**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,D1). 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-1+ cardiac progenitor cells (iCPCSca1), 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 41). 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 B1. Electron microscopy analysis of our samples revealed well preserved collagen fibrils and fibers, please see Figure 2 E,G1. 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 A1). 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/compounds2,3. Thus, exposing the cardiac tissue too lower SDS concentrations, we expect to prevent major ECM loss and fibers disruption (Figure 21). 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 intestine6 and on murine lung7 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-1+ cardiac progenitor cells (iCPCSca-1), at a density of 60-1500 cells/mm2 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 applied6.

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**

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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).