age.

NOTE: Avoiding bacterial contamination of the decellularized ECM scaffold gives the best imaging outcome and allows long-term sample storage. It is therefore important to keep all the steps sterile. As such, all instruments and surgical material, including suture, micro-suture, solutions, tubing, Luer connectors and catheters, must be sterile. Surfaces, including a polystyrene tray, must be disinfected with 70% ethanol, and the perfusion should preferably be carried out under a laminar flow hood. All procedures take place at room temperature unless otherwise indicated.

1. Post-mortem microsurgery

1.1. Euthanize the mouse using a CO_2 chamber.

1.1.1. Use a 4 L chamber, filled with 100% CO_2, starting at 0.2 L/min for 2 min and increasing until reaching a flow of 0.8 L/min after 3 min. The mouse should fall unconscious during the first 2 min, and then respiration should cease (usually around 5 min, but flow can be maintained as necessary).

1.2. Shave the thorax, abdomen and back of the mouse with the hair clipper and disinfect with

70% ethanol. Shaving greatly reduces the number of artifacts due to the presence of hair either on samples for imaging or biochemical analysis.

1.3. Pin the mouse to a polystyrene tray, extending its fore- and hindlimbs, as well as its head and tail. Place it under the microsurgery microscope.

1.4. Using a Mayo straight pattern scissors establish surgical access with a cutaneous incision running from the submandibular region to the lower abdomen and dissect subcutaneously to expose the thoracic wall and peritoneum.

1.5. Using microsurgical scissors, cut the pectoralis major and pectoralis minor muscles along the sixth intercostal space on both sides of the thoracic wall.

1.6. Using straight-pattern scissors, cut the sternum along the previous incisions, and then complete a sternotomy by cutting the sternum along its long axis, then elevate and pin both sides of the thoracic wall to expose the cardiopulmonary complex.

1.7. Using round-tipped micro-forceps (or Dumont micro-forceps), excise the thymus and surrounding adipose tissue by delicately pulling them off their attachments. This will reveal the major vessels.

1.8. Using the cautery, cauterize the descending cava vein and, using straight pattern scissors, cut the esophagus.

1.9. Using sharp micro-forceps, separate the brachiocephalic veins and the brachiocephalic, left common carotid and left subclavian arteries from the underlying tissue to facilitate ligation and cauterization.

1.10. Using micro-needle holder, sharp micro-forceps and 9-0 suture place stitches above the emergence of the brachiocephalic, left common carotid and left subclavian arteries.

1.11. Cauterize the brachiocephalic veins.

1.12. Separate the submandibular salivary glands along the midline to expose the neck muscles and the trachea. Separate the muscles to expose the cricothyroid ligament. Using micro-scissors, open an entrance by sectioning the ligament.

1.13. Introduce a 27 G catheter in the trachea and delicately push until the trachea branches into the bronchi (i.e., until resistance to the catheter is met, then retreat 3 mm). Be careful not to disrupt the bronchi. Using a 6-0 suture, place 3 stitches around the trachea to secure the catheter.

1.14. Section the mouse at the height of the 12th thoracic vertebra. The descending aorta runs anteriorly to the spine and should be sectioned here along with the spine. Set the lower half

apart.

1.15. Retrogradely catheterize the aorta and push the catheter until it reaches the aortic arc. Using 9-0 suture, place 4 stitches around the aorta, beginning 5 mm below the catheter tip.

2. Decellularization

2.1. Connect the mouse to a pump system using silicone tubing and Luer connectors. Perfuse with deionized water at 200 µL/min for 15 min. Maintain this flow rate during decellularization.

2.2. Change the perfusion agent to 0.5% sodium deoxycholate (DOC) diluted in deionized water and perfuse overnight.

2.3. Change the perfusion agent to 0.1% sodium dodecyl sulphate (SDS) diluted in deionized water and perfuse for 8 hours.

2.4. Perfuse with deionized water overnight to wash away SDS and DOC for 24 h.

2.5. Resect the decellularized heart and lungs by sectioning its attachments to the thorax using a curved scissors and store in a sterile cryo-tube with deionized water with 1% (v/v) penicillin–streptomycin and 0.3 µM sodium azide at 4 °C. ECM scaffolds can be stored for at least for 12 weeks[1]. If the scaffold will be used for biochemical analysis (e.g., mass spectrometry), snap freeze in liquid nitrogen.

3. Immunostaining

3.1. Plan the imaging: determine primary antibody (or antibodies) and the combination of fluorescently conjugated secondary antibodies to match each other and to fit the laser lines of the fluorescence microscope.

3.2. Block the sample by immersing it in a cryotube containing 6% (v/v) donkey serum - 3% (w/v) bovine serum albumin (BSA) overnight.

3.3. Incubate with primary antibody (or antibodies) in 3% donkey serum in PBS for 24 h.

3.4. Wash 5 times for 1 h each time in 0.05% tween 20 in PBS (PBST).

3.5. Incubate the sample with fluorescently conjugated secondary antibody (or antibodies) in 3% donkey serum in PBS for 24 h.

3.6. Wash 5 times for 1 hour in 0.05% (PBST). Wait 1 h between each wash.

3.7. Add deionized water and store at 4 °C away from direct light. At this point, the scaffold is ready to image.

4. Imaging

4.1. Place the sample in a glass-bottomed dish and humidify it with two droplets of storing solution (PBS or deionized water).

4.2. Prepare the objective. We recommend using a water immersion objective.

4.3. Inspect the sample using fluorescence light.

4.4. Switch to computer control. Turn on lasers and adjust laser intensity, pinhole aperture, detectors wavelengths, gain, resolution and zoom. Set the number and step size for z-stack and begin acquisition. We recommend using multiphoton laser excitation to increase tissue penetration and to minimize scattering of light, bleaching and tissue damage.

5. Hematoxylin-eosin staining

5.1. Excise 1 lung lobe from a euthanized mouse.

5.2. Place in a 10 mm x 10 mm x 5 mm cryomold and cover it with approximately 500 µL of OCT compound.

5.3. Freeze on dry ice (-70 °C) and maintain the sample at that temperature.

5.4. Excise one decellularized lung lobe from a processed mouse according to step 2.5.

5.5. Place in a cryomold with the largest surface area down and cover it with OCT compound as specified in step 5.2.

5.6. Freeze on dry ice (-70°C) and maintain the sample at that temperature until otherwise required. The sample can be stored for at least 12 weeks.

5.7. Section frozen tissue blocks at -20 °C in a cryostat with 5 µm thickness and place sections on adhesive glass slides and store at -80 °C.

5.8. Take slides to room temperature until air dried (approximately 20 min).

5.9. Shortly immerse in PBS and fix by immersing the slides in 4% paraformaldehyde in PBS for 15 min. Wash once in PBS for 5 min, then twice in distilled water for 5 min.

5.10. Immerse in Mayer's hematoxylin solution for 10 min. This time can be optimized according to tissue source and stain preparation.

5.11. Wash in a Coplin jar under running distilled water for 10 min.

5.12. Immerse in eosin solution for 7 min. This time can be optimized according to tissue source and stain preparation.

5.13. Dip in 50% ethanol to remove excess Eosin and dehydrate by shortly dipping in 70% ethanol, and in 96% and 100% ethanol for 30 seconds. Dip in xylene several times.

5.14. Apply few drops of DPX mounting medium and place a glass coverslip.

5.15. Leave slides to dry overnight under a chemical hood.

5.16. Scan slides in a slide scanner.

REPRESENTATIVE RESULTS:

Cardiopulmonary decellularization

After successfully completing the protocol, the heart and lungs, as well as annex tissue such as the aortic arc, will be free of cells. Decellularization can be validated by hematoxylin-eosin staining (**Figure 1**) of the ECM scaffolds showing removal of the nuclei comparing to the native tissue. These scaffolds retain the dimensions of fresh organs and its insoluble ECM structure is intact[2]. **Figure 2** shows a schematic representation of the key surgical steps required to successfully perfuse the mouse cardio-pulmonary complex.

ECM Imaging

In a standard setting, secondary antibodies can be used in green, red and far-red fluorescence channels (i.e., 488 nm, 555nm/594 nm and 647 nm wavelength detection); the addition of second harmonics generation (SHG) imaging using 2-photon excitation will reveal fibrillar collagen. Laser excitation can incite tissue autofluorescence and caution must be applied when using it with green fluorescence, as it may confound imaging data. A straightforward way to validate auto-fluorescence is to image an unstained control tissue and set laser intensity and detector gain accordingly and compare this with the antibody staining. However, this autofluorescence can be used as an advantage, as it can expose elastin in lungs scaffolds.

ECM scaffolds showed increased permeability and light penetrability[2]. Using this protocol with a motorized microscope stage allows for three-dimensional, tiled imaging of whole-mount) samples at submicron resolution (**Figure 3**). In case sectioning the tissue is necessary (e.g., to image cardiac walls or deep pulmonary parenchyma) tissue should be sectioned with a sharp scalpel before staining is conducted.

FIGURE LEGENDS:

Figure 1. Validating decellularization. Hematoxylin-Eosin staining of snap frozen samples from native and decellularized lungs and heart. Notice the absence of nuclei in decellularized samples. All scales in microns.

Figure 2. Micro-surgery schematic showing the key steps required to decellularize the cardio-

pulmonary complex.

Figure 3. Representative multiple protein immunostaining of decellularized PyMT mouse lungs from a 11-week-old female mouse. Tile mosaic showing the maximum projection of a z-stack. Inset 1 shows the pleura. Inset 2 shows normal parenchyma ECM. Inset 3 shows a bronchiole. The colors have been made accessible for the color blind. All scales in microns.

DISCUSSION:

Decellularization techniques based on tissue agitation alter ECM structure, making them unsuitable for ECM structure analysis[4]. Perfusion decellularization, using an anatomical route such as the aorta of the trachea, allows to reach the capillary bed, or terminal alveoli, and facilitates the delivery of decellularizing agents throughout the organ. The use of zwitterionic, anionic and non-ionic detergents to decellularize tissue is reported[4,5,6], however, sodium dodecyl sulphate (SDS, anionic) linearizes fibrillar collagen in the mouse fat pad[2] but not in the lungs; this suggests the choice of detergent must be optimized, adapting to the target tissue to maintain ECM structure. Tissue clearing methods could conceivably be used for ECM analysis, although they require chemicals that can change ECM cross linking, and tissue dimensions[7,8,9]. While acquired tissue transparency allows enhanced microscopic imaging, the presence of cells significantly worsens antibody penetration and may cover ECM epitopes/proteins. Isolating and imaging intact ECM permits quantitative analysis of its structure with analytical tools[2,10], mapping its composition[2] and opens the way for further ECM biochemical examination.

The dissection and ligation of major vessels and the consequent isolation of coronary and pulmonary circulation is necessary to achieve uniform pressure of perfused solutions throughout the tissues. Therefore, this protocol is dependent on the microsurgical expertise of the main operator. It is critical to operate with precision, so as to preserve vessels, lungs and heart intact. Executing this protocol repeatedly to understand the three-dimensional anatomy of the thorax is paramount to obtain consistent results.

The surgical procedure shown here sums the basic steps to access the mouse vasculature[1,2] and the organs in its territory. By changing the ligature pattern, it is possible to access the head and neck, the fore limbs and fat pads. Using the same skills, it is possible to decellularize the sub-diaphragmatic organs.

Equally as important is the careful design of the immunostaining setup. We have previously compiled a catalogue of validated antibodies against structural ECM proteins[2]. The standard setup can reveal up to three proteins and fibrillar collagen simultaneously, enabling cross-examination.

The significance of this method lies in the possibility of obtaining structurally and dimensionally intact ECM scaffolds. The deconstruction of a complex tissue into discreet components is one of the fundamental goals of bioengineering; while it is relatively straightforward to isolate cells, or blood, from an organ, there were no methods to obtain its ECM scaffolding. This was especially true of tumors, but the method presented here opens the way for ECM isolation in any mouse

strain for anatomical and biochemical analysis of the ECM.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Hynes, R.O. Extracellular matrix: not just pretty fibrils. *Science*. **326**, 1216-1219 (2009).

2 Mayorca-Guiliani, A. E. et al. ISDoT: in situ decellularization of tissues for high-resolution imaging and proteomic analysis of native extracellular matrix. *Nature Medicine*. **23**, 890-898 (2017).

3 Mayorca-Guiliani, A. E. et al. Decellularization and antibody staining of mouse tissues to map native extracellular matrix structures in 3D. *Nature Protocols*. **14**, 3395-3425 (2019).

4 White, L.J. et al. The impact of detergents on the tissue decellularization process: A TOF-sims study. *Acta Biomaterialia*. **50**, 207-219 (2017).

5 Ott, H.C. et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nature Medicine*. **14**, 213-221 (2008).

6 Uygun, B.E. et al. Organ re-engineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nature Medicine*. **16**, 814-820 (2010).

7 Susaki, E. A. et al. Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging. *Nature Protocols*. **10**, 1709-1727 (2015).

8 Tomer, R., Ye, L., Hsueh, B., Deisseroth, K. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. *Nature Protocols*. **9**, 1682-1697 (2014).

9 Erturk, A. et al. Three-dimensional imaging of solvent-cleared organs using 3DISCO. *Nature Protocols*. **7**, 1983-1995 (2012).

10 Wershof, E. et al. A FIJI Macro for quantifying pattern in extracellular matrix. *BioRxiv*. Doi.org/10.1101/867507 (2019)

Figure 1

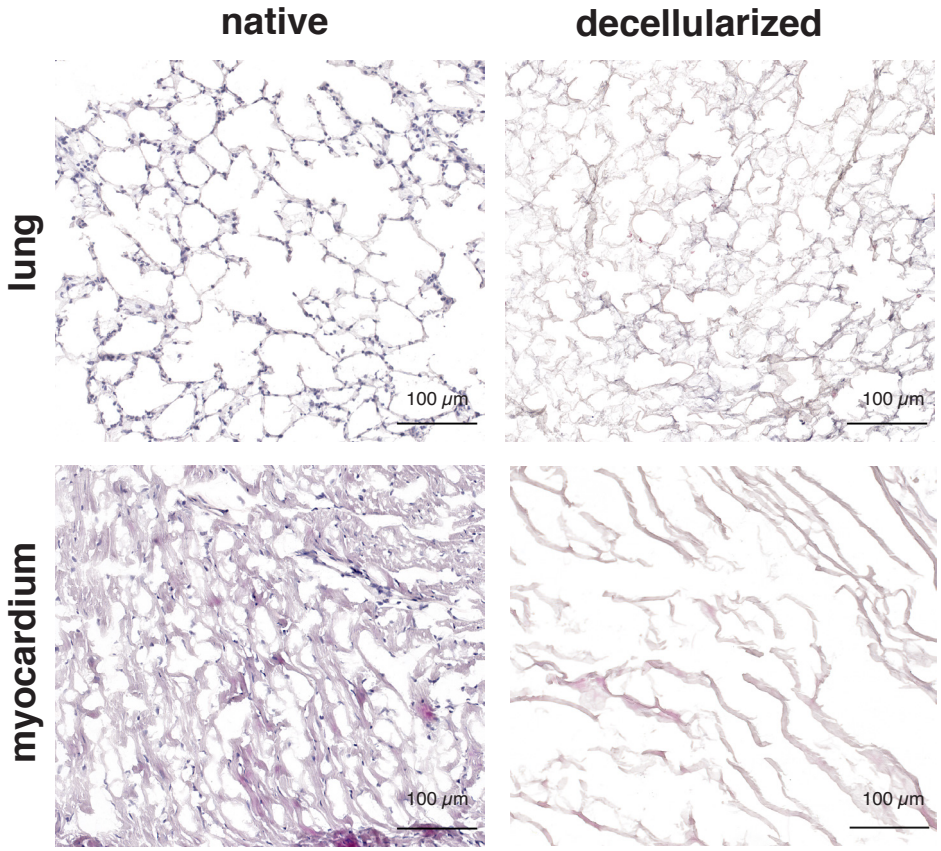


Figure 2

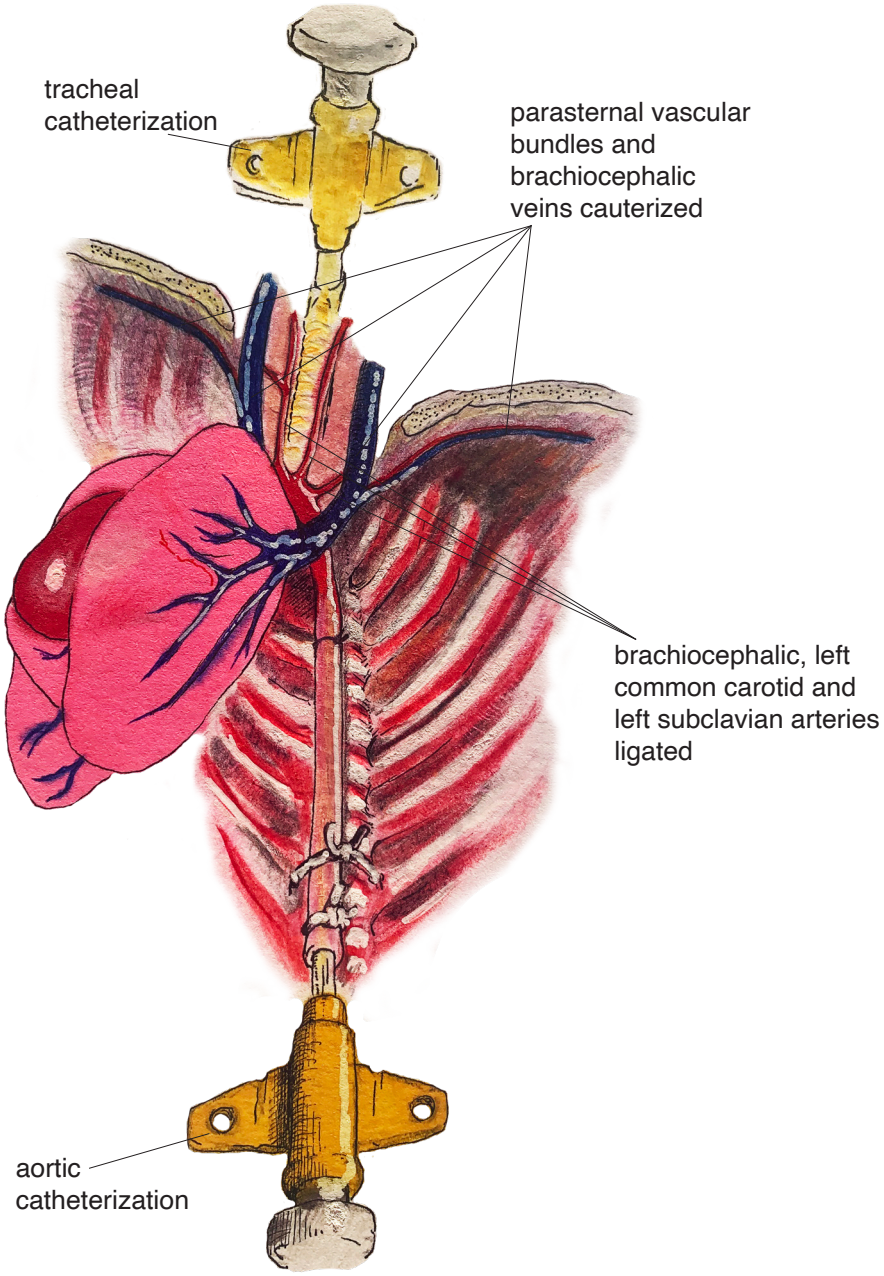
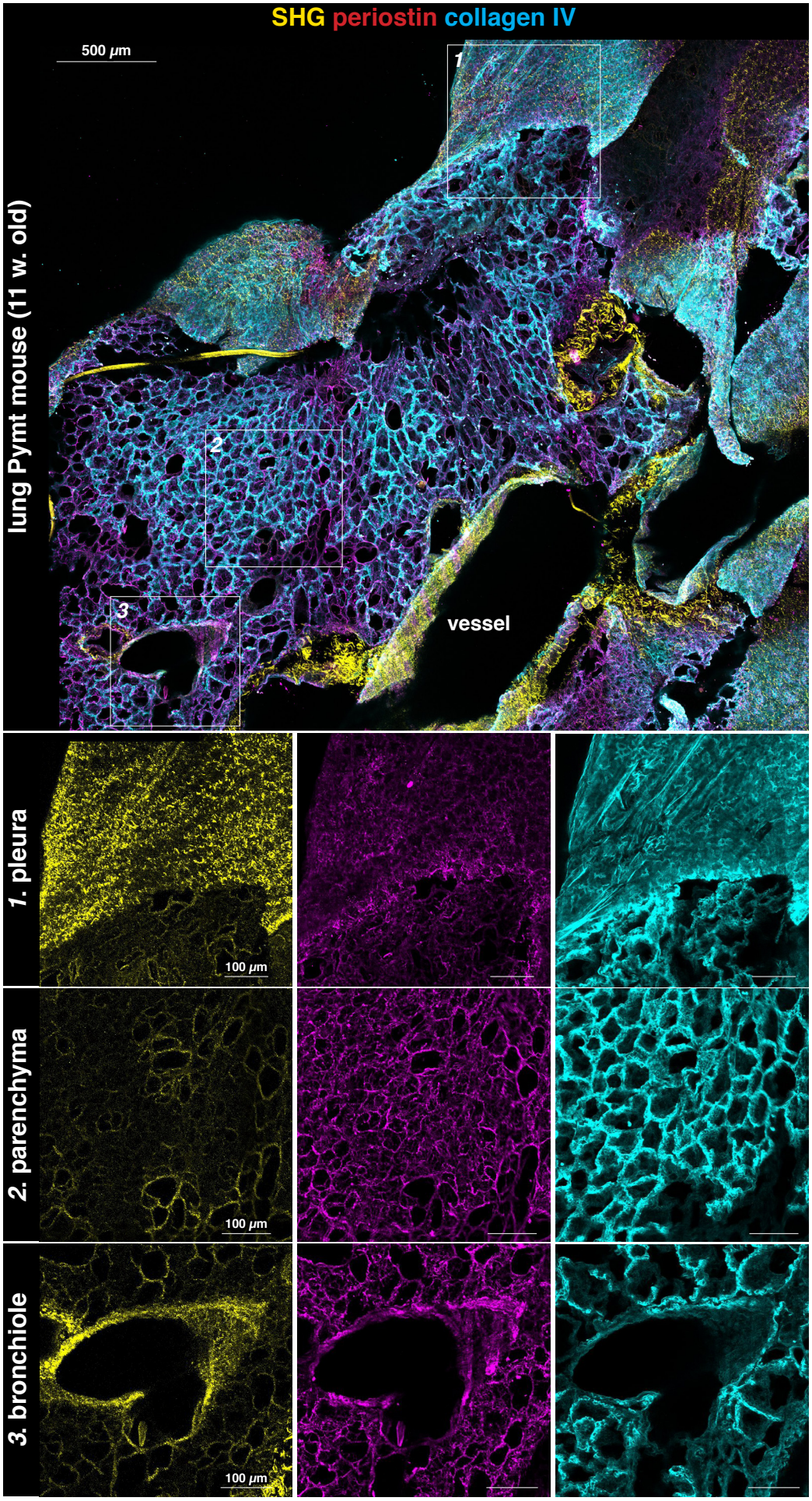


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
MICROSURGERY			
6-0 suture, triangular section needle (Vicryl)	Ethicon	6301124	
9-0 micro-suture (Safil)	B Braun	G1048611	
Adson forceps	Fine Science Tools	11006-12	
Adson forceps with teeth	Fine Science Tools	11027-12	
Castroviejo microneedle holder	Fine Science Tools	no. 12061-01	
CO_2 ventilation chamber for mouse euthanasia			
Deionized water (Milli-Q IQ 7000, Ultrapure lab water system)	Merck	ZIQ7000T0	
Disposable polystyrene tray (~30 × 50 cm)			
Dissection microscope (Greenough, with two-armed gooseneck)	Leica	S6 D	
Double-ended microspatula	Fine Science Tools	10091-12	
Dumont microforceps (two)	Fine Science Tools	11252-20	
Dumont microforceps with 45° tips (two)	Fine Science Tools	11251-35	

Hair clippers	Oster	76998-320-051
Halsey needle holder (with tungsten carbide jaws)	Fine Science Tools	12500-12
Intravenous 24-gauge catheter (Insyte)	BD	381512
Intravenous 26-gauge catheter (Terumo)	Surflo-W	SR+DM2619WX
Mayo scissors (tough cut, straight)	Fine Science Tools	14110-15
Microforceps with ringed tips (two)	Aesculap	FM571R
Micro-spring scissors (Vannas, curved)	Fine Science Tools	15001-08
Minicutter	KLS Martin	80-008-03-04
Molt Periostotome	Aesculap	D0543R
Needles (27 gauge; Microlance)	BD	21018
Paper towel (sterile) or surgical napkin		
Serrated scissors (CeramaCut, straight)	Fine Science Tools	14958-09
Spatula (Freer-Yasargil)	Aesculap	OL166R
Syringes (1 mL; Plastipak)	BD	3021001
Syringes (10 mL; Plastipak)	BD	3021110
Tendon scissors (Walton)	Fine Science Tools	14077-09

IMMUNOSTAINING

Alexa Fluor 488 donkey anti-guinea pig IgG	Thermo Fisher Scientific	A-11055	
Alexa Fluor 594 donkey anti-rabbit IgG	Life Technologies	A11037	
BSA(albumin bovine fraction V, standard grade, lyophilized)	Serva	11930.03	
Collagen IV polyclonal antibody (RRID: AB_2276457)	Millipore	AB756P	Host: rabbit
PBS (pH 7.4, 10×, Gibco)	Thermo Fisher Scientific	70011044	Host: goat
Periostin polyclonal antibody (a kind gift from Manuel Koch. RRID:AB2801621)			Host: guinea pig
Scalpel disposable with blade no.11 (pcs. 10)	VWR	233-5364)	
Serum (normal donkey serum)	Jackson ImmunoResearch	017-000-121	
Tween 20	Sigma-Aldrich	P9416-50ML	

IMAGING

Detectors (hybrid detector (Leica, HyD S model) and photomultiplier tubes (PMTs;)	Leica

Fluorescence light source	Leica	EL6000
Microscope (inverted multiphoton microscope)	Leica	SP5-X MP
Objective (lambda blue, 20×, 0.70 numerical aperture (NA) IMM UV)	Leica	HCX PL APO
Two-photon Ti–sapphire laser (Spectra-physics, Mai Tai DeepSee model)		
White-light laser (WLL)	Leica	

DECELLULARIZATION

70% Ethanol (absolute alcohol 99.9%); absolute alcohol must be adjusted to 70% (vol/vol) using deionized water	Plum	1680766
Deionized water (Milli-Q IQ 7000, Ultrapure lab water system)	Merck	ZIQ7000T0
Luer-to-tubing male fittings (1/8 inch)	World Precision Instruments	13158-100
PBS (pH 7.4, 10×, Gibco)	Thermo Fisher Scientific	70011044

Penicillin-streptomycin	Gibco	15140122
Peristaltic pump (with 12 channels)	Ole Dich	110AC(R)20G75
Silicone tubing (with 2-mm i.d. and 4 mm o.d.)	Ole Dich	31399
Sodium Azide	Sigma-Aldrich	08591-1ML-F
Sodium deoxycholate (DOC)	Sigma-Aldrich	D6750-100G
Sodium Dodecyl Sulphate	Sigma-Aldrich	L3771-500G

H&E STAINING

4% PFA	Fisher Scientific	15434389
96% Ethanol	Plum	201446-5L
Absolute ethanol	Plum	201152-1L
Coverslips (24x50mm; 1000 pcs)	Hounisen	422.245
Cryomolds Intermediate (15 x 15 x 5 mm; 100 pcs)	Tissue-Tek	4566
Cryostat	Leica	CM3050S
DPX mounting medium	Hounisen	1001.0025
Eosin Y solution alcoholic 0.5%	Sigma	1024390500
Feather microtome blac	Pfm medical	207500003
Fisherbrand Superfrost Plus slides (25 x 75 mm; 144 pcs)	Thermofisher	6319483

Mayers hematoxylin	Sigma	MHS32-1L
OCT compound	VWR	361603E
Slide scanner (Nanozoomer)	Hamamatsu Photonics	
Xylene	Sigma	534056-4L

Response to reviewer's comments

We thank the reviewers for their informed and diligent reading of our manuscript and their suggestions to improve it.

We have included two new figures: figure 2 depicts a schematic of the micro-surgery procedure and figure 3 (substituting figure 2 in the previous manuscript) shows a decellularized lung from a PyMT mouse with metastatic breast cancer immunostained for periostin and collagen IV. This is an original image and does not need to be referenced to a previous paper.

A detailed point-by-point response to reviewers' comments is provided below.

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 proof-read the manuscript and changed it to American English.

2. Please cite at least 10 publications.

New references, guided by reviewer's comments, are now included to complete 10.

3. Figure 1: Please add units to the scale bars in the figure: 100 microns?

Units have been specified in figures 1 and 3.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

All reference numbers are now in superscript.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have modified the protocol to make it clearer to the reader.

6. What is the age/gender/strain of the mouse used?

The age, gender and strain of the mice used in this manuscript are now specified in the protocol text.

7. 3.1: How is this excision done?

A description of the procedure needed to complete the excision is now in 5.1.

8. 3.2: Perfuse for how long?

A precise description of perfusion time is included in point 2.

9. 3.3/3.6: How much OCT compounds is used and for how long?

OCT compound is a wax-glycol blend that serves to embed snap frozen tissue to be sectioned for microscopic examination. We specify how much is used, but in principle the time it is used is equal to the time the tissue block is embedded and stored at -80°C.

10. 3.4/3.7: Freeze for how long? At what temperature?

The temperature and time are now specified.

11. 3.8: Dry for how long?

Drying time now included in the step.

12. 3.13: Wash for how long?

Washing cycles and time are now made clear.

13. Please discuss any limitations of the protocol in the discussion.

We insist in the importance of acquiring micro-surgical skills to complete the protocol. This is the main limitation we have noticed since we originally published the method in Nature Medicine in 2017. We expect that showing a video in JOVE will help overcome this obstacle.

Otherwise, we believe this protocol represents the state-of-the-art in ECM structure visualization and its limitations are inherent to its results: removing the cells prevents the study of cell-ECM interaction. We have mentioned these points in the discussion.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript described the protocol of decellularization for mouse heart and lung. However, the protocol described in the manuscript is difficult to be followed by other researchers. So, the authors should revise it. Specific comments are below.

We thank reviewer #1 for a detailed reading of our manuscript and agree on the complexity of the protocol. We have taken care to follow all recommendations.

Major Concerns:

1) The authors should mention the strain, sex, and age of used mouse.

Strain, sex and age of the mice used are now specified in the introduction to the protocol.

2) What was the increase rate of CO2 for euthanizing the mouse?

The euthanasia step is now explained in detail in 1.1.

3) For decellularization step, how did the authors keep sterile condition?

Instructions are detailed in the introduction to the protocol.

4) What was the solvent for SDS and DOC?

The solvent is deionized water, perfused as instructed in 2.4.

5) What was the perfusion rate and temperature during SDS and DOC treatments.

Flow speed and temperature for all steps are now specified. Briefly, decellularization is done at room temperature and samples storage can be done at either 4°C or -80°C depending on the procedure.

6) How did the authors wash decellularized tissues? Were the tissues perfused or stirred in the water? The authors should describe in detail. Also, the authors should give the temperature in wash process.

These steps are now specified in the protocol.

7) What is the storage condition, particularly temperature? Also, please give the storage period.

Storage conditions and time are specified in the protocol.

8) Please provide the provider of penicillin-streptomycin and sodium azide in the table.

This is now specified in 2.5.

9) What was the perfusion condition (temperature and perfusion rate) during OCT compound process?

OCT is not perfused. The sample is placed in a cryomold, and then covered with OCT. This is then frozen at -70°C as described in 5.

10) Please give the full name of PFA.

This is now specified in 5.9.

11) Please rewrite the manuscript in American English.

The manuscript is now edited in American English.

Reviewer #2:

Manuscript Summary:

The authors describe a microsurgical procedure of euthanized mice to access the heart and lungs as an approach to decellularize "in situ" these two tissues via perfusion-based decellularization using standard decellularization techniques based on DOC/SDS. The authors provide H&E staining of the resulting decellularized tissues and discuss the validity of this method in obtaining structurally and dimensionally intact ECM scaffolds. The reviewer has the following comments.

We thank reviewer #2 for a detailed reading of our manuscript. We have taken care to follow all recommendations.

Major Comments:

1. In the introductory section the authors present the different approaches that can be used for tissue decellularization, mentioning clearing methods and standard decellularization procedures. Can the authors elaborate on the discussion part of the manuscript the choice for DOC/SDS in favor of other widely used chemical decellularization methods based on other detergents and also detergent free methods? It would be interesting to the readership to have some other references to other methods that can be used for perfusion-based decellularization in comparison to that employed herein.

We now elaborate on the detergent choice for decellularization. In our experience, the detergent must be chosen after optimization, since the effects on ECM seem to be tissue-specific. This protocol has the aim of producing scaffolds that conserve their structure, we have found that

Sodium Deoxycholate and Sodium Dodecyl Sulphate fulfill this aim for the cardio-pulmonary complex.

2. Can the authors compare DOC/SDS methods for decellularization with others available, how does this method that is presented provide advantages?
Does it provide more collagen microstructure retention than for instance Triton-X100, CHAPS or other methods? Can the authors elaborate?
As with the previous comment, we elaborate in the discussion section that we rely on our experience to recommend a DOC-SDS combination.

3. The evaluation of the decellularization procedure can further be completed with lipid analysis, DNA analysis and also collagen content characterization/orientation analysis, etc. The authors could include relevant references for these protocols in the manuscript discussion so as to guide the readership to such additional characterization methodologies according to specific applications in addition to those associated with immunocytochemistry.
In our original publication we describe DNA analysis and fiber orientation and spacing analysis. We have referenced our Nature Medicine paper.

4*. The authors could include sketch anatomic images (medical procedure like illustrations) to better guide the readership through the procedure, this would aid especially those not highly skilled in such microsurgical procedures, which may be the case of JOVE readership that aims to learn new procedures.*
We thank the reviewer for this useful suggestion. We have included a new figure (Figure 2) to illustrate the key surgical steps required to complete this protocol.

Minor Comments:
1. Per journal Standards the manuscript should be written in English US, the authors should modify the text to accommodate such requirements. Please perform an English revision to the text on such grounds. Also include notations in SI units system particularly for decimals.
We have edited the manuscript to address these points.

2. Reference 1 and 5 are the same reference please correct.
The references have been corrected and expanded to 10.

Reviewer #3:
In this manuscript, the authors describe a protocol to decellularise the heart and lungs of mice, resulting in a scaffold of ECM that can be used as a platform to study the structure and function of

the ECM. The protocol is based on several excellent publications by the Erler group and would no doubt be very useful to the community. The protocol is overall very detailed and clear.

We thank reviewer #2 for a detailed reading of our manuscript and for highlighting the usefulness and clarity of our protocol. We have taken care to follow all recommendations.

A few minor issues should be addressed prior to publication:

1. In the abstract, it would be helpful if the authors start by stating the general purpose of this protocol, namely to decellularise the heart and lungs of mice to provide an experimental platform for research of the role of ECM, rather than the current statement: "We present a microsurgical procedure designed to catheterise the trachea and aorta of a euthanized mouse so as to access the heart and the lungs." These are just the experimental steps, not the purpose of the entire protocol.

The abstract now reflects this useful suggestion.

2. In the last two lines of the introduction the authors use "to our knowledge" twice. They may want to rephrase.

We thank the reviewer for bringing our attention to this. The introduction has been edited and corrected.

3. In the "Decellularisation" section 2.5: It should probably be 0.3uM rather than 0,3.

We thank the reviewer for bringing our attention to this. This is now corrected.

4. In section 3.8 the words "glass slides" are in larger font size.

This is now corrected.

5. In section 3.10: is it immersing in 4% PFA or applying it on the slides? The phrasing is not clear.

We now clearly state the slides must be immersed.

6. Time of incubation in Hematoxylin and Eosin may need calibrating, depending on the specific source and preparation of stains. This should be stated in the protocol.

This is now stated in 5.9 and 5.11.

7. Page 3-42 contain an empty table.

We apologize for this. One table of materials is attached to the protocol.