Utrecht, august 22 2018

*Rebuttal document for revision of manuscript JoVE58656*

**Dear editor and reviewers,**

We would like to thank you for thoroughly peer-reviewing and providing feedback on the manuscript. The comments are very useful and have improved the quality of the manuscript. We appreciate the time, work and precision spend on peer-reviewing the manuscript. The title is ‘Fabrication of decellularized cartilage-derived matrix scaffolds’ and the work is produced by K.E.M. Benders, M.L. Terpstra, R. Levato, J. Malda. Below, you will find a discussion and a response for each of the comments of the editor and reviewers in **bold**. Also, the adapted sentences according to the comments are presented in *italic with inserted words in italic and underlined.*

Yours sincerely,

Prof. dr. J. Malda

**Author’s response to peer-review comments**

Editorial comments

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

**The manuscript has now been thoroughly read to avoid the presence of spelling and grammar mistakes.**

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

**Thank you for your comment. We will ask for specific permission for each used and already published figure. Since one additional figure was added, we are currently asking for new permission. We will provide you with specific permission for all figures before the publication process.**

3. Figure 3: Please define the left and right panels in the figure legend.

**The left and right picture has been specified as follows:**

*The left and right picture display a scaffold from two different angles.*

4. Please provide an email address for each author.

**Each author is now provided with an e-mail address in section ‘AUTHORS & AFFILIATIONS’.**

5. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

**The centrifuge speed was changed from 4000 rpm to 3113 g for all of the five cases.**

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Picogreen, Triton, etc.

* **Triton is now changed to octoxynol-1.**
* **Picogreen is now refered to as:**
  + *8.2) Perform an assay with a fluorescence-based DNA quantification kit to measure the double-strand DNA content of the scaffolds to ensure complete decellularization. Follow the protocol provided by the manufacturer. Express the amount of DNA per dry weight of the scaffold.*
* **In the tabe of materials two trademark symbols have been removed.**

7. Please revise the protocol (lines 62-68, 1.2.1, 1.7, 3.4.1, etc.) to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

* **The imperative tense has been applied for the steps**
  + **1.1-1.14, 2.1-2.4, 3.2, 3.4, 5.7, 6.1-6.7, 7.1, 7.3 (some step-numbers have been changed due to adaptations to the manuscript)**
  + **In addition, extra information has been added below the steps as ‘Note: …’, which were added below the steps 1.3, 1.7, 1.9, 1.10, 1.14, 2.2, 3.1, 3.3.4, 4.2, 5.3, 6.3, 6.7, 7.2, 7.3, 7.7, 9.1, 9.3, 9.4.**
* **Safety procedures**
  + *1.2) Wear gloves and a lab coat during the entire harvesting procedure, since the donor can carry pathogens.*

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

**More information was added for the following steps**

* **1.2-1.8, 1.13, 2.2, 3.2, 9.3**

**A reference was added to provide information for the steps**

* **7.8, 8.1, 8.3, 9.4**

9. 1.1: What volume of washing solution is needed?

**The following volume has been added:**

*1.1) Ahead of the harvesting step, prepare 1 L of cartilage washing solution…*

10. 1.2: Please specify the source of knee joint and describe how to perform an arthrotomy.

**Extra information is now provided regarding performing the knee section, 1.4-1.8. Also, the joint source has been clarified.**

**1.3) …** *Note: for this protocol equine stifle cartilage was obtained from horses that had died from other causes than osteoarthritis, with permission from the owner. Beside horse stifle joints, also other joints from other species can be used in this protocol.*

11. 1.4: What container is used?

**For this step, 50 ml tubes were used.**

*1.11) Collect the cartilage slices in 50 ml tubes containing previously prepared cartilage washing solution (Figure 2A).*

12. 2.2: What can be used to sieve the cartilage particles?

**The maze size has been added (0,71 mm), as well as the product information in the materials list.**

13. 2.2.1: Please specify the size of the cartilage particles used in the protocol. Please specify at which temperature the particles can be stored.

**Regarding to the sieve that was used in this protocol, the particles size is not larger than 0,71 mm. In addition, the storage temperature has been added:**

*2.4) Store the particles in a dry place at room temperature.*

14. 3.5, 5.6: What volume of PBS solution is used to wash?

**The volume for washing has been added, which is 30 ml for both step 3.4 (former 3.5), and 5.6.**

15. 6.5: What is the source of the UV-light and what is the wavelength range of the UV-light?

**The UV light that was used is now specified in the materials list. In addition, the wavelength and distance are now added.**

6.6) … *cross-link them with ultraviolet (UV) light at 30 cm distance and 365 nm overnight.*

16. 6.6: Please specify how to do ethylene oxide gas sterilization.

**This was performed by an external company, from which the name is stated in the materials list.**

17. 7.2, 9.1: What is used to cut?

**The tool for cutting was added.**

*7.2) Cut the scaffolds in thin slices of approximately 3 mm with a scalpel.*

18. 7.3: How to cross-link using formalin?

**The method for cross-linking by using formalin has been specified below:**

*7.3) Embed the scaffolds in a drop of 4% (w/v) alginate and induce cross-linking by adding a similar volume of 3.7% non-buffered formalin that contains 20 mM CaCl2.*

*Note: alginate embedding makes the scaffold slices more rigid regarding to the washing steps prior to paraffin embedding. In case the scaffolds have been cell seeded or in vivo implanted, alginate embedding is not necessary, as the composition of the scaffolds will be resistant enough due to ECM incorporation by the cells.*

19. 7.4: Please specify the graded ethanol series used in this step.

**The procedure has been added:**

*7.4) Dehydrate the samples by placing them in a graded ethanol series, starting with one-hour cycles of 70%, 96%, 96%, 100% and 100% ethanol, followed by 2 one-hour cycles of xylene, and ending with 2 one-hour cycles of paraffin at 60 °C.*

20. 7.5: More details are needed regarding how to perform different stainings.

**For the staining protocols as well as the assays, references are added to former protocol. This is because the scope of this current protocol focuses on a new method for scaffold formation, rather than standardized laboratory protocols. However, references (11) are added to guide the reader to the corresponding protocols.**

21. 8.2: Please add more details here. How to measure DNA content?

**The DNA content was measured by a DNA quantification kit Picogreen that is specified in the materials list. Since no manufacturer’s names are allowed in the manuscript, the DNA assay is referred to as follows:**

8.2) Perform an assay with a fluorescence-based DNA quantification kit to measure the double-strand DNA content of the scaffolds to ensure complete decellularization. Follow the protocol provided by the manufacturer. Express the amount of DNA per dry weight of the scaffold.

22. 9.3.2: Please specify the medium used in the protocol.

**The medium has now been specified for this protocol.**

*9.3) Rehydrate scaffolds with cell culture medium by pipetting 1 ml of medium on top of the scaffold and let it soak for 30 minutes. Use either chondrocyte or MSC expansion medium.*

*Note: use the same medium for scaffold rehydration as was used for cell culture of the cells that will be seeded. Chondrocyte expansion medium consists of DMEM with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml fibroblast growth factor-2 (FGF-2). MSC expansion medium consists of α-MEM with 10% heat-inactivated FBS, 0.2 mM l-ascorbic acid 2-phosphate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml FGF-2.*

23. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

**The reference number are now all written as superscripts, and the order of the references are organized in the chronological order.**

24. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

**All the references have now been adapted to the above format.**

25. References: Please do not abbreviate journal titles.

**Adaptations have been made to the journal names, and they are now *italic*.**

26. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

**In the list of materials, two trademark/registered symbols have been removed.**

Reviewers' comments:  
Reviewer #1:  
  
Manuscript Summary:  
This manuscript describes a process to extract cartilage extracellular matrix and form it into scaffolds.  
  
Major Concerns:  
None  
  
Minor Concerns:  
Page 6 - last line. Safranin-O staining is said to confirm that there are no proteoglycans present in the extract, however this dye only stains the anionic glycosaminoglycans and provides no information of the presence of the proteoglycan protein cores. This sentence should be modified to indicate that no glycosaminoglycans are present. This error is also in the Figure 4 legend and should be corrected.

**Thank you for pointing out this scientifically important error. We have changed proteoglycans to glycosaminoglycans (GAGs) in three locations:**

* **First paragraph of the ‘Representative results’**
* **Description of figure 4**
* **Description of figure 6 (former figure 5)**

Page 8 - last paragraph. Explain how trypsin treatment 'leads to extensive loss of GAGs' as trypsin does not cleave glycosaminoglycan chains.

**Thank you for your suggestions. In the discussion section the following explanation was inserted:** *‘Even though trypsin does not cleave GAGs, the reason for the GAG-loss can be due to opening up the cartilage tissue by trypsin cleaving proteins that anchor or encapsulate GAGs.’*  
  
  
Reviewer #2:

Manuscript Summary:  
The authors present a detailed yet straight-forward method to both decellularise cartilage tissue and fabricate scaffolds from the resulting decell material. I feel that given the detail provided in this manuscript that the protocols detailed here could be readily reproduced by any tissue engineering lab as minimal specialist apparatus is required. Copy-editing of the manuscript is required as it contains a number of spelling and grammatical errors.  
  
I have provided some comments below, these are in order to provide further clarification to the reader and aid them in performing the protocol.  
  
Major Concerns:  
None.  
  
Minor Concerns:  
Line 70 - Clearly state that this is equine tissue used in this protocol - perhaps move part of the section from the discussion on different species (line 376) into the introduction to clarify. Due to the amount of processing involved I would assume that the protocol will work across various species.

**Thank you for your suggestion. We have inserted additional information about using equine tissue:**

**- Last paragraph of the introduction: *‘****This paper describes a protocol adapted from Yang et al. (2010)13 for the production of decellularized CDM scaffolds from equine stifle cartilage. These scaffolds are rich in collagen type II, devoid of cells and do not contain any glycosaminoglycans after decellularization. Both in vitro and in vivo experiments on (osteo)chondral defect repair can be conducted using these scaffolds.*

***-* Beginning of the protocol:point 1.3.** *‘Note: for this protocol equine stifle cartilage was harvested, however, also other joints from other species can be used.’*

Line 95 - Please provide more detail on the freeze drying parameters, temperature and vacuum set-points if applicable.

**Thank you for your suggestion. The following information has been added:**

*‘Set the freeze dryer at approximately 0,090 mbar while the ice condenser is -51 °C…’*

Line 97 - Are the slices stored at room temperate or at 4 degrees / -20 degrees?

**The storage conditions have been added:**

*‘1.14) Store the cartilage slices in a dry place at room temperature until further use.’*

Line 107 - "grinding automatically" can the authors please clarify what device they use here. Is this a cryo-mill system?

**More details have been provided now, and the automatic grinding machine is listed in the material’s list:**

*2.2) Directly grind the samples either manually or by a milling machine. When grinding the cartilage slices by hand, use a mortar and pestle and grind the slices for approximately 45 minutes until they are pulverized (Figure 2C-D) When grinding automatically, use a milling machine at its pre-set speed for a few seconds up to a minute, until the snap-frozen cartilage slices are pulverized.*

*Note: pre-cool the mortar or the milling compartment of the milling machine by adding liquid nitrogen before the cartilage slices.*

*Note: when using a milling machine, make sure that all particles are grinded, and no particles stay in the bottom of the grinding compartment.*

Line 114 - Are the particles stored at room temperate or at 4 degrees / -20 degrees?

**The storing conditions have been added:**

*2.3) Store the particles in a dry place at room temperature.*

Line 122 - Please specify a range of mass which can be used here (per tube). I assume that if doing a large prep you spread the cartilage particles out across a number of 50ml tubes?

**The following information about sample size has been added:**

*3.2) Fill up 50 ml tubes with the pulverized cartilage particles up to a volume of approximately 7.5 ml per tube.*

Line 129 - Can the authors provide more information as to what piece of equipment was used to vigorously agitate the sample, was it a vortex/shaker device?

**Thank you for the comment. The equipment that was used is to find in the materials list. The follow specification was added in the manuscript:**

*3.3.3) Incubate the samples for 4 hours at 37 °C on a roller.*

Line 151 - Is this water deionized? Perhaps re-phrase this sentence as demi-water would not be the most common term.

**Demi-water has been changed into deionized water.**

Line 195 - Are the cartilage particles lyophilised again or are they stored "wet"?

**The sentence has been clarified:**

*5.7) Leave the last PBS wash in the tubes, and store the* *decellularized cartilage particles at -80 °C.*

Line 201 - Is a defined mass of cartilage or concentration added to each well? What gives the optimal scaffold?

**The scaffolds are produced by adding as much cartilage in the mold as possible, while making sure to press all the air-bubbles out. No experiments have been done to investigate a higher particle density by applying more or less pressure. As for the protocol’s decellularization steps, each tube can be filled up to approximately 7.5 ml.**

*3.2) Fill up 50 ml tubes with the pulverized cartilage particles up to a volume of approximately 7.5 ml per tube.*

Line 214 - Please specify the UV light / lamp wavelength settings used to cross link the scaffolds. Are the scaffolds turned during the crosslinking procedure to expose all surfaces to the UV?

**The UV light that was used is now specified in the materials list. In addition, the wavelength and distance are now added.**

6.6) … *cross-link them with ultraviolet (UV) light at 30 cm distance and 365 nm overnight.*

Line 240 - Please state the % concentration of citric acid used here.

**The citric acid concentration has been added: 10 mM: step 7.7).**

Line 242 - Please state the section thickness used for slicing the samples.  
**The thickness of the histology slices has been added.**

*7.6) Cut the samples with a microtome in slices of 5 μm thick.*

Line 261 - Did the authors use a DMMB kit for this assay? If so please include kit details in materials list.

**In the current protocol no DMMB kit was used. Now a reference was added for performing the DMMB assay in step 8.3)**

*8.3) Perform a dimethylmethylene blue assay to quantify the remainder of the glycosaminoglycans (GAGs) within the scaffold, as was done before11. Express the amount in GAG per DNA.*

Line 266 - Do the scaffolds necessary have to be cut to 3mm thick or can they be cut to whatever size is required?

**Thank you for your comment. The scaffold size may have an effect on the dehydration protocol duration, as now clarified in a note. 7.2) …** *Note: if the scaffolds will be cut in larger or smaller sizes, the durations of the dehydration cycles need to be adapted.*

Line 290 - Do the authors use a chondrogenic induction medium here (with TGFb3)?If so please state this.

**The medium has now been specified for this protocol.**

*9.3) Rehydrate scaffolds with cell culture medium by pipetting 1 ml of medium on top of the scaffold and let it soak for 30 minutes. Use either chondrocyte or MSC expansion medium.*

*Note: use the same medium for scaffold rehydration as was used for cell culture of the cells that will be seeded. Chondrocyte expansion medium consists of DMEM with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml fibroblast growth factor-2 (FGF-2). MSC expansion medium consists of α-MEM with 10% heat-inactivated FBS, 0.2 mM l-ascorbic acid 2-phosphate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml FGF-2.*

Figure 4 - Please include images of native equine cartilage with staining for H&E, Saf-o and IHC for type II collagen to further demonstrate the effect of the decell procedure in terms of removal of cells and GAG.

**Thank you for your comment. Now, also a figure with healthy cartilage has been added, for the stains Safranin-O and collagen type II. The cells are also visible in the Safranin-O staining, for that the H&E staining was left out.**  
  
In addition, DNA removal is one of the key criteria that must be demonstrated in any decell protocol. The authors mention that the DNA levels are "diminished critically" but they do not state the DNA level obtained.  
Ref. 17 (Crapo et al.,) have a criteria of <50 ng dsDNA per mg ECM dry weight which is what multiple labs work towards when performing decell procedures.  
Please include (either as a definitive number in the text or graphically represented in figure 4) the exact DNA per mg of DRY weight tissue after the decell procedure.

**Thank you for this important comment. Regarding to this, two references have been added to state the criteria of decellularization, and DNA content is now included in the results section.**

For this specific decellularization method, DNA was below the detection range, which started at 57,8 ± 10 ng/mg DNA/dry weight (n=3).  
  
  
  
Reviewer #3:  
  
Manuscript Summary:  
The protocol is an interesting procedure for obtaining lab-made decellularized scaffold to be used in cell therapy or 3D culturing studies on cartilage cells. I think is interesting and suitable for publication.  
  
Major Concerns:  
The main concern is related with the steps used for the decellularization of the scaffolds. I think it would be interesting to state in the protocol which is the aim of every step. In addition, usually detergents are used for the breaking of cell membranes, so nuclease treatment could also be used after this, once the cell content have been released. It can be assumed that nucleases enters to the cell, but all of these should, at least be discussed in the discussion section.  
  
The presence of collagen type II is a key feature for these scaffolds and their presence has to be carefully validated. Figure 4c should include, in my view, a negative control to demonstrate that the straining of the presented image is specific staining and not background staining.

**Thank you for your comments on the manuscript. As for the explanation of protocol steps, details are now provided as stated at your related comment below. In addition, a Safranin-O and collagen II staining was now added for healthy cartilage. In the figure 5, an osteochondral plug was stained with collagen II, that shows a positive staining for cartilage, and a negative staining for bone. This confirms that the used collagen II staining does not show background, concluding that the decellularized scaffolds are not background staining.**

Minor Concerns:  
- A first part detailing the preparation of the solutions would help the reader

**An overview of the used solutions can help the reader to understand the protocol, however, since the protocol runs over several days, it may as well confuse the reader. This is because many of the solutions need to be prepared fresh right before the consecutive steps. Therefore, we left the explanation for preparing the solutions in the original order to provide a protocol that can be followed in chronological order.**  
  
- step 2.1.2. This step could be a little more detailed. Any comments on the speed of the grilling machine?

**Thank you for your comment. More details have been provided now, and the automatic grinding machine is listed in the material’s list:**

*2.2) Directly grind the samples either manually or by a milling machine. When grinding the cartilage slices by hand, use a mortar and pestle and grind the slices for approximately 45 minutes until they are pulverized (Figure 2C-D) When grinding automatically, use a milling machine at its pre-set speed for a few seconds up to a minute, until the snap-frozen cartilage slices are pulverized.*

*Note: pre-cool the mortar or the milling compartment of the milling machine by adding liquid nitrogen before the cartilage slices.*

*Note: when using a milling machine, make sure that all particles are grinded, and no particles stay in the bottom of the grinding compartment.*

- Step 2.2 It is stated that the particles could be sieved, but nothing is stated about which particle sizes could be used for which application… It also could include some details about the procedures used for the sieving.

**Now the sieve has been added to the material list, as well as the maze size (0,71 mm). Sieving was done for the purpose of removing large particles and clumps. The step has been changed like below.**    
*2.3) Sieve the cartilage particles in order get rid of larger parts by using a sieve with a 0,71 mm mesh size.*

- Related to the main concern mentioned above, it could be useful to include a brief explanation of the aim of every step (f.e. trypsin treatment is used for …)  
**Explanation of several steps have been added:**

* **3.1) ...** *Note: trypin is a protease that degrades proteins residing in the cartilage tissue. This enzymatic step will open up the dense cartilage structure.*
* **4.2) …** *Note: this step is performed to specifically degrade deoxyribonucleases and ribonucleases.*
* **5.3) …** *Note: This step breaks down cells by breaking up membranes.*

- Step 6.1. What does it exactly mean? Suspend the particles in warm water?

**This sentence has been clarified:**  
*6.1) If the particles have been stored at -80 °C, thaw the closed tubes that contain the cartilage particles in warm water before creating the scaffolds.*

- Step 7.3 should be a little more explained (f.e. cross link with formalize containing 20 mM Ca Cl2... how exactly?)  
**The method for cross-linking by using formaling has been specified, as below:**

*7.3) Embed the scaffolds in a drop of 4% w/v alginate and induce cross-linking by adding a similar volume of 3,7% non-buffered formalin that contains 20 mM CaCl2.*

*Note: alginate embedding makes the scaffold slices more rigid regarding to the washing steps prior to paraffin embedding. In case the scaffolds have been cell seeded or in vivo implanted, alginate embedding is not necessary, as the composition of the scaffolds will be resistant enough due to ECM incorporation by the cells.*

- Step 9.2. It depends on your experiment. P1 chondrocytes would be good for some applications with these scaffolds.

**P1 chondrocytes can indeed be useful in some cases, however, for this specific protocol we aimed for differentiated chondrocytes, since they were compared to MSCs in a former in vitro paper (11).**  
  
- Figure 5 can be completed with a figure showing scaffolds before and after the cell seeding and culture. It is important to show how the cells have produced the glycosaminoglycans. It could also include collagen type II immunostaining or some technique to show the expression of collagen type II.

**An extra figure has been added. This presents 4 and 6-week cultured scaffolds with mesenchymal stromal cells, stained for H&E, Safranin-O, collagen I and II. (figure 6)**