August 2, 2018

Re: Resubmission of manuscript *Novel Process for 3D Printing Decellularized Matrices*

The Editors

*Journal of Visualized Experiments*

1 Alewife Center, Suite 200  
Cambridge, MA 02140

Dear Editors,

Thank you for the opportunity to revise our manuscript, *Novel Process for 3D Printing Decellularized Matrices.* We appreciate the careful review and constructive suggestions.

Following this letter are the referee comments with our responses in italics, including how and where the manuscript was modified if applicable. Changes made in the attached manuscript are marked using track changes.

Sincerely,

Chia-Ying Lin, PhD

*Thank you for your thorough review of our manuscript and your thoughtful questions. We appreciate all of the reviewer comments and have addressed them below.*

**Editorial Comments:**  
  
• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

*Thorough review has been completed.*  
  
• Please avoid use of the pronouns “you” and “your” throughout the manuscript.

*All instances have been removed.*  
  
• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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. Some examples:

1. 1.1: What is the size (or size range) of the microspheres used? What is their composition (other than the encapsulated matrix)?

*The size range used is 53 – 106 µm. This has been clarified.*  
2) 2.2.1,5.1: Mention sputtering settings, coating thickness etc. Observe on an SEM? Mention magnification and all additional settings.

*The sputtering settings and SEM settings have been clarified.*

• **Protocol Numbering:** All steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

*This has been addressed.*  
  
• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE’s instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.  
  
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.  
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.  
4) Notes cannot be filmed and should be excluded from highlighting.  
  
• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail (3-6 paragraphs) : modifications and troubleshooting, limitations of the technique, significance with respect to existing methods, future applications and critical steps within the protocol.

*The authors have added details about modifications, troubleshooting, limitations, and critical steps.*  
  
• **Figures:** Please add scale bars to all micrographs.  
  
• **Figure/Table Legends:** Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

*The legends have been modified.*

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Cole Parmer), Harver & Boeker, CAPA 6506, Perstorp, Filabot EX2 (Filabot), Comfort Zone), FlashForge Dreamer,etc  
1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

*All instances have been removed.*  
  
• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as microspheres.

*Software has been added to the table.*   
  
• Please define all abbreviations at first use.

*This is done.*  
  
• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

*This has been addressed.*  
  
• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

*Not applicable.*

**Comments from Peer-Reviewers:**   
  
**Reviewer #1:**  
Major Concerns:  
My chief concern is that the authors do not discuss any methodology or provide data that detail whether the composite filament is biologically active. The authors state that the purpose of the composite filament is for use in osteochondral applications, but do not show any direct data that provide evidence for the mammalian cells to adhere, migrate, survive, or differentiate on the composite filament. Data showing that the DM is biologically active and/or that cells adhere to the filament is essential for showing that this methodology can be used for osteochondral, and more broadly tissue engineering applications.

*A protocol and results for a functional alkaline phosphatase (ALP) test has been added to show that encapsulated materials remain biologically active. While in vitro studies were not the focus of this protocol, it is a future aim and has been added as such in the discussion section. In addition, our previous study and a protocol submitted concurrently with this one shows the increased cellular activity, migration, and differentiation that resulted from decellularized matrix encapsulated in PLA microspheres.*   
  
Minor Concerns:  
1. General  
a. The authors state in the abstract that the melting temperature of PCL is 60 °C, whereas in the introduction the authors state "Polycaprolactone (PCL) is another FDA-cleared, bioresorbable polymer that can be 3D printed at a lower temperature (65 C)…" (Pg. 3, Paragraph 2, Lines 70-71). Which is correct? Please make sure values are consistent throughout the manuscript.

*In 3D printing, filament is usually printed just above the melting temperature of the material. Therefore, there is usually a slight increase from melting temperature to printing temperature. However, to maintain clarity, the language in the protocol was changed to only address the temperature at which the filament is printed to avoid confusion and since the print temperature is more critical.*

b. The use of imperative tense is not consistent throughout the Protocol Section of the Manuscript. There are several instances where the authors shift from imperative tense to second-person, which is distracting. Please make sure the use of imperative tense is uniform throughout the manuscript. Instances of second-person (You, your) are listed below:  
i. Ln 111  
ii. Ln 126  
iii. Ln 135  
iv. Ln 140  
v. Ln 141  
vi. Ln 143  
vii. Ln 148  
viii. Ln 154  
ix. Ln 164  
x. Ln 169  
*These instances have been changed.*

c. Please double-check grammar throughout manuscript.  
 *Grammar has been double checked.*

2. Methods  
a. Very little information is provided about the settings used for imaging via SEM. Only the brand of the SEM is provided. Information regarding voltage, magnification, and vacuum is revealed on the SEM micrograph in Figure 1 (Right Panel), but is otherwise not explicitly stated or referenced. Additional information would be greatly appreciated for replicating the protocol such as the model of the SEM and the detector that was used. Mentioning the SEM settings used in the Protocol would help strengthen the manuscript and increase the reproducibility.

*While the use of commercial language (such as detector and model of the SEM) was not allowed by JoVE, other settings for the sputter coating and imaging have been added to the protocol.*   
  
3. Figures  
a. Figure 2 could be enhanced with some annotations such as arrows that explicitly point out where the microspheres are in the filament, as it very difficult to distinguish microspheres from structural artifacts in the filament.  
b. Figure 2 could also be enhanced by labeling the components of the equipment displayed.  
c. Figure 2 could be enhanced by including an image that shows what a filament without microspheres looks like.  
d. I do not understand the purpose of Figure 3. Please provide more information in the figure legend, and annotations on the panels for

*The figures have been modified.*   
*The intention for Figure 3 was to show examples of what could be created from the 3D printer. The SEM images also show the absence of microspheres in the PCL(-) group and the presence of microspheres in the PLA-DM/PCL group. The legend has been updated.*  
  
**Reviewer #2:**  
Manuscript Summary:  
This article described a method for producing a PLA-Decellularized matrices (DM)/PCL filament with PLA-DM microspheres as 3D printing ink, and the authors introduced the use of the PLA-DM/PCL filament to print a designed geometry. By using the fused deposition modeling, the protocol prepared the PLA-DM/PCL filament in a gentle temperature. This approach avoids the exposures of DM to the high temperatures in the traditional procedure, which is significant for preserving the bioactivity of the DM and facilitating the tissue reconstruction in vivo. This novel and efficient method protocol may appeal to the readers of JoVE. However, there are some significant concerns to be solved and/or clarified. Therefore, I would recommend major revision for this manuscript.  
  
Major Concerns:  
1. Protocol 1.1  
The microspheres are the important materials in the preparation of filament, and the authors indicated that it is imperative to control the microspheres in uniform sizes. The protocol indicated that 53-micron sieve tray is final tray, so is it the microspheres smaller than 53 micron is usable? Or is there a limited range of the microspheres diameter?

*The authors have clarified in the manuscript the desired microsphere size range in the protocol steps. The desired size range is 53 – 106 µm. While hypothetically smaller microspheres could be used, it will change the flow dynamics of the molten powder. Smaller microspheres were not used in our studies, so the effects were not thoroughly studied or included.*

2. Protocol 3.4 Creating filament for 3D printing  
As the author mentioned, a "modified Filabot EX2" is used in the protocol, are there any necessary modification procedures should be introduced in the article to help the readers repeat the filament extruding process?

*Modification steps have been added to the protocol which include removing the insulating jacket and using desktop fans blowing ambient air for cooling.*

3. Protocol 4. Printing with the filament  
What is the temperature when the authors performing the 3D printing? As the authors mentioned, controlling low temperature is important for protecting the biological cues of the decellularized matrices. So, is it the printing temperature also gentle enough for the decellularized matrices? It is necessary to make it clear to the readers.

*The print temperature (65 – 70 oC) was added to those specific steps of the protocol. Also, a functional test and the results from that test were added to show that biologic activity is maintained.*

4. Discussion  
In the discussion section, the authors described a series of limitations of the decellularized matrices in the tissue engineering development so far. However, the judgements and language are subjective and ambiguous to the existing progress (eg. 215-218). The authors should clearly indicate the compared objects and describe their advantages and disadvantages. Also, it would be necessary for the authors to provide corresponding references.

*The discussion has been enhanced.*

5. Discussion  
As a method article, the authors should discuss the critical step of the protocol, and indicated clearly how would it affect the success of the experiment.

*A discussion of the critical step of the protocol (filament production) and how it could impact the success of the experiments (by producing non-uniform or low quality filament) was added.*   
  
Minor Concerns:  
1. The figures provided in the manuscript are in low quality. Please provide the scale bars in figures 1 and 3.

*The figures have been modified.*

2. Please check the reference are given in the correct output style. For example, there are a bunch of gibberish in ref 2.

*This has been addressed.*  
  
  
**Reviewer #3:**  
Manuscript Summary:  
Gruber et al describe a processing to make a 3D print filament with decellularized matrix.  
  
Major Concerns:  
The protocol title is "novel process for 3D printing decellularized matrices" but the authors only focused on the filament creation step and not detailed much about the procedures of decellularization and sphere creation from mixture of decellularized matrix and PLA. In order to grant publication of the methodology, it should have careful description of all steps involved, adding special attention to details.

*The decellularization and encapsulation steps have been detailed in a protocol that was co-submitted at the same time as this protocol, and therefore deemed unnecessary to include in this protocol. The authors have added summary steps at the beginning of the protocol.*

Furthermore, answers to the following points would be relevant:  
  
1. in protocol 1.1, it will help readers to understand how the decellularized matrix was encapsulated with brief description even that technique was published previously.  
*Although the matrix decellularization and encapsulation steps are detailed in a manuscript co-submitted with this one, the steps have been summarized at the beginning of the protocol.*

2. In protocol 2, the particle size evaluation should be also taken into consideration in QC step to ensure the step of sieve is successful.

*We typically use SEM imaging (detailed in this protocol) and SEM measurements to determine that the spheres are of adequate size for our procedure.*

3. In protocol 3.1, what size of microspheres is used in filament creation?

*This has been clarified in the protocol document (53 – 106 microns).*

4. In protocol 3.3, what's the model and manufacturer of the "miniature drum mixer"?

*The miniature drum mixer was custom built for our application by a graduate student. A machine which has a similar design has been added to the list of materials, and an image of the custom mixer was added to the protocol.*

5. In step 3 (creating filament), how do you ensure the sterility during the filament fabrication? Shouldn't you also consider the sterility evaluation in quality control?

*It is very difficult to ensure sterility in this process which involves such high surface area powders and microspheres. Instead, the scaffolds are disinfected after production with UV and ethanol.*   
6. The key part of the protocol is filament creation and it will be good to have the layout of whole system and label each components of the system.

*An image of the layout has been added.*   
7. Does the mixture of PLA-PLA affect the strength of printed products?

*This is currently being studied by our group, but was not the focus of this protocol. This has been added as a future direction in the discussion section.*

8. Do the encapsulated matrices affect the cell move in, and bio-recognition?

*This is also being studied by our group currently. This has been added as a future direction in the discussion section.*  
9. What are the characteristics of encapsulated DM? Like pole size, surface, ….

*This is also being studied by our group currently. This has been added as a future direction in the discussion section.*

10.3.10, please define the exact temperature needed to cool down the filaments.

*The exact temperature to cool the filaments is unknown. The cooling fans are desktop units that blow ambient air over the filament to increase the rate of cooling to room temperature. More detail about the fan placement and clarification of the ambient air which is blown has been added to the protocol.*  
  
Minor Concerns:  
In Figure 1, the scale bar is needed even in macroscopic observation

*This has been added.*

In Figure 3, the scale bar is needed for all sub-figures and the scale bar in SEM images is not clear.  
The picture of SEM on PLC(-) is missed.

*These concerns have been addressed. The PCL(-) image was present, but the labeling of the image was clarified to make it more clear.*