Point-by-point response to reviewers' comments

A Rapid and Robust Sample Preparation Method for Mass-Spectrometry-Based Proteomics Analysis of Ocular Microvessels

Natarajan Perumal, Lars Straßburger, Carsten Schmelter, Adrian Gericke, Norbert Pfeiffer, Franz H. Grus, Caroline Manicam

Department of Ophthalmology, University Medical Centre of the Johannes Gutenberg University Mainz, Mainz, Germany.

The authors appreciate the remarks and comments of the reviewers to further improve the clarity and content of this manuscript. We have addressed the reviewers' comments point-by-point. Our manuscript and corresponding files (i.e. figures and tables) have also been revised accordingly.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:

1) Comment: *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*
Response: The manuscript has been thoroughly proofread to ensure that the article is grammatically sound.

2) Comment: *Please revise lines 497-501 to avoid previously published text.*
Response: The highlighted lines have been revised accordingly.

3) Comment: *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. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: ProteoExtract, Eppendorf, Bullet Blender, Amicon Ultra, Multiskan Ascent, NuPAGE, ZipTip, Thermo Scientific, CTC Analytics AG, etc.*
Response: All commercial terms have been removed and replaced with generic names, as suggested.

4) Comment: *Please revise the protocol 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. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*
Response: The protocol has been revised to contain imperative sentences, as required.

5) Comment: *1.2 and 1.4: The Protocol should contain only action items that direct the reader to do something. Please write the text in the imperative tense in complete sentences.*
Response: Points 1.2 and 1.4 have been revised to complete imperative sentences, as required.

6) Comment: *3.6: Please specify the ratio of 0.5 mm and 1.0 mm zirconium oxide beads.*
Response: The ratio of the zirconium oxide bead mixture used has been specified in point 3.5 as the volume ratio relative to the sample and tissue protein extraction reagent (ratio of 1:1:2 = sample: beads: T-PER).

7) Comment: *Line 199: Is this a volume ratio? Please specify.*
Response: The volume ratio of the 0.5 mm and 1.0 mm zirconium oxide beads has been specified (please refer to comment 6).

8) Comment: *Lines 298-299, 344-345, 352-353, 437-438: Please include them as tables and upload them to your Editorial Manager account as an .xls or .xlsx file.*
Response: The highlighted texts are included in table format as excel files, as requested.

9) Comment: *Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.*
Response: Shorter protocol steps have been combined, as suggested.

10) Comment: *Please include single-line spaces between all paragraphs, headings, steps, etc.*
Response: The manuscript is formatted to single-line spacing, as required.

11) Comment: *After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.*
Response: The important steps in the protocol are highlighted in yellow for video purposes, according to the requirements.

12) Comment: *Figure 1: Please remove commercial language from figure (Bullet Blender, ProteoExtract, Amicon, ZipTip, etc.) and use generic terms instead.*
Response: All commercial terms have been removed and replaced with generic names in Figure 1, as suggested.

13) Comment: *Please consider combining Figure 2 and Figure 3 to reduce the total number of figures.*
Response: Figure 2 and Figure 3 have been combined (now Figure 2), as suggested.

14) Comment: *Figures 4, 5, 8: These figures of commercial products are not necessary because readers can find out the product information from the Table of Materials. Please remove them.*
Response: Figures 4, 5 and 8 have been removed, as suggested.

15) Comment*: Figure 6 and Figure 7: Please combine these figures if possible.*
Response: Figure 6 and Figure 7 have been combined (now Figure 3), as suggested.

16) Comment: *Figure 9: Please remove commercial language from figure (Eppendorf).*
Response: The commercial name has been removed and replaced with the generic name in Figure 9 (now Figure 4), as suggested.

17) Comment: *Discussion: Please discuss any limitations of the technique.*
Response: Two major limitations of the present study are discussed in the Discussion section, as suggested (page 15 line 580-592).

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript describes preparation of micro vessels from ocular tissues for mass spectrometric proteomics.

1) Major Concerns:
Comment: *There is not enough cross validation of isolated micro vessels. A combination of other methods such as DiI mediated identification of micro vessels prior to isolation has not been considered. The question is "are you isolating what you think you are isolating?". This has not been addressed in the manuscript. However, it still is a useful method for many researchers. Despite this concern manuscript still has utility in its current form and should be acceptable.*
Response: Thank you for highlighting this interesting point pertaining to vessel painting employing the lipophilic carbocyanine dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) method. In this study, we did not discuss in-depth about the anatomical aspects as well as the isolation procedures of the short posterior ciliary arteries (sPCA) owing to several factors. First, the description of the sPCA branches chosen for isolation has been provided in detail in our previous study (Manicam *et al*., 2016 DOI: 10.1038/srep38298), which is the basis of the current protocol and this has been highlighted in the Introduction section of the manuscript (page 3, line 87-92). The main focus of this article is the optimization steps for a rapid, robust and highly efficient protein extraction method from mass-limited samples and as such, the sPCA were used as exemplary ocular microvessels, as reiterated in the Discussion section (page 14 line 550-552). Second, although we did not elaborate on the verification steps of the identification of sPCA in the present study, this vascular bed was identified and isolated based on many other valid retrospective studies, which have provided excellent diagrammatic representation of the vascular bed being isolated based on detailed experimental works (Hayreh *et al.*, 2004 DOI:10.1167/iovs.03–0469; Ramirez *et al.*, 2012 DOI: 10.5772/47794; Erdogmus and Govsa, 2006 DOI: 10.1111/j.1600-0420.2006.00673.x). Third, the DiI technique is usually performed by direct manual intracardiac injection of the DiI solution followed by perfusion of fixative in rodents (Salehi *et al*., 2018 DOI: 10.1007/s12975-018-0632-0; Konno *et al*., 2017 DOI:

10.1038/s41598-017-09496-4; Li *et al*., 2008 DOI: 10.1038/nprot.2008.172; Hughes *et al*., 2014 DOI 10.1007/978-1-4939-0320-7_12). In the present study, we obtained our study samples i.e. the porcine eyes from the local abattoir and therefore, the standard method is not applicable in the current scenario. Consequently, the method may have to be modified to ensure its functionality to stain the porcine retrobulbar ocular vasculature *ex vivo*. Although we are in agreement with the reviewer that a verification stage would be better to further strengthen the identity of the isolated blood vessels, this procedure is beyond the scope of the present study and would be a highly suitable topic for another independent study. Nevertheless, we appreciate this highly useful and important suggestion and, we believe that future studies using this method would be feasible to provide such information.

2) *Minor Concerns:*
Comment: *The page 37-38 comments in pdf has gotten out of the table. Authors should reformat the original table so that there is no spill over of the information beyond page 36.*
Response: The original table has been reformatted to ensure no spill over to the next page, as requested.

Reviewer #2:
Manuscript Summary:
Well presented and useful method for ocular proteomic investigation.

1) Minor Concerns:
Comment: *two earlier and very relevant articles addressing proteomic methodology require brief discussion and reference. In order of importance:*
1. Ocular proteomics with emphasis on two-dimensional gel electrophoresis and mass spectrometry. Mandal et al. Biol Proced Online. 2009 Dec 24;12(1):56-88.
2. Analytical platforms in vitreoretinal proteomics. Cehofski et al. Bioanalysis. 2014;6(22):3051-66.

Response: Thank you for highlighting these highly relevant and interesting articles. As suggested by the reviewer, we have included these two papers in the reference list (page 16 reference 1 and 5) and, discussed several important points emerging from them in the Introduction (page 3 line 65-71) and Discussion sections (page 14, line 546-550; page 15, 583-585; page 15, line 588-592).