

Dear Professor Cao,
Dear Reviewers,

First of all, I'd like to thank you for your constructive revision, thoughtful comments, reasonable suggestions, and appreciation of our manuscript. We tried to answer all questions to the best of our knowledge and edited the manuscript.

Thank you for still considering our now revised manuscript for JoVE.

With best wishes and appreciation for your time,

A handwritten signature in black ink, appearing to read 'Ranjbar', with a stylized flourish extending from the end.

Mahdy Ranjbar

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.

R1: The manuscript has been proofread. Spelling as well as grammar issues were modified to the best of our knowledge.

2. Please revise lines 41-44 and 127-129 to avoid textual overlap with previously published work.

R2: Lines 41-44 have been revised. However, the technical description in lines 127-129 is difficult to rephrase as it simply states the characteristics of the device.

3. Authors and affiliations: Please provide an email address for each author in the manuscript.

R3: Email addresses have been added.

4. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10–50 words: “Here, we present a protocol to ...”

R4: The first sentence of our abstract, which summarizes our manuscript very well, has been moved to a separate summary section before the abstract section.

5. 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. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Spectralis®, Heidelberg Engineering GmbH, Carl Zeiss Meditec AG, Methocel, Tissue Tek, JenLab GmbH, etc.

R5: The manuscript has been modified accordingly.

6. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

R6: The required statement has been added.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1–2 actions and contain 2–3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

R7: The protocol has been reformatted.

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. Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

R8: The protocol has been modified.

9. Line 86: Please provide the composition of antiseptic solution. If it is purchased, please list it in the Table of Materials.

R9: The information has been added.

10. Line 96: Please describe how to adjust the pulse energy and focus point. What is the desired effect here?

R10: The sentence has been rephrased.

11. Line 100: Once cornea per well? Please also specify the incubation temperature and regenerative additives used in this step.

R11: The sentence has been rephrased.

12. Line 106: What container is used for fixation?

R12: The sentence has been rephrased.

13. Line 108: At what temperature is this done?

R13: The information has been added.

14. Line 117: Please describe how histological staining is done.

R14: The information has been added.

15. Please reference Figure 1 in the protocol.

R15: The reference has been added.

16. Please describe how to obtain data presented in Figure 4 in the protocol.

R16: The information has been added in the representative results section.

17. Figure 1: Please indicate what each color asterisk represents? Which focal length?

R17: The description has been added.

18. Figure 2 and Figure 3: Please describe what the arrowheads point to in the figure legend.

R18: The description has been added.

19. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg).

R19: Images have been reformatted to .eps

20. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

R20: The materials have been reordered.

Reviewer 1:

It would be useful if authors can provide a mechanical drawing of the porcine eye holder. The schematic could be part of Figure 1.

R: The porcine eye holder consists of different parts, which were partially 3D-printed and specifically arranged to hold the eye firm, without putting too much pressure on it. Instead of the suggested mechanical drawing we added a bigger image of the device to illustrate the setup.

Microscopy in Fig. 2 cannot be duplicated by many investigators since 2-photon microscopes are not commonly available in most research institutions. Perhaps authors could discuss alternative approaches to assess/tune the laser to ablate endothelial cells alone without damage to the adjacent cells and tissue.

R: We are aware that 2PM is not available everywhere. Therefore, we demonstrate in Figure 3 that the ablation of endothelial cells is also visible using common light microscopy after H&E staining.

Line 137 - How was the heat map calculated? Instead of graphical representation, is it more useful to provide the actual values of probability?

R: We used a single-blind review system, in which 3 reviewers who were blinded to the treatment parameters had to assign two-photon as well as histology images to three categories (no damage, too much damage, selective damage). Based on their evaluation the heatmap was calculated.

If possible, reproduce the histology micrographs at higher resolution in Fig. 3.

R: The resolution has been upscaled.

Reviewer 2:

1. In the abstract, the following phrase: "Precise application of lesions" is not self-explanatory. Please use a better phrase to relay your message.

R1: The sentence has been rephrased.

2. Line 48, it is better to state full corneal transplant, endothelial transplantation in the forms of DMEK and DSAEK rather stating lamellar graft.

R2: The sentence has been rephrased according to your suggestion.

3. Line 83, need to specify what DMEM abbreviation stands for.

R3: It has been written out in full.

4. Line 1010, what do you mean by regenerative additives of interest for up to 3 days? What do you mean? Did you already tell us what the regenerative additives of interests are?

R4: The sentence has been rephrased. We just wanted to state, that during these 3 days potential therapeutic agents can be added to the medium.

5. It is difficult to know how many eyes in total were used in this study based on the energy level and distance from the endothelium.

R5: In total we used 3 eyes for every constellation (12x21). 756 eyes in total were evaluated. This information has been added to the representative results section.

6. Was there any collateral damage to descemet's membrane? Was the damage to descemet membrane unacceptable? Only CEC without basement membrane insult was the target of your study?

R6: Yes, damage to Descemet's membrane (DM) ranged from small bumps to total rupture. During the review of the images minor damage to DM was acceptable, as long as there was no partial or complete rupture. Actually, minor damage to DM would be ideal for a disease model as this is common early stages of these diseases.

7. Author need to provide some range of energy and corresponding posterior distance from the endothelium that caused no damage except the desired specific insult to the CEC and nothing else. Please provide these in the result section instead of simply referring the reader to the figures.

R7: The range is illustrated in the heatmap, however we now added the required information also in the results section.

8. Was desired damage defined in terms of single cell damage or clusters of few cells without collateral damage?

R8: Clusters. We rephrased some passages for clarity.

9. Can author share some of the safe YAG laser setting in the discussion for other investigators who are interested in this field.

R9: Referring to R7.

10. Congratulate the authors on the great work. As mentioned in vivo animal study needs to verify the validity of their laser setting.

R10: Thanks for your appreciation of our efforts.

11. I think authors need to share their energy threshold and distance threshold that caused only CEC damage and then emphasize that the safety and validity of their work in the actual in vivo system needs to be cautiously performed.

[R11: Referring to R7+R9.](#)

12. I am curious to know about your custom designed holding apparatus. Such detail in the method section needs published references or more detailed information in the method section about the apparatus.

[R12: The porcine eye holder consists of different parts, which were partially 3D-printed and specifically arranged to hold the eye firm, without putting too much pressure on it. We added a bigger image of the device to illustrate the setup.](#)

13. For examples in the table of material, the holding apparatus company or manufacturing should be reflected.

[R13: Referring to R12.](#)