Journal of Visualized Experiments

Comparable decellularization of fetal and adult cardiac tissue explants as 3D-like in vitro microenvironments to dissect cell-ECM crosstalk

--Manuscript Draft--

Manuscript Number:	JoVE56924R1
Full Title:	Comparable decellularization of fetal and adult cardiac tissue explants as 3D-like in vitro microenvironments to dissect cell-ECM crosstalk
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Decellularization; extracellular matrix; 3D scaffolds; fetal heart; adult heart; cardiac tissue engineering
Manuscript Classifications:	10.1.293.140: Biomedical Engineering; 8.1.770.644.145: Biomedical Research
Corresponding Author:	Perpétua Pinto-do-Ó, PhD i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal Porto, PORTUGAL
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	perpetua@ineb.up.pt
Corresponding Author's Institution:	i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal
Corresponding Author's Secondary Institution:	Universidade do Porto Instituto de Ciencias Biomedicas Abel Salazar
First Author:	Ana C Silva
First Author Secondary Information:	
Other Authors:	Ana C Silva
	Maria J Oliveira
	Todd C McDevitt
	Mário A Barbosa
	Diana S Nascimento
Order of Authors Secondary Information:	
Abstract:	Current knowledge on extracellular matrix (ECM)-cell communication translates by large two-dimensional (2D) in vitro cultures studies where ECM components are presented as a surface coating. These culture systems, constitute a simplification of the complex nature of the tissue ECM which encompasses biochemical composition, structure and mechanical properties. To better emulate the ECM-cell cross-talk shaping-up the cardiac microenvironment, we have developed a protocol that allows the decellularization of whole fetal heart and adult left ventricle tissue explants at the same time for comparative studies. The protocol combines the use of a hypotonic buffer, a detergent of anionic surfactant properties and DNAse treatment, without requirement of specialized skills or equipment. The application of the same decellularization strategy across different age tissue samples is an alternative approach to perform comparative studies. The present protocol allowed the identification of unique structural differences across fetal and adult cardiac ECM mesh and biologic cellular responses. Furthermore, the herein methodology demonstrated a broader application being successfully applied in other tissues and species with minor adjustments, such as, in human intestine biopsies and mouse lung.
Author Comments:	This remark intends to justify our special request on the possibility of having the herein

	manuscript published before Oct-Nov 2017. We would be most indebted to your kind understanding that while the first author (and the one to be filmed by the JoVE Team while performing the protocol) has been developing her final work for the PhD dissertation in the Laboratory of Dr. McDevitt (PhD co-supervisor) at the Gladstone Institutes in San Francisco (CA), USA for the last year and a half, she might be returning to Portugal before the end of 2017. Attending to that the Iberian Peninsula is not yet in the JoVE roadmap for filming, it would be most wise to make the video before Ana Silva departure from McDevitt's lab. I thank you in advance for your original invitation which led to submitting our work to JoVE as well as for the kind and continued attention when we were not able to keep proposed deadlines.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	10-09-2017

Subject: Manuscript revision

Porto, 22nd September 2017

Dear Review Editor
Dr. Alisha DSouza

We would like to sincerely acknowledge the reviewer's comments and the opportunity to submit a revised version of our manuscript now entitled "Comparable decellularization of fetal and adult cardiac tissue explants as 3D-like *in vitro* microenvironments to dissect cell-ECM crosstalk" to JoVE.

We have carefully revised the manuscript taking into consideration all points raised by the reviewers. Please find enclosed a detailed response to reviewer one, addressing the comments that have been raised, a revised version of the manuscript with the alterations highlighted in blue and high-resolution figures files and Tables.

We hope that after this revision you can now appreciate our study and consider it suitable for publication at JoVE.

Sincerely,

Perpétua Pinto-do-Ó, PhD

Stem-cell Microenvironments in Repair/Regeneration Team, Coordinator
INEB - Instituto Nacional de Engenharia Biomédica & i3S - Instituto de Investigação e Inovação em Saúde, & INEB - Instituto Nacional de Engenharia Biomédica, Universidade do Porto, Porto, Portugal
Invited Professor, Lecturer in Stem Cell Biology, Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal
President of the Portuguese Society for Stem Cells and Cell Therapy (SPCE-TC

INSTITUTO
DE INVESTIGAÇÃO
E INOVAÇÃO
EM SAÚDE
UNIVERSIDADE
DO PORTO

Rua Alfredo Allen, 208
4200-135 Porto
Portugal
+351 220 408 800
info@i3s.up.pt
www.i3s.up.pt

Friday, June 30, 2017

Dear Senior Science Editor
Dr. Nandita Singh

Please consider our manuscript entitled "Parallel decellularization of fetal and adult cardiac tissue explants as 3D *in vitro* microenvironments to dissect cell-ECM crosstalk", for publication as an Invited Methods Article - JoVE Produced Video in *Journal of Visualized Experiments (JoVE)*.

Our laboratory main interest is to **contribute knowledge toward improving the anatomo-functional restoration of poorly regenerative organs**. In this context the heart, an organ with very low cellular turnover, whose function depends upon cells majorly generated during the embryonic development has been a challenge. In brief, for the last five years we have been addressing this problem in a comprehensive strategy directed to the **systematic identification of signals critical for** (i) initiation of the **cardiac molecular program** in the embryo ([1]Freire AG, 2017), (ii) **emergence and kinetics of the main cell lineages that compose the heart** ([2]Valente M, 2017, manuscript in revision & *Declaration of Invention*, 2015), and (iii) the **cellular cross-talk with the dynamic extracellular matrix (ECM) that supports the full organ development and growth** ([3]Silva AC, 2016). The latter publication has been the result of such concerted endeavor and tackles current limited data on microenvironmental cues that sustain survival, proliferation and functional proficiency of cardiac cells. Relative to recent developments, **we have advanced the field by showing unique features of close-to-native three-dimensional (3D) fetal (E18) and adult myocardial ECM and by demonstrating that fetal bioscaffolds are not only more efficiently recellularized with cardiomyocytes and cardiac progenitors as they also direct the cells toward a more regenerative-like profile**.

The herein manuscript is the new protocol, seminal to the work reported on *Biomaterials* (2016) above, developed for obtaining acellular ECM bioscaffolds from fetal- and adult-heart explants under parallel conditions, and thus ensure reliable comparison of the biochemical, biophysical and biological activity features of distinct

INSTITUTO
DE INVESTIGAÇÃO
E INOVAÇÃO
EM SAÚDE
UNIVERSIDADE
DO PORTO

Rua Alfredo Allen, 208
4200-135 Porto
Portugal
+351 220 408 800
info@i3s.up.pt
www.i3s.up.pt

microenvironments. Noteworthy, this protocol has by now endured testing on different systems and proved, among others, to be easily adaptable also for decellularizing human tissues[4] (normal and malignant colorectal samples) and murine lung[5] while retaining main ECM elements which include distinctive biochemical and structural meshwork features of the native equivalents, properties which, to the best of our knowledge, have not consistently been shown before. Thus, our experience legitimates the conviction that detailed sharing of this simple three-steps protocol with a broader scientific community will bring further insight into several other tissue-organ models and/or conditions.

A final remark to justify our special request on the possibility of having the herein manuscript published before Oct-Nov 2017. We would be most indebted to your kind understanding that while the first author (and the one to be filmed by the *JoVE* Team while performing the protocol) has been developing her final work for the PhD dissertation in the Laboratory of Dr. McDevitt (PhD co-supervisor) at the Gladstone Institutes in San Francisco (CA), USA for the last year and a half, she might be returning to Portugal before the end of 2017. Attending to that the Iberian Peninsula is not yet in the *JoVE* roadmap for filming, it would be most wise to make the video before Ana Silva departure from McDevitt's lab.

On the behalf of all authors, I thank you in advance for your original proposal which led to submitting our work to JoVE as well as for the kind and continued attention,

Sincerely,

Perpétua Pinto-do-Ó, PhD

Stem-cell Microenvironments in Repair/Regeneration Team, Coordinator
INEB - Instituto Nacional de Engenharia Biomédica & i3S - Instituto de Investigação e Inovação em Saúde, & INEB - Instituto Nacional de Engenharia Biomédica, Universidade do Porto, Porto, Portugal
Adjunct Professor, Lecturer in Stem Cell Biology, Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal
President of the Portuguese Society for Stem Cells and Cell Therapy (SPCE-TC

INSTITUTO
DE INVESTIGAÇÃO
E INOVAÇÃO
EM SAÚDE
UNIVERSIDADE
DO PORTO

Rua Alfredo Allen, 208
4200-135 Porto
Portugal
+351 220 408 800
info@i3s.up.pt
www.i3s.up.pt

[1]Freire AG, Waghray A, Soares-da-Silva F, Lee DF, Pereira CF, Resende TP, Nascimento DS, Lemischka IR*, Pinto-do-Ó P* (2017). Transient Hes5 activity instructs mesodermal cells toward a cardiac fate. *Stem Cell Reports* DOI: 10.1016/j.stemcr.2017.05.025 · License: CC BY-NC-ND 4.0

[2]Valente M, Resende TP, Nascimento DS, Cumano A*, Pinto-do-O P*. Unique signatures identify sequential stages of cardiomyocyte maturation differentially represented throughout life. (*Dev Cell*., in revision) & Valente M, Resende TP, Nascimento DS, Cumano A and Pinto-do-Ó P (2015). New surface markers discriminate different cardiac populations during development allowing their prospective isolation and the definition of the transcriptional profile at the single cell level. [**Declaration of Invention No I 2015-31 HC**] (Institut Pasteur, Paris, France)

[3]Silva AC, Rodrigues SC, Caldeira J, Barbosa MAOliveira MJ, Nascimento DS*, Pinto-do-P.* (2016). Three dimensional scaffolds of fetal decellularized hearts exhibit enhanced potential to support cardiac cells in comparison to the adult. *Biomaterials* DOI: 10.1016/j.biomaterials.2016.06.062. citations: 2 (RG), 1 (Scopus)

[4]Pinto ML, Rios E, Silva AC, Neves SC, Caires HR, Pinto AT, Durães C, Carvalho F, Cardoso AP, Santos N, Barrias C, Nascimento D, Pinto-do-Ó P, Barbosa MA, Carneiro F, Oliveira MJ (2017). Decellularized human colorectal cancer matrices polarize macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18. *Biomaterials* DOI.org/10.1016/j.Biomaterials.

[5]Garlíková Z, Silva AC, Rabata A, Potěšil D, Ihnatová I, Dumková J, Koledová Z, Zdráhal Z, Vinarský V, Hampl A, Pinto-do-Ó P*****, Nascimento DS*****. Generation of a close-to-native *in vitro* system to study lung cells-ECM crosstalk. (*Tissue Eng Part C*., in revision).

INSTITUTO
DE INVESTIGAÇÃO
E INOVAÇÃO
EM SAÚDE
UNIVERSIDADE
DO PORTO

Rua Alfredo Allen, 208
4200-135 Porto
Portugal
+351 220 408 800
info@i3s.up.pt
www.i3s.up.pt

TITLE:

Comparable decellularization of fetal and adult cardiac tissue explants as 3D-like platforms for *in vitro* studies

AUTHORS & AFFILIATIONS:

Ana C Silva[1,2,3,4], Maria J Oliveira[1,2,5], Todd McDevitt[4], Mário A Barbosa[1,2,3], Diana S Nascimento[1,2,*], Perpétua Pinto-do-Ó[1,2,3,*]

[1]i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal
[2]INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal
[3]Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal
[4]Gladstone Institutes, University of California San Francisco, San Francisco, California, United States of America
[5]Faculty of Medicine, University of Porto, Porto, Portugal

[*]Senior co-authorship

Corresponding Authors:
Perpétua Pinto-do-Ó
perpetua@ineb.up.pt
Tel: 00351 220 408 800

Diana S Nascimento
dsn@ineb.up.pt
Tel: 00351 220 408 800

Email Addresses of Co-authors:
Ana C Silva (ana.silva@ineb.up.pt)
Maria J Oliveira (mariajo@ineb.up.pt)
Todd McDevitt (todd.mcdevitt@gladstone.ucsf.edu)
Mário A Barbosa(mbarbosa@ineb.up.pt)

KEYWORDS:
Decellularization, extracellular matrix, 3D scaffolds, fetal heart, adult heart, cardiac tissue engineering

SHORT ABSTRACT:
The cardiac extracellular matrix (ECM) is a complex network of molecules that orchestrate key processes in tissues and organs while enduring physiological remodeling throughout life. Standardized decellularization of fetal and adult hearts permits comparative experimental studies of both tissues in a 3D context by capturing native architecture and biomechanical properties.

LONG ABSTRACT:

Current knowledge of extracellular matrix (ECM)-cell communication translates to large two-dimensional (2D) *in vitro* culture studies where ECM components are presented as a surface coating. These culture systems constitute a simplification of the complex nature of the tissue ECM that encompasses biochemical composition, structure, and mechanical properties. To better emulate the ECM-cell communication shaping the cardiac microenvironment, we developed a protocol that allows for the decellularization of the whole fetal heart and adult left ventricle tissue explants simultaneously for comparative studies. The protocol combines the use of a hypotonic buffer, a detergent of anionic surfactant properties, and DNase treatment without any requirement for specialized skills or equipment. The application of the same decellularization strategy across tissue samples from subjects of various age is an alternative approach to perform comparative studies. The present protocol allows the identification of unique structural differences across fetal and adult cardiac ECM mesh and biological cellular responses. Furthermore, the herein methodology demonstrates a broader application being successfully applied in other tissues and species with minor adjustments, such as in human intestine biopsies and mouse lung.

INTRODUCTION:

The extracellular matrix (ECM) is a dynamic network of molecules that regulate important cellular processes, namely fate-decision, proliferation and differentiation[1,2]. The investigation of cell-ECM interactions has been performed mainly in two-dimensional (2D) *in vitro* cultures coated with ECM components, which constitute a simplification of native ECM properties found *in vivo*. Decellularization generates acellular 3D-like ECM bioscaffolds that largely preserve the extracellular architecture and composition of native tissues and organs[3,4]. In addition to serving as bioactive scaffolds for tissue engineering, decellularized 3D ECM biomaterials are emerging as novel platforms to assess cell-ECM biology that parallel the *in vivo* environment.

Assessment of the differential role of the ECM components of distinct tissues, organs and age will benefit with the use of similar protocols of generating native bioscaffolds. In the heart, we have developed a versatile protocol for decellularization of fetal and adult-derived samples, as an alternative approach to perform comparative studies of the organ microenvironment. Using this methodology, we captured the native cardiac microenvironment and showed that fetal ECM promotes higher repopulation yields of cardiac cells[5]. Decellularization further provided identification of resident structural differences between fetal and adult ECM at the level of basement lamina and pericellular matrix mesh arrangement and fiber composition[5]. Prior to this work, head-to-head comparison of tissues at different ontogenic stages using the same decellularization approach has only been reported for rhesus monkey kidneys and rodent hearts. In addition, a limited number of studies report fetal tissue/organ decellularization *per se*[5-7]. This has been achieved using SDS as a unique decellularization agent; however, distinct SDS concentrations were used for the decellularization of fetal and adult cardiac tissue[7,8]. SDS is one of the most effective ionic detergents for clearance of cytoplasmic and nuclear material, and widely used in the decellularization of different tissues and specimens[9,10]. Solutions containing high SDS concentrations and extended periods of exposure have been correlated with protein denaturation, glycosaminoglycan (GAGs) loss and disruption of collagen fibrils[10,11], and therefore a balance between ECM preservation and cell removal is necessary. To apply the same procedure

to fetal and adult heart tissue, the protocol described herein is divided in three sequential steps: cell lysis by osmotic shock (hypotonic buffer); solubilization of lipid-protein, DNA-protein and protein-protein interactions (0.2% SDS); and nuclear material removal (DNase treatment).

Our protocol shows several advantages: i) the possibility of equivalent decellularization of age-specific cardiac tissues by the application of the same decellularization strategy; ii) no requirements for specialized methods or equipment; iii) ready adaptation to other tissues and species as it has been successfully applied with minor alterations in human intestine biopsies[12] and mouse lung[13]; and, importantly, iv) can address ECM biomechanical properties while enabling the assembly of 3D-like organotypic cultures that more closely mimic the molecular features of the native tissue microenvironment.

PROTOCOL:

All the methodologies described were approved by the i3S Animal Ethics Committee and Direção Geral de Veterinária (DGAV) and are in accordance with the European Parliament Directive 2010/63/EU.

1. Preparation of the decellularization solutions

Note: All decellularization solutions should be filtered through a 0.22 μm membrane filter and stored for a maximum of 3 months, except specified otherwise.

1.1. For 1x PBS: Mix 200 g of NaCl, 15.25 g of Na_2HPO_4 5 g of KCl, and 5 g of KH_2PO_4 with 900 mL of deionized water (DI water). Adjust solution pH to 7.5 and the final solution volume up to 1 L. Filter, autoclave, and store the solution at room temperature (RT).

1.2. For Hypotonic Buffer (10 mM Tris, 0.1% EDTA): Dissolve 1.21 g of TrisBASE and 1 g of EDTA in 900 mL of deionized water. Adjust solution pH to 7.8 with NaOH/HCl and the final solution volume to 1 L. Filter, sterilize by autoclave, and store at RT.

1.3. For 0.2% SDS solution (0.2% SDS, 10 mM Tris): Add 0.6 g of TrisBASE and 1 g of sodium dodecyl sulfate (SDS) in 400 mL of DI water and stir at 60 °C until complete solute dissolution. Let solution to cool down to RT. Adjust solution pH to 7.8 and the final solution volume to 500 mL. Sterilize solution by filtration.

Note: The SDS solution should be prepared prior to use. Filter solution only after complete SDS dissolution, otherwise the SDS insoluble particles will saturate the filter, changing the final SDS concentration and consequently affecting tissue decellularization efficiency.

1.4. For Hypotonic Wash Buffer (10 mM Tris): Mix 1.21 g of TrisBASE in 900 mL of DI water. Adjust solution pH to 7.8 and the final volume to 1 L. Filter, sterilize by autoclave and store at RT.

1.5. For DNase treatment (20 mM Tris, 2 mM $MgCl_2$, 50 U/mL DNase): Dissolve 2.42 g of TrisBASE and 2 mL of 1 M $MgCl_2$ in 900 mL of DI water. Adjust pH to 7.8 and the final solution volume to 1

L. Filter and sterilize solution by autoclave. Store solution at RT. Add DNase I (50 U/mL) prior to use.

1.6. For DNase I stock solution: Prepare a DNase buffer containing 50% glycerol, 20 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, and adjust to a final solution volume of 10 mL with DI water. In a laminar flow hood, add the DNase I powder to the DNase buffer and mix gently until complete dissolution. Sterilize solution through 0.22 μm filter. Prepare 1 mL aliquots and store at -20 °C.

2. Tissue harvesting and cryopreservation

2.1. Euthanize adult pregnant females at 18 days of gestation (E18 fetuses) via CO_2 asphyxiation. Perform a Cesarean section on the females with a surgical scissor and collect the uterine horns bearing the fetal mice to a Petri dish with ice-cold 1x PBS using two serrated tweezers with curved tips (one tweezer to each horn end).

2.1.1. Open the uterine horns and amniotic sac to isolate the fetuses. Transfer the fetuses to a new Petri dish with ice-cold 1x PBS to anesthetize them and decapitate with a scissor.

2.1.2. Euthanize 7-8 weeks old male C57BL/6 mice using CO_2 asphyxiation. Perform an incision on each mouse chest and collect the heart.

2.1.3. Briefly rinse fetal and adult hearts with ice cold 1x PBS.

2.2. Remove the atria with the help of a scalpel. Perform an incision on the right ventricle and remove it using straight small scissors. Expose the left ventricle inner wall by removing the septum. In a new Petri dish, divide the left ventricle free-wall in longitudinal strips with ± 2 mm thickness and remove the papillary muscle using a scalpel and serrated tweezers with curved tips.

2.2.1. Excise small tissue explants with the help of a scalpel using a 2 mm x 2 mm grid (Detailed description in supplementary **Figure 1** [5]). Briefly rinse tissue explants with 1x PBS.

2.3. Add ~250 µL of the optimal cutting temperature (OCT) compound to autoclaved 1.5 mL microcentrifuge tubes. Transfer whole fetal hearts and adult cardiac explants to the tubes with 250 µL of OCT and freeze them with dry ice-cooled isopentane and store at -80 °C (to a maximum of 6 months).

Note: The use of cardiac tissue explants cryopreserved for more than 6 months will significantly reduce the decellularization efficiency, especially in the adult heart tissue explants.

3. Tissue decellularization

Note: Cardiac tissue decellularization is performed in a 24-well tissue culture plate with one sample per well. 1 mL of each decellularization solution is added to each individual well. All decellularization steps should be performed with agitation at 165 rpm (incubator shaker with an

orbital diameter of 20 mm) and at 25 °C, unless specified otherwise. For more details, please consult the scheme on **Figure 1A**. Amphotericin B (*e.g.* fungizone) and gentamicin are freshly added to all decellularization solutions before use to a final concentration of 2.5 μg/mL and 0.01 μg/mL, respectively. To quantify the amount of DNA retained within decellularized tissues, the sample mass needs to be determined before starting the decellularization protocol. The DNA quantification protocol is further detailed in section 5.1.

3.1. **DAY 1**

3.1.1. Remove tissue samples from the -80 °C freezer. Leave the 1.5 mL microcentrifuge tubes at RT until OCT becomes partially melted. Transfer the cardiac tissue block of the still frozen OCT to a Petri dish with 1x PBS.

3.1.2. After the OCT melts, move the cardiac tissue to a new Petri dish with 1x PBS. Wash samples in 1x PBS and at 60 rpm with a shaker for 10-15 min. Repeat at least twice.

3.1.3. Add 1 mL of working Hypotonic Buffer per well of a sterile 24-well tissue culture plate. Transfer one sample per well with the help of forceps.

3.1.4. Move the 24-well tissue culture plate with the samples to an incubator shaker at 25 °C and start the 18 h incubation with Hypotonic Buffer.

3.2. **DAY 2**

3.2.1. Prepare the 0.2% SDS solution as described in section 1.3.

3.2.2. Aspirate the Hypotonic Buffer and wash the samples with 1x PBS for 1 h. Repeat 3 times.

Note: PAUSE POINT: Tissue under decellularization can be kept in 1x PBS at 4 °C for 18 h in static conditions.

3.2.3. Remove the 1x PBS, add 1 mL of freshly made SDS solution (step 1.3) per well and incubate samples for 24 h.

Note: (CHECK POINT) Post SDS incubation, ensure that samples exhibit a white to translucent appearance. At this step, SDS treated samples are drenched in DNA, showing a gelatin-like consistency. Remove SDS solution slowly to avoid sample adhesion to the pipette tip.

3.3. **DAY 3**

3.3.1. Wash the samples with Hypotonic Wash Buffer (1 mL per well) for 20 min. Repeat 3 times.

Note: At this step, it is normal to observe a decrease in the sample size due to the removal of cell remnants. PAUSE POINT: Tissue under decellularization can be kept in Hypotonic Wash Buffer at

4 °C for 18 h in static conditions.

3.3.2. Add 1 mL of DNase Treatment solution per well and incubate the samples for 3 h at 37 °C.

3.3.3. Aspirate the DNase Treatment solution and wash the samples with 1x PBS (1 mL per well) for 20 min. Repeat 3 times. Perform a final wash with 1x PBS (1 mL per well) overnight at 25 °C and shaking at 60 rpm.

3.3.4. (Check point) After DNase treatment, ensure that the decellularized samples have lost the gelatin-like consistency.

Note: For the DNase treatment, remove and add 1x PBS gently since samples can be easily entrapped and attached to the pipette tip.

4. **Assessment of decellularized tissue cell removal**

4.1. Fix the samples in a 24-well tissue culture plate, 1 sample per well, by adding 1 mL of freshly made 10% formalin neutral buffer with 0.03% aqueous eosin for 2.5-3 h at RT.

Note: Eosin is added to the fixative to stain the decellularized tissue and to ease sample visualization during sectioning and histological staining. The addition of the eosin neither interferes with the histological stains nor with immunofluorescence techniques. Alternatively, the fixation can be performed over night at 4 °C.

4.2. Remove the fixative solution and add 1 mL of 1x PBS to wash the sample.

4.3. Encapsulate the fixed bioscaffolds in histology processing gel using a disposable vinyl specimen mold according to the manufacturer's instructions.

4.4. Remove the histology gel with the sample embedded from the mold and transfer to Biopsy Processing/Embedding Cassettes with Lid.

Note: An alternative to tissue embedding in histology and processing gel is to process each sample in two biopsy sponges with small pores. The samples should be localized to the center of the biopsy sponges to enable detection. Of note, some samples can be difficult to localize using this approach.

4.5. Process the samples for paraffin embedding through successive incubations (30 min per solution) in series of alcohol concentrations (70%, 80%, 90%, 100%, 100%), isoparaffinic aliphatic hydrocarbon solution (2 incubation stations) and paraffin (2 incubation stations) at 56 °C.

4.6. Mount the samples in a paraffin block.

4.7. Cut 3 μm-thick paraffin sections on a microtome and collect the sections on glass microscope slides.

4.8. Dry paraffin sections overnight at 37 °C.

Note: (PAUSE POINT) Paraffin sections are stored at 4 °C until further use.

4.9. To verify protocol efficiency, perform Hematoxilin and Eosin (H&E) and Masson's Trichrome staining (MT) of the decellularized sample sections and to sections of non-manipulated tissue according to manufacturer's instructions.

Note: H&E staining stains cell nuclei blue/purple, cytoplasm dark pink/red and collagen pale pink and MT stains nuclei black, cytoplasm red, and collagen and mucin blue. Efficient decellularization is achieved when a porous light pink (H&E, MT) and blue (MT) network is observed, in the absence of a nuclear stain.

5. Assessment of decellularized tissue nuclear material removal

Note: The quantification of the DNA content on decellularized tissue must be performed in comparison to the respective non-manipulated tissue.

5.1. Before decellularization, weigh a 1.5 mL microcentrifuge tube on a high precision digital scale. Transfer the cardiac tissue to the tube, remove the extra amount of 1x PBS and weigh the tube with the samples. Calculate the wet weight of the samples:

$$Sample_{wet\ weight} = weight_{\ tube\ with\ samples} - weight_{\ empty\ tube}$$

5.2. Decellularize the samples as described in Step 3.

5.3. After decellularization, collect the decellularized tissues into a microcentrifuge tube.

5.3.1. Due to the small dimensions and mass of the individual cardiac samples and kit specifications, pool decellularized tissues of each sample condition (fetal heart: ≥ 5 whole hearts; adult LV explants: ≥ 15 explants). Perform the same procedure with the non-manipulated tissue.

Note: (PAUSE POINT) The samples can be stored at -20 °C until DNA extraction and quantification are performed.

5.4. Cut the non-manipulated and decellularized tissues in small explants with a help of a scalpel in a clean Petri dish.

Note: Use an individual scalpel and Petri dish for each sample. Mechanical disruption of the samples with a scalpel facilitates their posterior enzymatic digestion and subsequent DNA extraction.

5.5. For DNA extraction, use a spin-column based DNA extraction method according to the manufacturer's instructions.

5.5.1. Collect the mechanically dissociated samples from the Petri dish with the master digestion buffer (digestion buffer and proteinase K) according to the manufacturer's instructions using a wide orifice pipette tip.

Note: Tissue lysis with Proteinase K digestion is faster in decellularized samples. To process distinct samples at the same time, keep lysed samples on ice, until all samples are lysed to minimize degradation. Complete samples lysis is achieved between 4-6 h.

5.5.2. Proceed with the protocol according the manufacturer instructions.

5.5.3. To increase the yields of extracted DNA of decellularized and non-decellularized tissue samples, elute the DNA on 25-50 μL and 100 μL of elution buffer, respectively. Collect the eluted DNA and load the spin-column. Incubate for 1 min at RT. Centrifuge the samples according to the manufacturer's instructions.

Note: (PAUSE POINT) The DNA extracted from the samples can be store at -20 °C until further use.

5.6. Quantify the extracted DNA from the samples with a fluorescent dsDNA detection kit following the manufacturer's instructions.

5.7. Normalize the DNA content as nanograms of DNA per milligram of initial sample wet weight (before decellularization).

6. Decellularized scaffolds cell seeding

Note: All solutions/reagents need to be sterile and the entire procedure performed at sterile conditions.

6.1. Prepare 1x DPBS with 2.5 μg/mL of amphotericin B and 1% P/S (penicillin, 100 I.U.; streptomycin 100 μg/mL).

6.2. Remove the 1x PBS solution from the last decellularization washing step (step 3.3.3) and add 500 μL per well of the freshly prepared 1x DPBS/2.5 μg/mL of amphotericin B/1% P/S solution. Store samples at 4 °C from 1-7 days.

Note: Samples storage at 4 °C is important to maintain sterility.

6.3. Add P/S to cell basal media (without FBS and supplements) to a final concentration of 1%.

6.4. Aspirate 1x DPBS/2.5 μg/mL of amphotericin B/1% P/S solution from the decellularized samples. Add 500 μL per well of cell basal media/1% P/S and let samples equilibrate for 1 h at 37 °C, or at 4 °C for more than 1 h.

6.5. Split the cells of interest and prepare small aliquots of cells at the cell density desired.

Note: The decellularized tissue seeding must be performed under a stereoscopic microscope and in sterile conditions. Cell culture media should contain 1% P/S.

6.6. Pipette 3-4 μL of DPBS to the center of a well of a 96-well plate. Using thin and straight tweezers, transfer the decellularized scaffolds to the DPBS drop, remove the extra DPBS by aspiration, and make sure that the sample is not folded or wrinkled. Let it become dry at the edges to adhere to the well surface.

Note: Detached scaffolds have lower seeding efficiency.

6.7. Add cells to the scaffold by pipetting the single-cell solution slowly against the edges of the well.

Note: For example, neonatal rat cardiomyocytes are seeded with at a density of 7500 cells/mm^2 and immortalized mouse Lin$^-$ Sca-1$^+$ cardiac progenitor cells (iCPC$^{Sca\text{-}1}$) at a density of 60-1500 cells/mm^2. Adjust cell density according the experimental rationale.

6.8. Add DPBS to the neighboring wells to avoid fast media evaporation.

6.9. 24 hours post cell seeding, if the cell type used adhered to the tissue culture polystyrene plates (TCPS), transfer bioscaffolds to a new well. Otherwise, replace the medium to remove non-adherent cells.

6.10. Change carefully the media according to specific requirements of the cell type.

REPRESENTATIVE RESULTS:

The decellularization efficiency should be assessed through three main techniques: macroscopic observation, histology and DNA quantification. The macroscopic appearance of samples post-SDS treatment indirectly affects the efficacy of cell removal. After SDS incubation, samples should appear as translucent to whitish (**Figure 1C**). Fetal (E18) decellularized tissues are characterized by a highly translucent structure while adult explants have a translucent to white appearance. A whiter appearance is generally correlated to an ECM network exhibiting higher fiber and collagen content, *e.g.* the adult ventricular and vascular vessels ECM mesh (**Figure 1C**). Hematoxylin & Eosin (H&E) and/or Masson's Trichrome (MT) stains are performed to confirm efficient cell

removal by the observation of a porous mesh (light pink, H&E; light pink and blue, MT) (F**igure 1C**). In addition, the MT stain highlights the collagen meshwork in blue[5]. Clearance of nuclear material after decellularization is accessed by DNA quantification and a reduction of approximately 99.8% is generally obtained, when compared to non-manipulated tissues (**Figure 1C**). The presence of nuclear material on decellularized scaffolds has been described as a trigger of undesired inflammatory response upon implantation[14]. For this reason, confirmation of efficient decellularization is essential prior to native 3D scaffold repopulation experiments. Decellularized scaffolds may be stored in sterile conditions up to the seeding with the cells of interest. Cell viability is monitored throughout *in vitro* culture by calcein staining (**Figure 2A**). Nevertheless, calcein stain can be cytotoxic to sensitive cell types. Terminal analysis of cell repopulation and distribution across scaffolds is performed post-paraffin processing; a snapshot of the bioscaffold repopulation assessed by H&E staining at a central section of bioscaffolds is shown (**Figures 2B, 2C**).

FIGURE AND TABLE LEGENDS:

Figure 1. **Fetal (E18) and adult cardiac tissue decellularization procedure and confirmation of decellularization efficiency. (A)** Protocol overview. **(B)** Decellularization protocol detailed. **(C)** Macroscopic analysis of cardiac fetal and adult tissue before and after decellularization. Scale bar: 2 mm. **(D)** Quantification of nuclear material in decellularized versus non-manipulated tissue. Data expressed as mean ± SEM. Student's t-test, two-tailed *p<0.05. **(E)** H&E and Masson's Trichrome histological analysis of cardiac fetal and adult tissue before and after decellularization. Scale bar: 100 μm.

Figure 2. Repopulation analysis of decellularized scaffolds seeded with Lin- Sca-1^{+} cardiac progenitor cell line (iCPC$^{Sca\text{-}1}$). (A) High cell viability observed at scaffolds surface during *in vitro* culture by calcein stain (green). Scale bar: 100 μm. **(B)** Cell number and distribution across scaffolds assessed via H&E staining. Scale bar: 100 μm. **(C)** Orthogonal view of cells embedded in the decellularized ECM. Confocal image of 50μm-thick paraffin section. Scale bar: 10 μm (orthogonal view XZ, YZ) and 40 μm.

Table 1. Troubleshooting table for parallel decellularization of fetal (E18) and adult mouse cardiac tissue.

DISCUSSION:

The extracellular matrix (ECM) is a highly dynamic and complex meshwork of fibrous and adhesive glycoproteins, consisting of a reservoir of numerous bioactive peptides and entrapped growth factors. As the major modulator of cell adhesion, cytoskeleton dynamics, motility/migration, proliferation, differentiation and apoptosis, ECM actively regulates cellular function and behavior. Knowing that cellular behavior differs in 2D and 3D cultures, there have been efforts to develop novel organotypic models that can accurately replicate natural tissue environments. In the last years, tissue decellularization has emerged as an alternative technique for tissue engineering and regenerative medicine. Thus, tissue and organ decellularization is

currently the tool of choice to better dissect tissue-specific microenvironmental parameters (biochemical, structural and mechanical) and biological activity *in vitro* and *in vivo*.

We developed a protocol that combines the use of a hypotonic buffer with a detergent of anionic surfactant properties followed by a DNase treatment[5,12,13]. The present protocol constitutes a simple and reproducible method to perform comparative analysis between decellularized fetal and adult mouse cardiac tissue. Our experience with other tissues, namely with tumor samples derived from cancer patients' surgical resections and mouse lung tissue, shows that this protocol is easily adaptable and successful in other conditions and models[12,13]. The application of the same decellularization procedure on different samples allows comparative studies of the ECM composition, biomechanical properties, architecture and cellular modulatory properties in a 3D context.

One of the most difficult challenges of tissue decellularization is the balance between outright cell removal, ECM meshwork preservation and tissue biocompatibility. Hence, several critical steps need to be carefully considered, such as the time of tissue cryopreservation before decellularization, the correct explant size, the use of fresh solutions, and the manipulation of the final decellularized tissue. During our studies, we observed a direct correlation between long storage time of cryopreserved tissue and the use of longstanding SDS solutions with decellularization inefficiency. Long term tissue storage leads to inefficient cellular content removal rendering remnants of thick cellular areas (without nuclei) among the complex ECM meshwork. A similar undesirable effect is attained when long stored SDS solutions are used for tissue decellularization, likely because SDS solutions have a short stability due to reduced solubility, hydrolysis, and pH alterations over time[15,16]. The use of explants of the correct size (~1.5 mm x 1.5 mm x 1.5 mm) is also essential for successful adult tissue decellularization, since larger tissue resections are more difficult to decellularize, displaying cell debris entrapped at the surface and arrested between the dense ECM network. Although this protocol provides an efficient method of cardiac tissue decellularization, a minor fraction of adult explants may present cell remnants, in particular those of a larger size. Histological analysis of these samples eases their identification and subsequent exclusion from the study. Ultimately, the protocol herein is versatile and readily applicable to distinct specimens with slight adjustments to the tissue explant size, SDS concentration (0.1-0.2% SDS) or solution incubation time[5,12,13].

The major limitation of the present method is that the manipulation of small size samples, *i.e.* murine fetal heart and adult heart explants, requires some handling skills. In fact, both fetal and adult decellularized tissues are delicate structures that can be permanently deformed when dried during the procedure or entrapped by the thin pipette tip or during handling with forceps[5].

The major novelty of this protocol, besides the parallel decellularization of fetal mouse heart and adult left ventricle explants for comparative assessment of the ECM composition (biomechanical analyses) and associated biological function, is its simple translation to distinct tissues and models[5,12,13]. The decellularization of tissues of distinct ages by applying standardized methodology was described only on the rhesus monkey kidney, and the transverse sections of fetal, neonatal and adult tissues, which were subjected to a 1% SDS treatment for 10 days[6]. In a

cardiac setting, although fetal, neonatal and adult tissues have been decellularized, the methods differed on the degree of mechanical dislodging, SDS concentrations and time of application[7,8]. As each decellularization procedure affects the ECM in a unique manner, the comparison of decellularized samples obtained from different protocols may lead to misleading conclusions. Hence, applying the same decellularization procedure across different samples enables reliable comparative analysis.

The large majority of the commercially available decellularized tissues derive from adult specimens[17]. Despite the growing recognition for an increased ability of fetal microenvironments in providing pro-regenerative signals in comparison to their adult counterparts, decellularization of fetal tissues was only reported in few studies[5,7,18-21]. Understanding ECM dynamics during the aging process will be crucial to identify unique features of pro-regenerative microenvironments which, in turn, will impact the development of higher efficiency biomimetic-materials.

ACKNOWLEDGMENTS:

The authors are indebted to all members of Pinto-do-Ó laboratory for relevant critical discussion. This work was supported by Programa MIT-Fundação para Ciência e Tecnologia (FCT) under the project "CARDIOSTEM-Engineered cardiac tissues and stem cell-based therapies for cardiovascular applications" (MITP-TB/ECE/0013/2013). A.C.S. is a recipient of a FCT fellowship [SFRH/BD/88780/2012] and M.J.O. is a FCT Fellow (FCT-Investigator 2012).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Frantz, C., Stewart, K. M. & Weaver, V. M. The extracellular matrix at a glance. *Journal of Cell Science.* **123** (24), 4195-4200, doi:10.1242/jcs.023820 (2010).

2 Rozario, T. & DeSimone, D. W. The extracellular matrix in development and morphogenesis: a dynamic view. *Dev Biol.* **341** (1), 126-140, doi:10.1016/j.ydbio.2009.10.026 (2010).

3 Badylak, S. F., Taylor, D. & Uygun, K. Whole-Organ Tissue Engineering: Decellularization and Recellularization of Three-Dimensional Matrix Scaffolds. *Annu Rev Biomed Eng.* **13** (1), 27-53, doi:10.1146/annurev-bioeng-071910-124743 (2011).

4 Ott, H. C. *et al.* Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med.* **14** (2), 213-221, doi:10.1038/nm1684 (2008).

5 Silva, A. C. *et al.* Three-dimensional scaffolds of fetal decellularized hearts exhibit enhanced potential to support cardiac cells in comparison to the adult. *Biomaterials.* **104** 52-64, doi:10.1016/j.biomaterials.2016.06.062 (2016).

6 Nakayama, K. H., Batchelder, C. A., Lee, C. I. & Tarantal, A. F. Decellularized rhesus monkey kidney as a three-dimensional scaffold for renal tissue engineering. *Tissue Eng Part A.* **16** (7), 2207-2216, doi:10.1089/ten.TEA.2009.0602 (2010).

7 Williams, C., Quinn, K. P., Georgakoudi, I. & Black, L. D., 3rd. Young developmental age cardiac extracellular matrix promotes the expansion of neonatal cardiomyocytes in vitro. *Acta Biomater.* **10** (1), 194-204, doi:10.1016/j.actbio.2013.08.037 (2014).

8 Fong, A. H. *et al.* Three-Dimensional Adult Cardiac Extracellular Matrix Promotes Maturation of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. *Tissue Eng Part A.* **22** (15-16), 1016-1025, doi:10.1089/ten.TEA.2016.0027 (2016).
9 Tapias, L. F. & Ott, H. C. Decellularized scaffolds as a platform for bioengineered organs. *Curr Opin Organ Transplant.* **19** (2), 145-152, doi:10.1097/MOT.0000000000000051 (2014).
10 Crapo, P. M., Gilbert, T. W. & Badylak, S. F. An overview of tissue and whole organ decellularization processes. *Biomaterials.* **32** (12), 3233-3243, doi:10.1016/j.biomaterials.2011.01.057 (2011).
11 Gilbert, T. W., Sellaro, T. L. & Badylak, S. F. Decellularization of tissues and organs. *Biomaterials.* **27** (19), 3675-3683, doi:10.1016/j.biomaterials.2006.02.014 (2006).
12 Pinto, M. L. *et al.* Decellularized human colorectal cancer matrices polarize macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18. *Biomaterials.* **124** 211-224, doi:10.1016/j.biomaterials.2017.02.004 (2017).
13 Garlikova, Z. et al. Generation of a close-to-native in vitro system to study lung cells-ECM crosstalk. *Tissue Eng Part C: Methods*. September 2017, ahead of print. doi: 10.1089/ten.TEC.2017.0283 (2017).
14 Wong, M. L. & Griffiths, L. G. Immunogenicity in xenogeneic scaffold generation: Antigen removal vs. decellularization. *Acta Biomaterialia.* **10** (5), 1806-1816, doi:10.1016/j.actbio.2014.01.028 (2014).
15 Motsavage, V. A. & Kostenbauder, H. B. The influence of the state of aggregation on the specific acid-catalyzed hydrolysis of sodium dodecyl sulfate. *J Colloid Sci.* **18** (7), 603-615, doi:10.1016/0095-8522(63)90054-0 (1963).
16 Rahman, A. & Brown, C. W. Effect of pH on the critical micelle concentration of sodium dodecyl sulphate. *J Appl Polymer Sci.* **28** (4), 1331-1334, doi:10.1002/app.1983.070280407 (1983).
17 Brown, B. N. & Badylak, S. F. Extracellular matrix as an inductive scaffold for functional tissue reconstruction. *Transl Res.* **163** (4), 268-285, doi:10.1016/j.trsl.2013.11.003 (2014).
18 Ieda, M. *et al.* Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. *Dev Cell.* **16** (2), 233-244, doi:10.1016/j.devcel.2008.12.007 (2009).
19 Whitby, D. J. & Ferguson, M. W. The extracellular matrix of lip wounds in fetal, neonatal and adult mice. *Development.* **112** (2), 651-668 (1991).
20 Brennan, E. P., Tang, X. H., Stewart-Akers, A. M., Gudas, L. J. & Badylak, S. F. Chemoattractant activity of degradation products of fetal and adult skin extracellular matrix for keratinocyte progenitor cells. *J Tissue Eng Regen Med.* **2** (8), 491-498, doi:10.1002/term.123 (2008).
21 Coolen, N. A., Schouten, K. C., Middelkoop, E. & Ulrich, M. M. Comparison between human fetal and adult skin. *Arch Dermatol Res.* **302** (1), 47-55, doi:10.1007/s00403-009-0989-8 (2010).

Figure 1

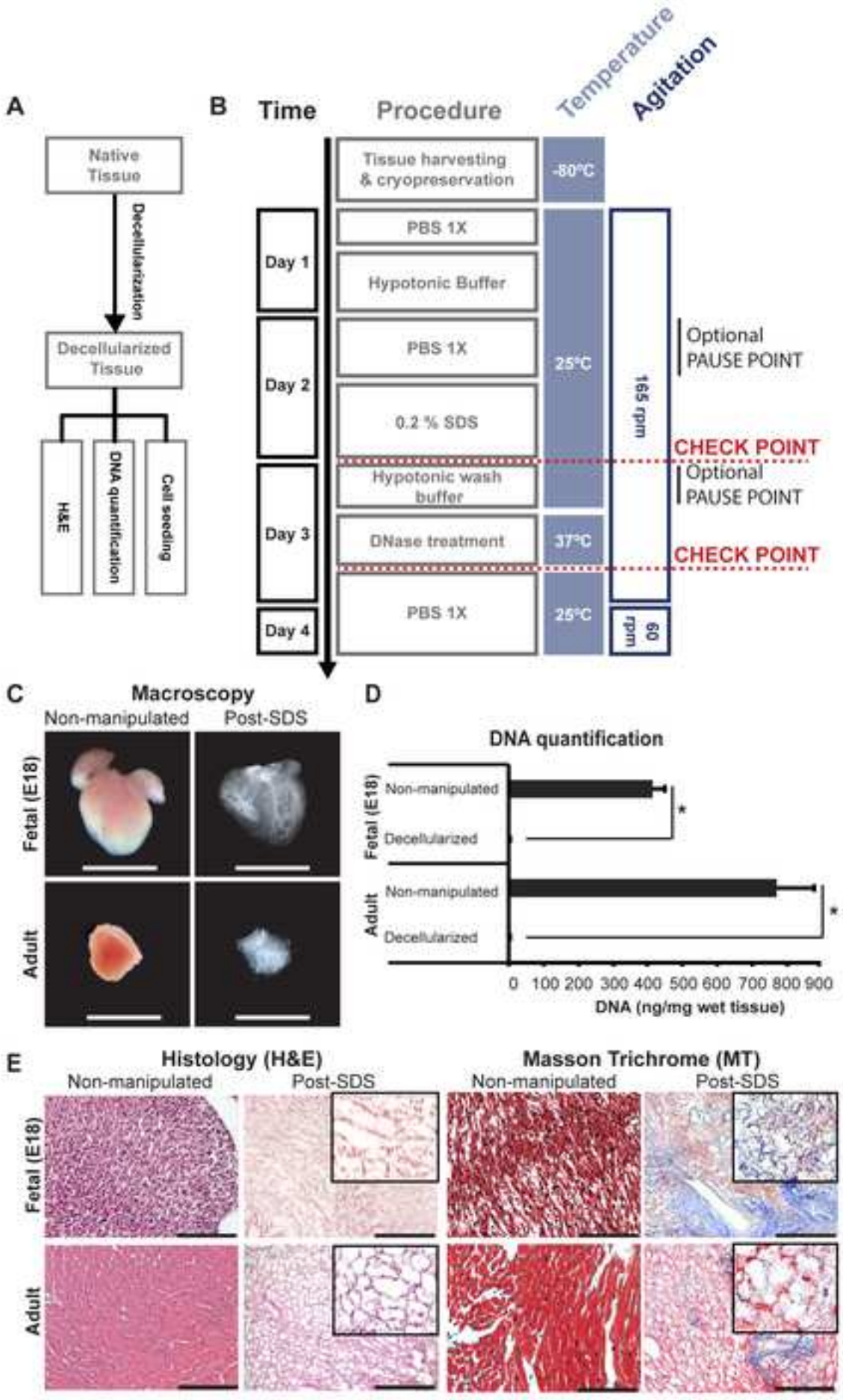

Figure 2

Click here to download Figure Figure2_56924_update.tif

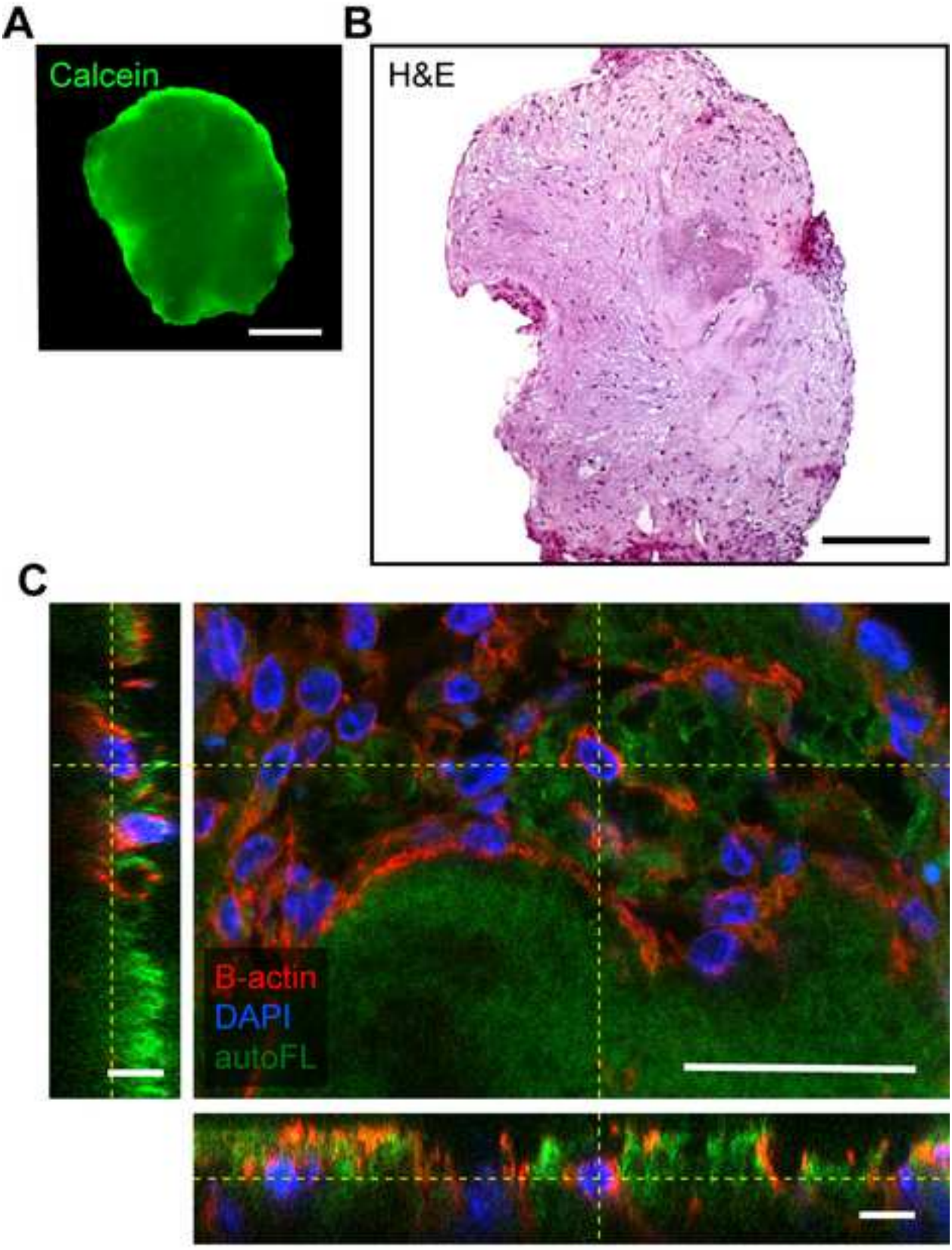

Table 1

Step	Observation	Possible reason
3.1.2.	Tissue with a brownish color	Sample cryofixation; unstable freezing temperature
3.2.4.	Pink-to-white opaque samples	SDS solution was not well prepared; adult LV explants too big
3.3.4.	Samples with a gelatin-like appearance	Inefficient DNA removal
4.6.	Histology gel compaction	Long waiting time in PBS1X/ethanol before paraffin processing
6.6.	Dried decellularized samples	Samples were left to dry for too long
6.7.	Decellularized sample detachment during seeding	Samples were not sufficient dried prior to seeding

Problem	Solution
Sample cryofixation will lead to inefficient removal of cytoplasmic proteins	Store frozen tissue during shorter periods. Check freezer temperature and stability
Inefficient removal of cytoplasmic proteins	Ensure complete SDS dissolution. Decellularize samples of smaller size.
Inefficient nuclear material clearance	Increase DNase concentration and/or incubation time.
Alteration of decellularized tissue structure	Start histological processing as soon as possible
Permanent collapse of decellularized tissue. Inefficient cell seeding	Allow samples to slightly dry only in the periphery in order to become attached to the bottom of the well during seeding
Inefficient cell seeding	Increase ECM drying time to allow better adherence prior to cell seeding

Materials Table

Click here to download Materials Table JoVE_Materials-1.xls

Name	Company	Catalog Number	Comments
Equipment			
Incubated Benchtop Shaker	Orbital Shakers	IKA:3510001	Recommended
Fluorimeter	-	-	Equipment available
Digital weight scale	-	-	Equipment available
Inverted Microscope	-	-	Equipment available
Cell culture incubator	-	-	Equipment available
Fridge (4ºC)	-	-	Equipment available
Deep freezer (-80ºC)	-	-	Equipment available
Microtome	-	-	Equipment available
Cirurgical Instruments			
Vannas Spring Scissors - 2.5mm Cutting Edge	Fine Science Tools	5000-08	Recommended
Dumont 5 Fine Forceps - Biologie/Inox	Fine Science Tools	11254-20	Recommended
Dumont 7 forceps	Fine Science Tools	11272-30	Recommended
Dissecting Scissors, straight	-	-	Tool available
Forceps, serrated, curved	-	-	Tool available
Materials			
24 well plates, individually wrapped	VWR	29442-044	-
96 well plates, individually wrapped	VWR	71000-078	-
Steriflip-GV, 0.22µm, PVDF, Radio-Sterilized	Millipore	SE1M179M6	-
Eppendorff	-	-	Material available
15 mL Falcon tubes	Fisher Scientific	430791	-
50 mL Falcon tubes	Fisher Scientific	430829	-
Four-Compartment Biopsy Processing/Embedding Cassettes with Lid	Electron Microscopy Science	70075-B	-
Fisherbrand Superfrost Plus Microscope Slides	Thermo Fisher Scientific	22-037-246	-
Tissue cryopreservation			
Shandon Cryomatrix embedding resin	Thermo Scientific	6769006	-
2-METHYLBUTANE ANHYDROUS 99+% (isopentane)	Sigma-Aldrich	277258-1L	-

Dry ice	-	-	-
Decellularization			
NaCl	BDH Prolabo	27810.364	-
Na_2HPO_4	Sigma-Aldrich	S-31264	-
KH_2PO_4	Sigma-Aldrich	P5379-100g	-
KCl	Sigma-Aldrich	P8041-1KG	-
TrisBASE	Sigma-Aldrich	T6066-500G	-
Sodium dodecyl sulfate	Sigma-Aldrich	L-4390	-
$MgCl_2$	MERCK	1.05833.1000	-
DNAse I	AplliChem	A3778,0050	-
Gentamicin	Gibco	15710-049	-
Fungizone	Gibco BRL	15290-026	-
Deionized water (DI water)	-	-	-
Histology			
10 % formalin neutral buffer	Prolabo	361387P	-
Eosin Y AQUEOUS	Surgipath	01592E	Can be replaced by alcoholic eosin
Richard-Allan Scientific HistoGel Specimen Processing Gel	Thermo Fisher Scientific	HG-4000-012	-
Ethanol ethilic alcohol 99,5% anydrous	Aga	4,006,02,02,00	-
Deionized water (DI water)	-	-	-
Clear Rite 3	Richard-Allan Scientific	6915	-
Shandon Histoplast	Thermo Fisher Scientific	RAS.6774006	-
Kits			
PureLink Genomic DNA Mini Kit	Thermo Fisher Scientific	K182001	-
Quant-iT PicoGreen dsDNA kit	Invitrogen	P11496	-
Cell culture			
DPBS	VWR	45000-434	-
Penicillin-Streptomycin Solution 100X	Labclinics	L0022-100	-
Fungizone	Gibco BRL	15290-026	-

Cell culture media of the cell of interest	-	-	-

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Parallel decellularization of fetal and adult cardiac tissue explants as 3D in vitro microenvironments to dissect cell-ECM crosstalk

Author(s): Ana C Silva, Maria J Oliveira, Todd McDevitt, Mário A Barbosa, Diana S Nascimento, Perpétua Pinto-do-Ó

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Perpétua Pinto-do-Ó
Department:	INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Porto 4200-135, Portugal
Institution:	i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto 4200-135, Portugal
Article Title:	Parallel decellularization of fetal and adult cardiac tissue explants as 3D in vitro microenvironments to dissect cell-ECM crosstalk
Signature:	Perpétua Pinto do Ó
Date:	1 July 2017

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Reply to Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

In this manuscript, Silva et al. a "new" protocol presented a new developed decellularization protocol in which a hypotonic buffer was combined with anionic surfactants and DNAse treatment. This new protocol was applicable across different age tissue samples and assured in the authors view more reliable comparative analysis.

Major Concerns:

1. General remark, the authors state that 2D in vitro cultures are an oversimplification of the complex nature of the tissue ECM. There approach will result in a 3D approach. To my opinion the seeding method the authors choose will in the case for heart tissue never result in a real 3D culture environment. With the static seeding the cells will only be cultured on top of the scaffold, since the fiber organization of decellularized heart tissue doesn't allow cell ingrowth. So this is a 2.5D in vitro culture setup and not the resemblance of native 3D. Figure 2A and 2B also don't show a 3D culture environment, only the presence of cells.

We appreciate the comments and concerns of the reviewer #1. Because of the small size (fetal heart ±4-5mm, adult explants ±1.5-2mm) of the decellularized scaffolds, passive cell seeding was the only feasible method. Other seeding methods were tested, such as cell injection, but showed little success. We agree with Reviewer#1 that the nature of fiber organization can work as a barrier to cell ingrowth, especially in the adult heart, where the basal membrane is a thick and well-defined network (Figure 1, and Figure 2 A,C,D[1]). In addition, the nature of seeded cells, i.e. their motile and enzymatic potential, also influences the capacity to colonize the interior of the decellularized scaffold. However, applying our decellularization and seeding strategy, with rat neonatal cardiomyocytes and immortalized mouse $Lin^{-}Sca\text{-}1^{+}$ cardiac progenitor cells ($iCPC^{Sca1}$), we were able to observe cells on the surface and in the interior of cardiac decellularized scaffolds, in close contact with the ECM (Silva *et al.* 2016, Figure 3 and 4[1]). For a more comprehensive illustration, a detail of cells surrounded by the ECM mesh was added to the Figure 2C. Although this data indicates cells become embedded in the ECM, we are aware that, due to the sample size and seeding mode, our *in vitro* setup is in the limit between

2.5D and 3D. In line with this, and according to reviewer #1 comment, we substituted 3D by 3D-like *in vitro* culture system along the present revised version of the manuscript.

2. General remark, sometimes it is unclear when a new part of the protocol starts, since the numbering goes from 3.10 - 4.1 -3.11 so not really logically. The protocol would be more clear if the authors would divide it in sections, e.g. 3. Tissue decellularization, 4. Assessment of decellularization efficiency, 5. Scaffold characterization.
We appreciate the suggestion and alterations to the protocol were performed according to Reviewer#1 comments.

3. In the title "parallel" means "occurring or existing at the same time or in a similar way, but for me it is more at the same time. Since the protocol is more based on a similar way I would choose a different word, e.g. equivalent.
According to Reviewer# 1 suggestion, and considering the sample size and seeding strategy, the title of the manuscript was changed to: "*Comparable decellularization of fetal and adult cardiac tissue explants as 3D-like in vitro microenvironments to dissect cell-ECM crosstalk*".

4. In the introduction I think the use of references is poor and references to literature could be used more often, e.g. in the second sentence, the beginning of the second paragraph.
We followed Reviewer#1 suggestion and increased the number of references to literature, please see edited manuscript.

5. In the introduction the authors state that "decellularization of fetal tissues has been poorly explored and reported only for the decellularization of fetal rhesus monkey kidney and rodent heart´. The described protocol in the current manuscript is based on fetal and adult mouse heart tissue, which are also rodents and the group published already a manuscript on decellularized fetal mouse heart in 2016. So this statement of "poorly" is incorrect, in my opinion.
According to Reviewer #1 comment, the sentence was changed to: "Prior to this work, head-to-head comparison of tissues at different ontogenic stages using the same decellularization approach has only been reported for rhesus monkey kidney and rodent heart. In addition, a limited number of studies report fetal tissues/organs decellularization *per se.*"" (lines 83-86).

6. In the introduction the authors state that "long exposure to SDS have been correlated with protein denaturation, GAGs loss and disruption of collagen fibrils. In the current protocol incubation with 0.2% SDS solution is 24h. Is the combination of the hypotonic buffer with the lower amount of SDS for 24h, "gentle" enough to prevent GAGs loss and collagen fibril disruption? There is no proof that this new protocol prevents this loss compared to previously published protocols.

We acknowledge Reviewer#1 comment; indeed, we do not have any data comparing our protocol with other previously described protocols. We observed a reduction of sulfated GAG content after decellularization as reported in other publications, although we were able to preserve essential ECM molecules and their distribution upon decellularization, please see, Figure 2A and B[1]. Electron microscopy analysis of our samples revealed well preserved collagen fibrils and fibers, please see Figure 2 E,G[1]. During protocol development, as described previously on Silva *et al*. 2016, different SDS concentrations were tested and 0.2% SDS showed best efficiency regarding network preservation and cell removal (Supplementary Figure 2 A[1]). The majority of heart tissue decellularization protocols reported in the literature are focused on adult tissues and based on higher SDS concentrations (0.5%, 1% SDS) and combination of SDS with other detergents/compounds[2,3]. Thus, exposing the cardiac tissue too lower SDS concentrations, we expect to prevent major ECM loss and fibers disruption (Figure 2[1]). However, when decellularizing adult intestine using a similar protocol but with lower SDS concentrations (0.1%) (ML Pinto, Biomaterials, 2017), we were not able to avoid significant (approx. 80%) GAGs loss. This is expected since GAGs, apart from being easily leachable components of the ECM, are integrated within cell membranes, being partially removed with the decellularization procedure. To clarify the introduction, we altered the manuscript (line 90-97) to: “Solutions containing high SDS and extended periods of exposure have been correlated with protein denaturation, glycosaminoglycan (GAGs) loss and disruption of collagen fibrils, and therefore a compromise between ECM preservation and cell removal is necessary. To apply the same procedure to fetal and adult heart tissue, the protocol described herein is divided in three sequential steps: cell lysis by osmotic shock (Hypotonic Buffer); solubilization of lipid-protein, DNA-protein and protein-protein interactions (0.2 % SDS); nuclear material removal (DNAse treatment). “

7. In the introduction the authors state that this protocol can be straightforwardly adapted to other tissues and species. Although there is no proof of this straightforward adaptation with clear provided results. From literature is known that decellularization methods for different species and organs need specific protocols and reagents.

We appreciate the comments of Reviewer#1 and the word “straightforward” was substituted by “readily” for a better comprehension (line 100). Although data concerning the adaptation of this protocol to other organs was not included in this manuscript, results on human intestine[6] and on murine lung[7] have been published.

8. The Protocol section is not as extensively described as I'm used from manuscripts published in JoVe.

In order to fulfill the Reviewer#1 request, the protocol section was edited for a better and detailed description of each procedure.

9. In the Protocol section 2.1, "euthanize adult female at 18 days of gestation, in my opinion strangely written, I would add the word pregnant "euthanize adult pregnant females at 18 days of gestation". Followed by the sentence that adult left ventricles were isolated from 7-8 week old male C57BL/6 mice.

Alterations in the text were performed accordingly to Reviewer#1 suggestions.

10. In the Protocol section 2.3, what is OCT? The Tissue Tec OCT? What is the rationale behind the use of the Tissue Tec OCT? Could cryopreservation of the hearts be a better solution to store them?

The definition of the acronym OCT mentioned by the Reviwer#1 refers to the optimal cutting temperature (OCT) compound. The acronym definition was added to the manuscript (line 161). The rationale behind the use of OCT is to preserve tissue structure during cryopreservation. We have not tested cryopreservation of the tissue without the OCT compound. After defrosting, washing of tissue samples is required to efficiently remove the OCT compound prior to decellularization.

11. The authors state that 1ml of reagents is used to 6-8mg of cardiac tissue? Is this the same amount that the authors used, for me that is not clear? Is the volume of Hypothonic Wash Buffer and PBS the same as the Hypothonic Buffer and SDS?

We addressed the comments of the Reviewer#1 and edited accordingly the manuscript to clarify the decellularization procedure: "Cardiac tissue decellularization is performed in 24-well tissue culture plate with one sample per well. A final volume of 1mL of each decellularization solution is added to each individual well" (lines 171-173).

12. For me the part of the Quantification of DNA content can be explained in more detail. What is the size of small explants? Is the protocol the same for non-manipulated samples and decellularized samples, since the weight will be different and lysis may be faster and in decellularized samples.

To maximize DNA extraction efficiency from small samples we used a pool of samples of the same condition. The tissue length and mass were ±4-5mm and ±6-8mg for the fetal heart and ±1.5-2mm and ±1-2mg for the adult LV explant, respectively. The same DNA isolation and quantification protocol was applied to native (non-manipulated tissue) and decellularized tissues. Indeed, we observed a faster lysis of the decellularized tissues during DNA extraction step but, at the end of the procedure, the quality yield of DNA extraction was similar in both conditions. The protocol section "5. Assessment scaffold nuclear material removal" was edited for a better and detailed description, as suggested by Reviewer#1.

13. The authors correct the samples for wet weight, the decellularized tissue is known for to collect lots of fluid and is harder to remove the excess fluid compared to non-manipulated fluids. Why did authors not choose to correct for dry weight?

The quantity of DNA present post-decellularization was corrected to the initial wet weight of the tissue (before decellularization) and not to the decellularized tissue which collect high amount of fluids. The rational for this normalization was to quantify the reduction of the quantity of DNA after decellularization in comparison to the initial tissue (before decellularization). Furthermore, the decellularization procedure described on the present manuscript was developed for wet

tissues. Thus, samples dehydration for the initial wet weight measurement (before decellularization) would interfere with sample integrity and protocol efficiency.

14. The authors are not clear about concentrations of cells.

We acknowledge Reviewer #1 for this comment. Different cells types have been used for passive seeding the decellularized tissues generated through this protocol, namely, neonatal rat cardiomyocytes postnatal day 1 to 3, using a cell density of 7500 cells/mm2, and immortalized mouse $Lin^{-}Sca\text{-}1^{+}$ cardiac progenitor cells ($iCPC^{Sca\text{-}1}$), at a density of 60-1500 cells/mm^2 [1]. Information regarding cell density was added to the revised manuscript (lines 349-351). Of note, in future experiments cell densities must be adjusted by the user according to their experimental set up and cell type of interest.

15. What is TCPS?

We acknowledge Reviewer #1 for this note. In the present manuscript TCPS means Tissue Culture Polystyrene Plates, the definition was added to the revised manuscript.

16. Regarding the decellularization efficiency in the Results. For cardiac tissue it is known that due to the highly organized tissue architecture, DNA or Benzonase treatment will lead to removal of nuclei, but cell membranes and cytoplasm might be still present. The Masson Trichrone and HE pictures lack magnification to really show porous mesh. The DNA content data look promising but might not represent real decellularization due to presents of cell membranes and other cell remnants. Since the organization in the fetal heart is less organized decellularization will be more efficient and less cell remnants will be present.

We appreciate the comments and concerns of the Reviewer #1. Of note, in the first submitted version of the present manuscript we have not included Masson Trichrome data but only Hematoxylin and Eosin stains. Masson Trichrome data was added, please see, Figure 1E. We agree with reviewer #1 that histological staining is insufficient to confidently show removal of cytoplasmic and membrane remnants yet we consider it can be a good indicator. In fact, to the best of our knowledge, no experimental approach can accurately quantify the amount of cell membranes and cytoplasm remnants and thus histological staining are commonly applied to demonstrate decellularization efficiency. We have included an inset with more detail of the

decellularized ECM structure (please see Figure 1E in the revised manuscript). Moreover, in our previous work [1] we confidently confirmed removal of cytoplasmic components by showing lack of abundant cardiac proteins (vimentin and cardiac Troponin T) after decellularization by immunofluorescence.

17. Regarding the highly organized tissue architecture of the heart and the small pores and stated in remark 1, the results are not convincing that cells are really in a 3D configuration.

We appreciate the comments and concerns of the Reviewer#1 and, as mentioned in the reply to Reviewer#1 comment 1, we are aware of our cell seeding and sample size limitations. However, we could observe cell ingrowth to the interior of decellularized scaffolds, where cells are completely surrounded by the ECM mesh. To address Reviewer#1 comment, Figure 2C was edited by the addition of a confocal image of a thick central section of a repopulated cardiac scaffolds demonstrating that cells in the interior of the scaffold are embedded in the ECM, in a 3D-like configuration.

18. In the Discussion, the suggestion is to vary with SDS concentrations to improve loss of cell remnants, although the protocol was designed to use less SDS and protect the samples from protein and GAG loss.

The paragraph mentioned by Reviewer#1 in the discussion was edited for a better interpretation: "Ultimately, the protocol herein demonstrates to be versatile and readily applied on distinct specimens through slight adjustments on tissue explants size, SDS concentration (0.1-0.2% SDS) or solutions incubation time [5,12,13]." (lines 441-443). This suggestion is based on our experience in the decellularization of other tissue matrices, as the case of human intestine samples, for which a 0.1% SDS concentration was applied[6].

19. In the end, I'm not convinced that this protocol is faster, simple and more protective then other published decellularization methods.

We acknowledge the opinion of the Reviewer#1 about the comparison between our protocol and other published decellularization methods. As mentioned before, we are not disregarding the scientific rigorous work published by other colleagues in the field, but rather proposing a

different approach for comparative studies across specimens using a similar decellularization method. The text in the revised manuscript was edited to clarify this message (line 58-59).

Reviewer #2:

Manuscript Summary:

The authors describe an interesting and robust method to generate fetal and adult ECM from cardiac tissue with many potential future applications in cell and stem cell biology.

References

1 Silva, A. C. *et al.* Three-dimensional scaffolds of fetal decellularized hearts exhibit enhanced potential to support cardiac cells in comparison to the adult. *Biomaterials.* **104** 52-64, doi:10.1016/j.biomaterials.2016.06.062, (2016).

2 Ott, H. C. *et al.* Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nature Medicine.* **14** (2), 213-221, doi:10.1038/nm1684, (2008).

3 Oberwallner, B. *et al.* Preparation of cardiac extracellular matrix scaffolds by decellularization of human myocardium. *J Biomed Mater Res A.* **102** (9), 3263-3272, doi:10.1002/jbma.35000, (2014).

4 Gilbert, T. W., Sellaro, T. L. & Badylak, S. F. Decellularization of tissues and organs. *Biomaterials.* **27** (19), 3675-3683, doi:10.1016/j.biomaterials.2006.02.014, (2006).

5 Crapo, P. M., Gilbert, T. W. & Badylak, S. F. An overview of tissue and whole organ decellularization processes. *Biomaterials.* **32** (12), 3233-3243, doi:10.1016/j.biomaterials.2011.01.057, (2011).

6 Pinto, M. L. *et al.* Decellularized human colorectal cancer matrices polarize macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18. *Biomaterials.* **124** 211-224, doi:10.1016/j.biomaterials.2017.02.004, (2017).

7 Garlikova, Z. et al. Generation of a close-to-native in vitro system to study lung cells-ECM crosstalk. *Tissue Engineering Part C: Methods*. September 2017, ahead of print. doi: https://doi.org/10.1089/ten.TEC.2017.0283, (2017).