The Journal of Visualized Experiments

Dear Editor Vineeta Bajaj,

I am pleased to submit a revised version of our manuscript entitled "*Ex vivo* tissue culture model for fibrovascular complications in proliferative diabetic retinopathy" (manuscript # JoVE59090).

We appreciate the careful review of our manuscript by you as the editor and by the expert referees. The numerous points raised were constructive and helpful for revising and improving the manuscript. We have now addressed all the comments, and provide detailed point-by-point answers in the attached document for the comments and responses. We hope that the manuscript now meets the criteria for publication in JoVE.

All authors are aware of and agree to the content of the paper and concur with the submission of this revised version.

Thank you for considering our revised work for publication in The Journal of Visualized Experiments.

Yours sincerely,

Kaisa Lehti, Ph.D.
Research Director, Tissue Invasion Research Group
Genome-Scale Biology Research Program
Faculty of Medicine, University of Helsinki, Finland

Genome-Scale Biology Program
Biomedicum Helsinki
University of Helsinki

P.O. Box 63 (Haartmaninkatu 8), FIN-00014 University of Helsinki, Helsinki, Finland
Tel. +358-294125034
kaisa.lehti@helsinki.fi

Editorial comments:

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.

Re: We have now thoroughly proofread the entire manuscript.

2. Please provide an email address for each author.

Re: An email for each author has now been added in the "AUTHORS & AFFILIATIONS" paragraph.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Re: Thank you for this comment, we have now rephrased the Short Abstract/Summary accordingly.

4. Please do not cite any literature in the abstract section. Please move the citation 1 to the introduction instead.

Re: We have now removed the citation 1 and included it in Introduction (now citation 11).

5. VE 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: Apotome, ZEN software, Triton X, Plan Apochromat, Zeiss AxioImager.Z1, Plan-Neofluar, Hamamatsu Orca R2 1.3, etc.

Re: We have now removed all company names and substituted them with generic terms.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Re: We have now reviewed the entire protocol and written all steps in the imperative tense and added all other remarks as notes. We have also mentioned the use of safety procedures where needed.

7. Please leave a single line space between the sub step and the note following it.

Re: We have now added a single line space between every sub step and the note following it.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. e.g., Line 211-219.

Re: Thank you for this comment. We have now thoroughly checked the protocol and included numbered steps where necessary, including lines 211-219. We have made the text in line 165-166 "Repeat steps 3.5 and 3.6 for all the FT pieces." as step 3.7.

9. Please add more details to your protocol steps. 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.

Re: Thank you for this comment which helps us to improve the protocol. More details have now been provided.

10. 1.1. Prepare how?

Re: See comment #9.

11. 1.3: Please remove the quoted from the name of the media.

Re: We have now removed the statement "This ready medium is hereafter called "ex vivo culture medium" and instead named the medium already in the first sentence of section 1.3.

12. 2.1: How much tissue is excised?

Re: The size of the excised tissue is very variable, ranging between 3 and 50 mm^2. This information has now been added as a note to step 2.1.

13. 2.3: Image and look for what? Please explain in a one-liner note.

Re: We have now added in a one-line note the purpose of the imaging and some properties of the tissue that can be visualized.

14. 2.4. How much PBS is present in one well. Please provide volumes throughout the protocol.

Re: We have now revised the entire protocol and added volume information where relevant.

15. 3.3: Volume?

Re: See comment #14.

16. 3.4. Please provide the step number for the aliquoted fibrinogen solution to bring out clarity.

Re: We have now added this notion in the text.

17. Lines 157-161: We cannot have two notes section together. Please consider moving some part to the discussion and make one note only.

Re: We have now added the first note to the protocol step since this is most commonly the case when handling the FTs.

18. 3.6: How long do you incubate this for? Does the droplet forms immediately?

Re: Thank you for this comment. We have now added the following statement to step 3.6 "Ensure also that mixing, dispensation and pipetting are performed quickly as the fibrin gel will form within 1.5 minutes, as tested in step 3.4.".

19. Line 188: Please make it a substep. Image how? Please provide citations if any.

Re: We have now made it a sub step and added notion on the quantification, as performed in Gucciardo *et al., The Journal of Pathology*, 2018. The citation has also been added.

20. 5.1: The difference between native and ex vivo culture is not very clear in section 3. Please bring out the difference.

Re: Thank you for this comment. We have now added a statement concerning the native FTs and the FT *ex vivo* cultures at the beginning of the protocol step 5 as "The FT/fibrin gels can be cultured ex vivo for the desired time period (FT *ex vivo* cultures) or fixed on the same day (native FT) for native FT characterization[11]". In order to better clarify this difference we have also substituted the term "fresh", sometimes referring to native FTs, with the term "native" throughout the manuscript. The term "fresh" was instead used only where it referred to the FTs prior to dissection and fibrin embedding.

21. Once formatted, please ensure that the highlight is no more than 2.75 pages including heading and spacings and forms a cohesive story. This is our hard-cut limit for filming.

Re: We have now revised the highlighted text to fit within the limit.

22. 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]."

Re: Thank you for this note. According to Wiley, publisher of the article in The Journal of Pathology, permission is not required for reusing a part of a figure while appropriate acknowledgement and citation must be given (please see https://onlinelibrary.wiley.com/page/journal/10969896/homepage/permissions.html). I uploaded this link to the editorial policy as a .docx document in our Editorial Manager account. The Video 1 of the current manuscript shows the 3D volume reconstruction of a native FT stained by whole-mount immunofluorescence. A snapshot/frame is included in Figure 1B of the above mentioned

published article (Gucciardo et al., 2018). Citation as “Snapshot/frame in Gucciardo et al., The Journal of Pathology, Volume 245/ Issue 2 Copyright © 2018 Pathological Society of Great Britain and Ireland, first published by John Wiley & Sons Ltd.” is included in the Video 1 legend.

23. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.

Re: Thank you for this comment. We have now revised all Figure legends and added all missing information.

24. Table 1 and 2: Please remove the manufacture’s detail and move it to the materials table. If possible please merge table 1,2 with the Table of Materials.

Re: We have now merged Tables 1 and Table 2 with the Table of Materials. We also included additional information in the comments/description field.

25. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Re: We have now revised the entire discussion to include the above-mentioned topics and transferred the removed information to the protocol section.

26. Please do not abbreviate the journal title in the reference section.

Re: We have now revised the references format to include full journal titles.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript by Gucciardo et al described a new protocol for 3D culture of fibrovascular tissue explants obtained from the diabetic patients suffering proliferative retinopathy. The protocol is very well-described and detailed in all the steps and it will for sure allow the researcher set it up straightforwardly. This 3D culture of diseased diabetic retinal tissue may facilitate the understanding of the pathogenesis of this long-term diabetes complication and also open avenues to try new inhibitors and therapies aimed at stopping cell proliferation and clear up the fibrotic tissue to improve/restore visual capacity.

Major Concerns:

None.

Minor Concerns:
1. Point 2.2., line 128. The authors should mention as a guide the longest time the explant is still viable after vitrectomy since it will be very difficult for most of researchers to get the tissue in such a short time (1-2 minutes).

Re: This is a valid point. We have tested as long as 2 hours after vitrectomy and this did not affect the *ex vivo* growth. However, we cannot provide a different estimate, as longer transfer times remain to be tested. We have now modified point 2.2 of the protocol to also include this info as well as info that was in discussion (see comment #9 of this reviewer) as "In our case the transfer takes less than 5 minutes and the explants are embedded within fibrin usually in 1 - 1.5 hours (maximum 2 hours) after surgical removal. The impact of longer transfer times on the 3D culture would need to be tested.". Nevertheless, we think that when team work is involved, longer transfer times can still be compensated by shortening the times *e.g.* for TA solution preparation, fibrinogen sterile-filtration and fibrin gel formation testing. We have added this notion in Discussion. Yet, we consider the close physical proximity of the research unit to the hospital instrumental for the use of these small tissue specimens.

2. Point 2.4., line 132. The authors say: 'Cut the FT into ~ 1mm2 pieces…'. Is the FT thick? Should this thickness be taken into account when cutting the pieces?

Re: This is a valid point. The FTs do not usually grow as a thick mass, but rather as a sheet at the vitreoretinal surface. However, they can fold during transfer and handling, prior to dissection. Therefore we added a note to step 2.4 advising to gently spread the FT prior to performin cuts.

3. Point 3.3 (and also 3.9.). Of interest to mention for what purpose is aprotinin added to the thrombin/fibrinogen reaction…to avoid gel degradation by 'proteases'?

Re: This is a good point. We have added this information in step 3.3 and revised step 3.10.

4. Point 3.4., lines 150-151. 'One tenth of the initially used volume of thrombin…'. Just of thrombin or of the TA solution? No HBSS plus only thrombin has been prepared during the protocol.

Re: Thank you for this comment. If needed, we usually add one tenth of the initially used volume of thrombin. If the volume of thrombin used was e.g. 30 µL, we start by adding 3 µL of thrombin stock solution to the ready TA solution (this leads roughly to a 10% increase in the final concentration of thrombin), and continue doing so until the fibrin gel formation occurs within 1.5 minutes. We have now clarified this statement as follows "The fibrin gel formation should take place in ~1 minute. If this takes over 1.5 minutes, the concentration of thrombin in the TA solution prepared in step 3.3 can be increased by adding more thrombin stock solution. One tenth of the initially used volume of thrombin stock solution can be added, repeatedly, until the fibrin gel formation occurs within 1.5 minutes.".

5. Point 3.6. (and also 3.4.), line 164. When pipetting the gel and particularly the FT into the gel which type of pipette should be used (a p100?)?

Re: The upright fibrin gel droplet is cast by dispensing the fibrinogen/TA solution mix onto the FT already placed on the plate well. To better clarify this we have modified this step as "Add 25 µL of TA solution to the 25 µL fibrinogen solution aliquoted in step 3.2, mix in the tube by pipetting, dispense onto the FT piece, placed on the plate well,……." and "Ensure that the FT is not aspirated during pipetting….". The fibrinogen mixed with the TA solution (50 µL in total) is pipetted with a 10–100 µL or 20–200 µL pipette. Same applies if the fibrin gels are cast on a 48-well plate.

6. Point 6.1. Please give an indicative/estimated volume of acetone:methanol and blocking solutions to be prepared.

Re: We have now added indicative volumes of all solutions to be prepared. See also editorial comment #14.

7. Points 6.3. and 6.4 and so on. Indicate the recommended volume for post-fixation and for the washes.

Re: We have now added indicative volumes also for these steps.

8. Point 6.20. The authors may include here the microscope settings/details recommended for image acquisition (although some details are further developed in the Figure legends).

Re: We have now added some details as "……equipped with an optical sectioning function and 20x or 40x objective. Use tile function to capture a greater area of the tissue at once.".

9. Discussion. The reviewer recommends to remove from discussion the second and third paragraphs since they are included mostly in the protocol and refer to M&M (from lines 406 to 421). The comment on longer transfer times to be tested (line 421) can be included as a note in the protocol and also mention if the authors have actually tested longer transfer times and their impact on 3D culture success (see also point 1).

Re: We thank the reviewer for this comment. We have now revised the discussion and included the info, mentioned in lines 406-421, in points 2.2, 2.4, 3.4, 3.5 and 3.6 of the protocol section (see comment #1 of this reviewer).

10. Discussion, line 449. Mention some of the identified growth factors in the vitreous of PDR and include the reference.

Re: We have now included this info and reference. In Gucciardo *et al.,* 2018, we quantified angiogenic and fibrotic growth factors VEGFA, bFGF, VEGFC and TGFβ as well as tested the *ex vivo* culture responsiveness to these growth factors.

11. Discussion, line 452. Include the reference.

Re: Thank you for this comment. We have now added the references.

12. Mention if the authors have tried to embed the FT into Matrigel or COL I and if the 3D culture and staining work as well as in fibrin.

Re: We have now added this notion in the discussion as follows "These matrices are generally suitable for 3D culture and whole-mount immunofluorescence but their effects on the PDR FTs remain to be tested and considerations on the timing of 3D matrix formation will need to be made in order to stay within the limits for preventing 2D growth[18,19]"

13. Mention if the 3D FT culture is amenable for time-lapse microscopy (have they tried?).

Re: This is an interesting point. We think that with the appropriate instrumentation this 3D *ex vivo* culture model is amenable for time-lapse microscopy, even though we have not yet tried performing such analysis. We have now included this notion in the discussion.

14. Of interest to mention that PDR FT displays proliferation (Gucciardo et al, 2018). But what about signs of 'hypoxia/hyper-glucose' signaling? Is it possible/have the authors tried to perform western blot/qPCR analysis from the tissue explants after culture?

Re: This is an interesting point. We have not tested hypoxia even though this should be doable with commercially available hypoxia probes or CA9 staining, for example. Western blot or qPCR analysis of the tissue explants after culture is possible, by first retrieving the FTs from the fibrin gel by fibrinolysis using *e.g.* plasmin/ nattokinase treatment. We have added this notion to Discussion.

15. Maybe of interest to mention whether there are reliable PDR mouse models and if ex vivo eye cultures from these models have been established/are feasible so molecular pathways can also be explored/identified in mice and compared/validated in the human 3D cultured-FT samples.

Re: This is an interesting point. However, mouse models of diabetes do not develop proliferative changes of human PDR, which makes the 3D *ex vivo* culture of patient-derived PDR FTs described in this manuscript, essential for the study of PDR pathophysiology. We have now clarified this concept in the Discussion as follows "Existing diabetic mouse models develop many features of early stage DR but fail to comprehensively recapitulate the progressive changes occurring in human PDR, thus hindering the studies of the PDR disease mechanisms[7,8]. Moreover, the murine eye is fundamentally different from the human eye, in that it lacks the macula, further emphasizing the importance of studying the human disease[24].

Reviewer #2:

Manuscript Summary:

This manuscript reports a method to suspend patient-derived fibrovascular tissues in polymerized fibrin matrices to study vessel outgrowth in the presence of various stimuli.

Major Concerns:

1. The temperature of all solutions should be indicated when handling all reagents.

Re: We have now revised the entire protocol and added this info.

2. The volumes of all solutions should be indicated throughout the protocol.

Re: We have now revised the entire protocol and added this info. See also editorial comment #14.

3. The heterogeneity in response is concerning in Figure 5. Also, have the authors ensured that the tissue is not contacting the bottom surface of the tissue culture plate. This will enhance cell outgrowth and potentially lead to variability in other explant models, such as aortic rings.

Re: We thank the reviewer for this comment. Majority of the *ex vivo* cultures develop outgrowths already after 2 days of culture (72%; 22%, day 4; 6%, days 6–13, Gucciardo et al., 2018). In some cases, the FTs do outgrow in 2D and such explants are excluded from growth quantifications. Most often, these explants outgrowing in 2D reach sooner the fibrin border. Such explants are also excluded. We have included this notion in Discussion.