Jan 3, 2019, Beijing

Alisha DSouza   
Senior Review Editor

*Journal of Visualized Experiments*

Dear Dr. Alisha DSouza,

Thank you very much for your thoughtful review and consideration of our manuscript entitled “*Morphological and Functional Evaluation of Ribbon Synapses at Specific Frequency Regions of the Mouse Cochlea* (59189 \_R1)” for publication in the *Journal of Visualized Experiments*.

In accordance with the comments and suggestions of the editor, which have helped us to substantially improve the quality of our manuscript, we have revised the text in preparation for resubmission as requested. In the revised manuscript, we have incorporated our responses to the editor, which are appended at the end of this letter. We believe that our responses have adequately addressed all editorial concerns and hope that our revised manuscript is now suitable for publication in your esteemed journal.

Additionally, we made a change in the order of the corresponding authors in the author list of the article, which is in accordance with the requests and contributions of the corresponding authors. And all other authors have agreed with this change.

Please feel free to respond with any further questions or concerns. Thank you again for your assistance during the editorial process, and we look forward to your response.

Sincerely,

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**Replies to Editorial Comments**

*Please see the attached word document. In-text comments have been made; these require your attention. Please address the comments by editing your manuscript/figures. Please maintain the current format and track all your edits.*

**Response:** We have revised the manuscript in accordance with the comments and suggestions. We have addressed each of the editorial comments individually, which are appended at the end of this letter.

*-Line 167:* *I restructured this for clarity. How exactly is this fit in? is it simply place in the ear canal? How is it secured?*

**Response:** The tip is cone-shaped, which can be tightly fitted into the external ear canal with its entrance. There is no space to move for the tip, therefore it is secured.

*-Line 184: Reference fig 1 here?*

**Response:** We have referenced fig 1 here.

*-Line 197: Mention surgical tools used.*

**Response:** The relevant information has been included as follows: “Using a fine forceps, remove the temporal bones, sever the stapes artery, remove the stapes from the oval window, and rupture the round window membrane.”

*-Line 199: What size?*

**Response:** The relevant information has been included as follows: “Make a small hole at the apex of the cochlea by gently rotating the tip of the needle (13 mm, 27 G).”

*-Line 225: Citing a reference is not sufficient for filming. Please mention dissection steps involved. Mention surgical tools used.*

**Response:** The relevant information has been included as follows: “Use #3, #5 Dumont forceps and the 27 G needle to dissect the apical, middle and basal cochlear regions in turn and then dissect the cochlea out of the bone under a stereo dissection microscope, as previously described.”

*-Line 226: Cut how? Mention surgical tools used. Please size of the cut.*

**Response:** The relevant information has been included as follows: “Make a series of small cuts along the spiral ligament using a razor blade , and remove the tectorial membrane and Reissner’s membrane.”

*-Line 238: Nothing to film here so I am unhighlighting this.*

**Response:** In accordance with your recommendation, the text has been unhighlighted.

*-Line 242: Nothing to film here so I am unhighlighting this.*

**Response:** In accordance with your recommendation, the text has been unhighlighted.

*-Line 246: Nothing to film here so I am unhighlighting this.*

**Response:** In accordance with your recommendation, the text has been unhighlighted.

*-Line 246: Can you provide the conversion formula here for completeness? If so, we could then include this step for filming and show the formula in the video.*

**Response:** The conversion formula has been included as follows: “d(%) = 1 - 156.5 + 82.5 × log(f), with a slope of 1.25 mm/octave of frequency, where *d* is the normalized distance from the cochlear apex in percent, *f* is the frequency in kHz.”

*-Line 248: Reference fig 2 here?*

**Response:** We have referenced fig 2 at step 3.5.

*-Line 255: The goat serum with TX100?*

**Response:** Yes, the blocking/permeabilization solution is 10% goat serum/PBS/0.1% Triton X-100.

*-Line 291: Excitation?*

**Response:** The text has been corrected.

*-Line 292: Excitation?*

**Response:** The text has been corrected.

*-Line 293: Excitation?*

**Response:** The text has been corrected.

*-Line 295: Mention stack height?*

**Response:** The relevant information has been included as follows: “Acquire confocal z-stacks over a distance of 8 μm from each cochlear turn using a 63x high-resolution oil immersion lens.”

*-Line 304: Is this done in image processing software? Please mention all button clicks and selections in explicit detail.*

**Response:** The number of synaptic puncta for each IHC is calculated manually. The image processing software is only used for the counting of total synaptic puncta at specific frequency regions.

*-Line 304: This is a bit vague. Please clarify the steps.*

**Response:** We apologize for the lack of clarity in the original text, which has been revised as follows: “Divide synaptic counts in each z-stack at specific frequency regions by the number of IHCs (equal to the DAPI nuclear manual counts) to calculate the number of synaptic puncta for each IHC.”

*-Line 305: Is the counting done manually? It appears that the counting is described below, is that correct?*

**Response:** The DAPI nuclear count is performed manually, which is equal to the number of IHCs. The counting of total synaptic puncta is performed by image processing software, which is described in the 5.5.1 and 5.5.2. The text has been revised as follows: “Divide synaptic counts in each z-stack at specific frequency regions by the number of IHCs (equal to the DAPI nuclear manual counts) to calculate the number of synaptic puncta for each IHC.”

*-Line 308: I made this substeps instead of a note and rewrote in the imperative voice. I think it should be moved up before 5.4. Please check and clarify.*

**Response:** We have checked the substep carefully and moved it up at 5.4. The description of “Experimenters should remain blinded as to whether the slide is from the apex, middle, or basal turn of the cochlea” should be considered as a note.

*-Line 313: Reference fig 3 here?*

**Response:** We have referenced fig 3 at step 5.7.

*-Line 319: Set to what levels? Please mention all button clicks and selections in explicit detail.*

**Response:** The relevant information has been included as follows: “To visually assess synaptic structure and distribution, we used Photoshop (Adobe) to manually isolate individual IHCs from their neighbors by the Brush Tool to better visualize the cytoskeletal architecture and synaptic localization.”

*-Line 320: How? Please mention all button clicks and selections in explicit detail. This needs more details.*

**Response:** The relevant information has been included as follows: “To visually assess synaptic structure and distribution, we used Photoshop (Adobe) to manually isolate individual IHCs from their neighbors by the Brush Tool to better visualize the cytoskeletal architecture and synaptic localization.”

*-Line 323: Please mention all button clicks and selections in explicit detail. Unclear what is being done here, so we cannot film as written.*

**Response:** The relevant information has been included as follows: “To inspect the juxtaposition of presynaptic ribbons (CtBP2) and postsynaptic receptor patches (GluR2), we used Photoshop extracts the voxel space around ribbon by the Rectangular Marquee Tool and isolate individual ribbon by Image Cutting.”

*-Line 325: Please mention all button clicks and selections in explicit detail.*

**Response:** The relevant information has been included as follows: “Through clicking Image > Image Size, acquire a thumbnail array of these miniature projections, which can then be used to identify paired synapses (appeared as closely juxtaposed pairs of CtBP2-positive and GluR2-positive puncta) versus orphan ribbons (lacking postsynaptic glutamate receptor patches).”

*-Line 326: Unclear how you define paired synapses and orphan ribbons. Please clarify and all the necessary details.*

**Response:** The relevant information has been included as follows: “Through clicking Image > Image Size, acquire a thumbnail array of these miniature projections, which can then be used to identify paired synapses (appeared as closely juxtaposed pairs of CtBP2-positive and GluR2-positive puncta) versus orphan ribbons (lacking postsynaptic glutamate receptor patches).”

*-Line 348: There is nothing to film here unless you describe some actions to perform. I have unhighlighted this section.*

**Response:** In accordance with your recommendation, the text has been unhighlighted.

*-Line 350: Define P1 and N1.*

**Response:** The relevant information has been included as follows: “ABR wave I amplitude is defined as the difference in voltage between Ip (the positive peak of wave I) and In (the negative peak of wave I).”

*-Line 350: Reference fig 4 here?*

**Response:** We have referenced fig 4 here.