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Title: Decellularization of the Murine Cardiopulmonary Complex

Authors and Affiliations:

Alejandro E Mayorca-Guiliani[1]#, Maria Rafaeva[1], Oliver Willacy[1], 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

☒ All author names and affiliations are correct.

Corresponding Authors:

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

Email Addresses for All Authors:

alejandro.mayorca@bric.ku.dk
maria.rafaeva@bric.ku.dk
oliver.willacy@bric.ku.dk
chris.madsen@med.lu.se
raphael.reuten@bric.ku.dk
janine.erler@bric.ku.dk

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   no

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Leica S6D

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes.**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as possible.

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

   ☐ Interviewees self-record interview statements. JoVE can provide support for this option.

   ☐ Interview Statements are read by JoVE's voiceover talent.

   ☐ Author interview statement opt out. Statements removed completely.

4. **Filming location:** Will the filming need to take place in multiple locations? **yes**

   If **Yes**, how far apart are the locations? Two different buidings approx. 500 m. from each other. In the first, surgical procedures are carried out in the basemen and interviews in

the 4th floor. In the second building we will perform the imaging in a room of approx. 30 sq. meters, with a free space of approx. 15 sq. meters

To ensure that your script can be filmed in one day, the Protocol section is restricted to **55 shots** (shots are the 3-digit numbers like 2.1.1, 2.1.2…etc)

Current Protocol Length

Number of Steps: 24
Number of Shots: 55

Introduction

1. Introductory Interview Statements

Your answers to these questions will become author interview statements, which authors will memorize and then deliver on camera.

- Enter the **full name** of the author who will deliver the statement.
- Fill out **both** required statements.
- Answer in full sentences, in a style suitable for being spoken aloud.
- Limit the length of each statement to **30 words or fewer**.
- Answers will be edited for length, clarity, and consistency with journal style guidelines.

REQUIRED: Why is your protocol significant? *OR* What key questions can this method help answer?

1.1. **Alejandro E Mayorca Guiliani:** this protocol is the gateway to the extracellular matrix, its complexity and its structure, down to sub-micron scale.

REQUIRED: What is the main advantage of this technique?

1.2. **Alejandro E Mayorca Guiliani:** The key advantage here is to obtain an extracellular matrix that retains its structural integrity, thus opening the way for biochemical and anatomical analysis

Introduction of Demonstrator on Camera

Complete this statement **ONLY** if any of the individuals who will be demonstrating the procedure on camera will not be delivering an Introductory Interview Statement.

1.3. **Janine T Erler:** Demonstrating the procedure will be Alejandro Mayorca Guiliani, Raphael Reuten, and Maria Rafaeva currently in my laboratory, and Chris Madsen who was in my laboratory and continued the work at Lund University.

1.3.1. INTERVIEW: Author saying the above.

1.3.2. The named demonstrators looks up from workbench or desk or microscope and acknowledge the camera.

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the ethical committee regulating experimental medicine in the University of Copenhagen.

Protocol

Please review this section to make sure that it accurately describes your protocol. Use **Track Changes** when making edits or revisions.

- The one-digit numbers represent **sections** of the video. The text will appear onscreen.
- The two-digit numbers (e.g. 2.1., 2.2.) represent **steps** of your protocol. The text will be recorded by a professional voiceover talent.
- The three-digit numbers (e.g. 2.1.1., 2.2.2.) represent the **shots** that our videographer will capture at your lab.
- To ensure that your protocol can be **filmed in one day**, the protocol is restricted to **25 steps** and/or **55 shots**.

Please use this draft script to help you prepare for filming day.

- Filming should take no more than 10 minutes per step. If a step will take more than 10 minutes, prepare the product from that step in advance.

2. Post-mortem microsurgery

2.1. Begin by shaving the thorax, abdomen and back of the mouse with a hair clipper **[1]**. Disinfect the area with 70% ethanol **[2]**, then pin the mouse to a polystyrene tray, extending its fore- and hindlimbs as well as its head and tail **[3]**. Place it under the microsurgery microscope **[4]**.

2.1.1. WIDE: Establishing shot of talent shaving the mouse.

2.1.2. Talent disinfecting the area.

2.1.3. Talent pinning the mouse to a polystyrene tray.

2.1.4. Talent placing the tray under the microscope.

2.2. Using a Mayo straight pattern scissors, make a cutaneous incision running from the submandibular region to the lower abdomen and dissect subcutaneously to expose the thoracic wall and peritoneum **[1]**.

2.2.1. SCOPE: Talent making the incision and exposing the thoracic wall and peritoneum.

2.3. Then, use microsurgical scissors to cut the pectoralis major and pectoralis minor muscles along the sixth intercostal space on both sides of the thoracic wall **[1]**.

2.3.1. SCOPE: Talent cutting the muscles.

2.4. Cut the sternum along the previous incisions with straight-pattern scissors, then complete a sternotomy by cutting the sternum along its long axis **[1]**. Elevate and pin both sides of the thoracic wall to expose the cardiopulmonary complex **[2]**.

2.4.1. SCOPE: Talent performing the sternotomy.

2.4.2. SCOPE: Talent elevating and pinning the thoracic wall.

2.5. Use round-tipped micro-forceps to excise the thymus and surrounding adipose tissue by delicately pulling them off their attachments **[1]**. Then, cauterize the descending cava vein and cut the esophagus with straight pattern scissors **[2]**.

2.5.1. SCOPE: Talent excising the thymus and adipose tissue.

2.5.2. SCOPE: Talent cauterizing the cava vein and cutting the esophagus.

2.6. Separate the brachiocephalic veins and the brachiocephalic artery with sharp micro-forceps **[1]**, then separate the left common carotid and left subclavian arteries from the underlying tissue to facilitate ligation and cauterization **[2]**.

2.6.1. SCOPE: Talent separating the brachiocephalic veins and the brachiocephalic artery.

2.6.2. SCOPE: Talent separating the left common carotid and left subclavian arteries from the underlying tissue.

2.7. Using a micro-needle holder, sharp micro-forceps, and a 9-0 suture to place stitches above the emergence of the brachiocephalic, left common carotid, and left subclavian arteries **[1]**. Cauterize the brachiocephalic veins **[2]**.

2.7.1. SCOPE: Talent placing the stitches.

2.7.2. SCOPE: Talent cauterizing the brachiocephalic veins.

2.8. Separate the submandibular salivary glands along the midline to expose the neck muscles and the trachea, then expose the cricothyroid ligament **[1]**. Using micro-scissors, open an entrance by sectioning the ligament **[2]**.

2.8.1. SCOPE: Talent exposing the trachea, then the cricothyroid ligament.

2.8.2. SCOPE: Talent sectioning the ligament.

2.9. Introduce a 27-gauge catheter into the trachea and delicately push until the trachea branches into the bronchi, taking care to not disrupt the bronchi **[1]**. Using a 6-0 suture, place 3 stitches around the trachea to secure the catheter **[2]**.

2.9.1. SCOPE: Talent introducing the catheter into the trachea.

2.9.2. SCOPE: Talent placing the stitches around the trachea.

2.10. 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 **[1]**.

2.10.1. SCOPE: Talent sectioning the mouse.

2.11. Retrogradely catheterize the aorta and push the catheter until it reaches the aortic arc **[1]**. Using a 9-0 suture, place 4 stitches around the aorta, beginning 5 millimeters below the catheter tip **[2]**.

2.11.1. SCOPE: Talent catheterizing the aorta.

2.11.2. SCOPE: Talent placing stitches around the aorta.

3. Decellularization and Immunostaining

3.1. Connect the mouse to a pump system using silicone tubing and Luer connectors **[1]**. Perfuse with deionized water at 200 microliters per minute for 15 minutes **[2-TXT]**, then change the perfusion agent to 0.5% DOC diluted in deionized water and perfuse overnight **[3-TXT]**.

3.1.1. Talent connecting the mouse to a pump system.

3.1.2. Talent perfusing the mouse with water. **TEXT: Maintain 200 microliters/min flow rate during decellularization**

3.1.3. Talent changing the perfusion agent. **TEXT: DOC: sodium deoxycholate**

3.2. On the next day, change the perfusion agent to 0.1% SDS diluted in deionized water and perfuse for 8 hours **[1-TXT]**. Then, perfuse with deionized water for 24 hours to wash away the SDS and DOC **[2]**.

3.2.1. Talent changing the perfusion agent to SDS. **TEXT: sodium dodecyl sulphate**

3.2.2. Tissue perfusing with water.

3.3. Resect the decellularized heart and lungs by sectioning its attachments to the thorax **[1]**. Store the tissue in a sterile cryo-tube in deionized water with 1% penicillin–streptomycin and 0.3 micromolar sodium azide at 4 degrees Celsius **[2]**.

3.3.1. Talent resecting the heart and lungs.

Added shot: Talent putting the tissue in a cryotube.

NOTE: Shot 3.3.1. and the added shot filmed together in a single take. For the added shot use "Store the tissue in a sterile cryo-tube" as the VO.

3.3.2. Tissue close-up

3.4. To perform immunostaining, block the sample by incubating it in a cryotube containing 6% donkey serum and 3% BSA overnight **[1]**. Then, incubate it with primary antibody in 3% donkey serum in PBS for 24 hours **[2]**.

3.4.1. Talent immersing the sample in 6% donkey serum and 3% BSA.

3.4.2. Talent transferring the sample to primary antibody in 3% donkey serum in PBS.

3.5. After the incubation, wash the sample 5 times in PBST for 1 hour per wash **[1]**. Incubate the sample with fluorescently conjugated secondary antibody in 3% donkey serum in PBS for 24 hours, then repeat the washes in PBST **[2]**. Add deionized water and store the sample at 4 degrees Celsius away from direct light **[3]**.

3.5.1. Talent washing the sample in PBST.

3.5.2. Talent incubating the sample with the secondary antibody.

3.5.3. Talent storing the sample in a refrigerator.

3.6. To image the sample, place it in a glass-bottomed dish and humidify it with two droplets of storing solution **[1]**. Set the objective and inspect the sample using fluorescence light **[2]**.

3.6.1. Talent dropping storing solution on the sample.

3.6.2. Talent choosing the objective and viewing the sample.

3.7. 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, then begin acquisition **[1]**.

3.7.1. Talent setting the imaging parameters and starting the imaging.

4. Hematoxylin-eosin staining

4.1. Excise 1 lung lobe from a euthanized mouse and place it in a 10 by 10 by 5-millimeter cryomold **[1]**. Cover it with approximately 500 microliters of OCT compound **[2]** and freeze it on dry ice. Maintain the sample at that temperature **[3]**.

4.1.1. Talent placing an excised lung in a cryomold.

4.1.2. Talent covering the tissue with OCT.

4.1.3. Talent putting the sample on dry ice.

4.2. Excise one decellularized lung lobe from a processed mouse, place it in a cryomold with the largest surface area down **[1]** and cover it with OCT compound **[2]**. Freeze the sample on dry ice and maintain it at that temperature until otherwise required. The sample can be stored for at least 12 weeks **[3]**.

4.2.1. Talent placing a decellularized lung in a cryomold.

4.2.2. Talent covering the lung with OCT.

NOTE: Shots 4.2.1. and 4.2.2. were filmed together in a single take.

4.2.3. Talent freezing the sample on dry ice.

4.3. Use a cryostat to section frozen tissue blocks into 5-micrometer sections at -20 degrees Celsius **[1]** and place the sections on adhesive glass slides **[2-TXT]**. Transfer the slides to room temperature until air dried **[3]**.

4.3.1. Talent cutting the tissue block.

4.3.2. Talent placing a section on a slide. **TEXT: Store at -80 °C**

4.3.3. Slides at room temperature.

NOTE: The entire step 4.3 was filmed in a different room.

4.4. Briefly immerse the slides in PBS **[1]**, followed by 4% paraformaldehyde in PBS for 15 minutes **[2]**. Wash once in PBS, then twice in distilled water for 5 minutes per wash **[3]**. Immerse the slides in Mayer's hematoxylin solution for 10 minutes **[4]**.

4.4.1. Talent immersing the slides in PBS, with the PBS container labeled.

4.4.2. Talent immersing the slides in PFA, with the PFA container labeled.

4.4.3. Talent washing the sample in PBS or water.

4.4.4. Talent immersing the slides in hematoxylin, with the hematoxylin container labeled.

4.5. Next, wash the slides in a Coplin jar under running distilled water for 10 minutes **[1]** and immerse in eosin solution for 7 minutes **[2]**. Dip in xylene several times **[4]**.

4.5.1. Talent washing the slides under running water.

4.5.2. Talent immersing the slides in eosin, with the eosin container labeled.

4.5.3. Talent dipping the slides in 50% ethanol, with the other ethanol jars in the shot and labeled.

NOTE: Shot number 4.5.3. was dropped and hence not filmed.

4.5.4. Talent dipping the slides in xylene.

4.6. Apply a few drops of DPX mounting medium and place a glass coverslip **[1]**. Leave the slides to dry overnight under a chemical hood, then scan them in a slide scanner **[2]**.

4.6.1. Talent applying mounting medium and placing a cover on the slide.

4.6.2. Talent scanning the slide.

Protocol Script Questions

Authors: Please use the **step numbers from the script above** (not step numbers from the manuscript) when answering the questions below. Please do not include steps that will be screen-captured and do not list entire sections.

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.

1. Steps 2.3 to 2.9 are the most important

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.

2.3 to 2.9 are crucial to success of the procedure.

Results

Please review this section to make sure that it accurately reflects your findings.

- Use **Track Changes** when making edits or revisions.
- If you would like the video to include different results, please revise this section.
- When revising, please keep the length of the voiceover below 200 words. (Voiceover is the text that follows the two-digit numbers.)
- Please note that the video cannot include voiceover without an accompanying visual.

5. Results: Multiple protein immunostaining of decellularized PyMT mouse lungs from a 11-week-old female mouse

5.1. After successfully completing the protocol, the heart and lungs, and annex tissue will be free of cells **[1]**. Decellularization can be validated by hematoxylin-eosin staining of the ECM scaffolds **[2]**. These scaffolds retain the dimensions of fresh organs and its insoluble ECM structure is intact **[3]**.

5.1.1. LAB MEDIA: Figure 1.

5.1.2. LAB MEDIA: Figure 1. *Video Editor: Emphasize the decellularized images.*

5.1.3. LAB MEDIA: Figure 2, just the top image.

5.2. ECM scaffolds showed increased permeability and light penetrability. Using this protocol with a motorized microscope stage allows for three-dimensional, tiled imaging of whole-mount samples at submicron resolution **[1]**.

5.2.1. LAB MEDIA: Figure 2.

Conclusion

6. Conclusion Interview Statements

Below are prompts for interview statements that can be used to further emphasize the significance of your protocol.

- Answer **one** of the prompts below.
- Limit the statements to **30 words**.
- Answer the questions in full sentences; you will need to memorize and deliver the interview statements during filming.
- Indicate the **full name** of the author who will deliver the statement.

What is the most important thing to remember when attempting this procedure? Please indicate the steps (*e.g.*, 2.4., 2.5.) in the Protocol section of the script that this advice applies to.

6.1. **Alejandro Mayorca Guiliani:** (2.3; 2.4; 2.5; 2.6; 2.7; 2.8; 2.9) It is essential to shunt flow toward the organs of interest to achieve complete, uniform decellularization. The microsurgical dissection and ligation of vessels must be effective and, at the same time, respect the target tissues to maintain the intact native ECM structure.

Following this procedure, what other methods can be performed? What questions would these additional methods answer?

6.2. **Alejandro Mayorca Guiliani:** the scaffolds will be enriched for structural ECM proteins, and can be used for mass spectrometry or tissue engineering.

After its development, did this technique pave the way for researchers to explore new questions within a specific scientific field? If so, how?

6.3. **Alejandro Mayorca Guiliani:** this technique is opening the way to high resolution mapping of the ECM in mammalian organs, both during health and disease.

Thank you for addressing our questions. We will incorporate your answers and suggestions, and send you the final script before your filming day. You will also receive detailed preparation instructions in the email accompanying the final script.