**Point-by-point reply**

**Manuscript: 59249\_RO\_101618 entitled ”Visualizing *in vivo* blood-brain barrier permeability and gelatinase activity in brain sections of mice with experimental autoimmune encephalomyelitis”**

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Dear Editor and dear Reviewers

Please find our point-by-point answers to your queries below:

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*Answer: this has been done*

2. Figure 3: Please replace commercial language “DQ” with a generic term.

*Answer: We have used now the generic term “dye-quenched gelatine”.*

3. Please shorten the title if possible.

*Answer: this has been done*

4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

*Answer: This has been changed as requested.*

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 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: Eppendorf, c0mpeteTM, DQTM, Tissue-Tek, Superfrost®, Mowiol, EnzChekTM, etc.

*Answer: This has been addressed accordingly.*

6. 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. See examples below.  
7. 2.1.3: Please split into two steps.

*Answer: this has been done.*

8. 2.2.1: Please specify the concentration of isofluorane and mention how proper anesthetization is confirmed.

*Answer: This information has been added.*

9. 2.2.5: Please describe the composition of maintenance diet and breeding diet.

*Answer: this information has been included*

10. 4.6: What volume of PFA is used?

*Answer: Has been added.*

11. 4.7: Please specify the surgical instruments used.

*Answer: We have rephrased this paragraph.*

12. 6.6: What is used to cut tissue sections?

*Answer: This information has been added.*

13. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

*Answer: This has been addressed.*

14. Please include single-line spaces between all paragraphs, headings, steps, etc.

*Answer: this has been done.*

15. 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.

*Answer: this has been done*

16. 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.

*Answer: this has been respected.*

17. 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.  
*Answer: this has been done*

18. Discussion: Please discuss any limitations of the technique.

*Answer: this has been done.*

19. 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.

*Answer: this has been done*

20. 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. See the example below:Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

*Answer: addressed*

21. References: Please do not abbreviate journal titles. If there are six or more authors, list the first author and then “et al.”.

*Answer: addressed*

22. Table of Materials: Please sort the items in alphabetical order according to the Name of Material/Equipment.  
*Answer: This has been addressed*

**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
Engelhardt and Tietz present valuable methodologies for neuroimmunological research. The way of presentation is clear and contains sufficient details. I am convinced that video that is going to be produced will complement the manuscript perfectly.  
  
Major Concerns:  
none  
  
Minor Concerns:  
There are some parts of the text that are highlighted. I am not aware of the purpose of the highlighting, but these are essential parts of the protocols, and they should stay in the final version of the manuscript.

*Answer: This is the part of the manuscript highlighted on request of the journal to be considered for making the movie.*   
"end-feed" should be replaced with "end-feet" throughout the manuscript.

*Answer: has been corrected*  
There are other typos and grammar errors in the manuscript, please check carefully.

*Answer: has been corrected*  
Please, explain "breeding diet" and its purpose regarding EAE induction.

*Answer: This information has been included*  
  
  
**Reviewer #2:**  
Manuscript summary:  
In this manuscript Tietz and Engelhardt describe detailed protocols to investigate the impairment of the neurovascular unit in an animal model of neuroinflammation. They describe:  
1. how to induce EAE in C57BL/6 mice by active immunization,  
2. how to analyze blood brain barrier permeability by using a combination of exogenous fluorescent tracers of two different molecular weights.  
3. A protocol for combining in situ zymography and immunofluroscence for simultaneous detection of gelatinase activity and immune cell infiltration across the basement membranes.  
The manuscript is well-written, well-organized and for the most part clearly presented. In particular, the authors should be commended for explaining their rationale for using these assays in the particular way and combination presented in this manuscript, specific reagents for their chemical, non-immunogenic, or other properties, and the importance of the accomplished scientific observations for deciphering underlying biological events.  
However, a few issues still need to be addressed that should substantially improve the impact of this publication:  
  
Major issues:  
1. Representative results need to be provided for the BBB permeability assay comparing uninjured with EAE conditions, and clearly demonstrating how this protocol detects BBB disruption and why the combination of these two molecular weight tracers is advantageous. Fig.2 currently does not demonstrate leakage in the CNS and does not clearly explain what the advantage of using these two tracers in combination is.

*Answer: A revised figure has been included to address this request accordingly.*

2. In addition to the instructions provided on how to most efficiently make essential solutions, the concentrations of final and intermediate solutions should also be provided for all protocols.

*Answer: This information has been included.*

3. 2.2 EAE induction: point 2.2.3: The authors should detail the different injection spots with the exact volumes used here and not in the discussion. A schematic would also help if the authors would not mind preparing one.

*Answer: A schematic figure has been included*

4. The EAE scoring scale should be better presented and explained so an inexperienced experimenter can learn what signs to look for. The weight correlation is rather confusing too. What is the meaning of "indicative of second check"? It currently appears on two pages with missing text. Details regarding when they start to weigh and score would also help.

*Answer: This has been explained in more detail in the revised manuscript. Please note that each explanation in the text will be correlated to a video showing exactly what to look for.*

5. 4-In vivo permeability assay: point 4.6: The protocol needs to be more clear regarding fixation, post-fixation and cryopreservation of the tissue (with sucrose?)

*Answer: this has now been addressed in paragraph 5. Please note that in situ zymography is not performed on the same tissue sections analyzed for BBB permeability*

6. 7-In situ zymography: point 7.9: If the sections are already fixed with PFA, do they need to be fixed again with methanol? This seems to be in contrast to what the authors describe in their recently published JAMB in EAE study.

*Answer: Please our explanation to point 5.*

7. Is a permeabilization reagent used during the washes or the incubation with the primary or secondary antibodies?  
*Answer: No permeabilization reagent is used during the washes or the incubation with primary or secondary antibodies.*

Minor issues:  
1. The language needs some professional editorial work to improve readability and clarity, and correct some typos and grammatical errors

*Answer: this has been addressed.*

2. The numbering of steps needs to be checked for consistency

*Answer: this has been addressed.*

3. Abbreviations should always follow the terms they are meant to abbreviate (ex: ECM)

*Answer: this has been addressed.*

4. 2.1.3: Clarify the language: adjust the emulsion volume?

*Answer: done.*

5. Explain the rationale behind the diet change in EAE mice

*Answer: this information has been included.*

6. 4.6 the sentence is confusing. Clarify the order of steps and how the two syringes are used and what for (not for opening the atrium presumably)

*Answer: this has been addressed.*

7. 5.7 place brain slices with the front facing down should be rephrased to the anterior side of each brain pieces you cut in a previous step

*Answer: This has been changed.*

8. 6-Preparation of frozen tissue sections: point 6.1: describe for how long should pre-warming the frozen tissue in the cryostat last

*Answer: this has been included.*

9. 6.4 clarify the parenthesis

*Answer: The explanation has been added to now paragraph 7.2.*

10. Some additional references for original findings discussed throughout the manuscript and particularly in the discussion could be included for proper scientific etiquette

*Answer: We have added additional references to address original findings on investigating BBB integrity in vivo using FITC-albumin (KRUEGER 2015) and Evans Blue (GRAESSER 2002).*