Dear Editor,

Thank you giving us the opportunity to submit a revised draft of our manuscript titled "**3D Characterisation of Endoplasmic Reticulum-Organelle Membrane Contact Sites in Hepatocytes using Serial Section Transmission Electron**" [JoVE63496]. We appreciate the time and effort that you and the reviewers have dedicated. We are grateful for the constructive and insightful comments and we have been able to incorporate changes to accommodate most of the suggestions.

We have uploaded the revised version, with all changes accepted, as well as a tracked changes copy should you need it.

Below is a point-by-point response to the editorial and reviewers' comments.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have corrected all the spelling and grammar mistakes.

2. Please shorten the title along the lines of "3D Characterization of Inter-Organelle Contacts in Hepatocytes using Transmission Electron Microscopy"

Response: We have changed the title to "3D Characterisation of Inter-Organelle Contact Sites in Hepatocytes using Serial Section Electron Microscopy"

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our", "your" etc.).

Response: We have excluded the use of all personal pronouns in the manuscript.

4. 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 (For example: TAAB, Devcon, etc.)

Response: All commercial terms have been removed and only included in the *Table of Materials.*

5. ml should be mL, µl should be µL, and minutes should be min throughout the manuscript. Please include a single space between the numeral and the unit, e.g., 24 h, 4 °C, etc.

Response: All units are following the correct SI/metric format now.

6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts without brackets after the appropriate statement(s), but before the punctuation.

Response: All in text citations are now following the JoVE format.

7. Please revise the Introduction to also include the following:

a) A description of the context of the technique in the wider body of literature

b) Information to help readers to determine whether the method is appropriate for their application

Response: We have extended our paragraphs so that the key features of various volumetric EM techniques are discussed, and included a comparison table. This will help readers to decide which techniques are suitable for their application.

8. Lines 43-51: Please provide relevant citations.

Response: This section has been re-written in response to Reviewer 1's comments, and we have included relevant citations, please see the last paragraph of the revised Introduction.

9. Please refrain from using bullets or dashes in the Protocol section.

Response: We have removed all bullets point in the Protocol section and have limited our use of dashes.

10. Please use a single line spacing between steps and sub steps of the protocol.

Response: Format changed accordingly.

11. 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.

a. Step 1.2: how warm is the fixative (i.e., temperature) and what is its composition?

b. Steps 2.4-2.6: how many times is the washing in each of these steps repeated?

Response: We have included details for the fixative temperature and composition and the number of times the washing steps should be repeated.

12. Step 5.4: Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Response: The originally Step 5.4 has been simplified and broken down into new Step 5.4 and Step 5.5.

13. Step 7.1: Please cite the source of Amira. Please include the download/source links for the software/plugins used in this study in the Table of Materials.

Response: Done.
14. Line 423: please fix the typos ("?") in tissue dimensions.
Response: Typos has been corrected, see representative results paragraph 1.
15. Panel 4D is not discussed in Results section. Please do.
Response: We have included the description for Figure 4D.
16. Figures 2-4: please include scale bars in all the microscope images.
Response: All microscope images now include a scale bar.
17. Figure 4: Please include the scale corresponding to the scale bars in panel B. Please also mention what the far-right image of panel E denotes.
Response: We have included the values for scale bars and labelled all the reconstructed 3D models.
18. Remove the embedded Table of Materials.
Response: A separate Table of Materials is provided in the modified manuscript.

19. As we are a methods journal, please revise the Discussion to also cover the following explicitly in detail in 3-6 paragraphs with citations:
Response: We have endeavoured to include these aspects in the following paragraphs.
a) Critical steps within the protocol
Response: Discussion paragraph 3 and 4
b) Any modifications and troubleshooting of the technique
Response: Discussion paragraph 3 and 4
c) Any limitations of the technique
Response: Discussion paragraph 2 and Overview table
d) The significance with respect to existing methods
Response: Discussion paragraph 2 and Overview table
e) Any future applications of the technique
Response: Discussion paragraph 1 and 7

20. Please revise the Table of Materials to include all the supplies (chemicals, reagents, equipment, consumables, software, etc.) used in this study.
Response: We have updated the Table of Materials accordingly
21. Please ensure that the references are numbered in the order of appearance and are formatted as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 5 authors, list only the first author then et al. Please expand journal names.
Response: The bibliography now follows the JoVE format.

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In the manuscript entitled '3D characterization of Endoplasmic Reticulum-Organelle Membrane contact sites in hepatocytes using serial section transmission electron microscopy, Burden et al propose a robust and efficient way to collect serial sections for electron microscopy and conduct quick 3D analysis of biological compartments of optimized volumes.
In the abstract, the author describes carefully the scientific context of their work and the advantageous reason for conducting serial section TEM to investigate 3D properties of dynamic and highly plastic biological sub-compartment such as the ER.
The proposed manuscript is highly detailed, close to a book chapter level, and nicely written so it's easy to read through and could become a bench protocol to any user with experience in the field.
Response: We thanks the reviewer for the kind and constructive comments.

Major Comments:

1. The title inspires a biology paper with the biological outcomes, while the content is focused on a methodology presentation. I would suggest transforming significantly the title to reflect more the actual purpose of the paper and avoid confusion of the readership.
Response: As this protocol was invited to be part of a special issue entitled "Emerging models and methods to investigate liver regeneration and disease", we aimed to write a balance manuscript to include significant

details relating to both the method and the biology. However, we thank and appreciate your comments and as such have tried to modify the Abstract, Introduction and Discussion to also demonstrate the breadth of the application. With respect to the title, we have changed the title from “3D Characterisation of Endoplasmic Reticulum-Organelle Membrane Contact Sites in Hepatocytes using Serial Section Transmission Electron” to “3D Characterisation of Inter-Organelle Contact Sites in Hepatocytes using Serial Section Electron Microscopy” to hopefully broaden its readership and better reflect the protocols applications.

2. The same comment applies to the abstract (first half is about biology, second half starting line 27 about the method), and even more to the introduction (two paragraphs on biology, one on methodology that could be the actual abstract, no presentation on serial sectioning and existing literature).

Response: We agree with the reviewer and in light and agreement with comment 1, have revised the Abstract and Introduction to emphasize the methodological elements, including references to serial sectioning approaches, modifications and tips and tricks.

3. To me, this is the main limitation of this article so far: strong discrepancy between abstract/introduction and content.

Response. We agree with the reviewer’s comment so that we have revised the Abstract and Introduction so that the theme of this manuscript is more coherent.

4. The paper present no novelty in method nor in results, but has the quality of synthesizing several technical and software tools into one readable and detailed workflow.

I would empathize that the strong place of the biological topic could even drag away readers not from the biological field but with a great interest for the method that is applicable to a wide range of questions.

In the 'representative results' section, the structure is correctly presenting the outcome of the method. In the second paragraph, 431-432, I would suggest rephrasing in a way that the ER has been used as a case study to support the benefit of the method. It currently reads more to me as a bio paper on ER analysis (logical with the abstract but see comments above). The third paragraph used adequately the biological topic to illustrate the method.

Response: We thank the reviewer for the critical comments. We have changed the focus of the introduction and discussion, put more attention to the details for the serial sectioning technique and toned down the focus of our biological case study.

In the discussion, other 3D techniques are presented that are now spreading rapidly. I would suggest to find a way to illustrate the benefit of the authors' methods compared to other (FIB-SEM, 3View, Array Tomo) in regard to the 'volume of acquisition/speed of acquisition/equipment available at hand/cost/time to run the experiment/ease of the experiment/ease to analyse the results (maybe not all at once, but those elements are of interest). It would illustrate nicely the benefit of their approach and would help facility managers to guide their users towards one method of the other according to their project.

Maybe a reference to the first full eukaryotic cell imaged by electron tomography of a serial section would also be wise (Höög et al, dev cell).

Response: We have created an overview table giving a comparison of the different vEM techniques. This includes resolutions achievable, average volumes acquired, costs to purchase and costs to maintain, to help guide the readers depending upon their research needs. We have additionally included relevant references and reviews of the techniques. We did not extensively compare speed and ease of acquisition between the techniques, because this varies significantly depending upon the volume of data required, but have referenced this aspect in Discussion paragraph 2. We have included the beautiful Höög et al, reference.

Minor Comments:

1. What is the function of the Triton X-100? I am familiar with the use of Chloroform to spread and unfold sections, but I never used Triton, what advantage does it bring?

Response: We have included why the use of Triton X-100 is useful during sectioning and collection.

2. Line 250, paragraph 3.14, the authors mention the use of a ball-dropper. I am not familiar with this tool. A commercial reference of a picture could be of interest

Response: We have changed the text to better reflect the catalogue name of “Glass applicator rod” and included its ordering details in the Table of Materials.

3. Nice serial sections are easier to collect with highly parallel bloc faces top and bottom. This can be achieved using a trimming cryo-knife, but then the very sharp and clean bloc face is causing difficulties to get attaching ribbons. Trimming with razor blades is less clean and helps to get sections attached, but not always. To get nice serial sections, some glue or wax could be deposited below the bloc face to help keep the ribbons together.

There is no mention of it, and I think it could be of interest to the readership. Some literature already mentions and discusses that (methods in cell biology and journal of microscopy to my memory).
Response: In the protocol section we have described the use of a razor blade and included the use of trimming diamond knives as an alternative option. We have also included the use of contact cement to aid ribbon stability as an alternative. We have also briefly mentioned this in the Discussion paragraph 4 of the revised manuscript.
4. The protocols to use trackEM2 and Amira are nicely described and will satisfy many users.
Response: We thank the reviewer for the kind comment.

Reviewer #2 comments:
The manuscript by Chung et al presents a well-detailed protocol based on Serial Section Transmission Electron Microscopy that can be used to identify spatial interactions between organelles, including contact sites. Overall, I found the manuscript interesting and the protocol very clear. I think that it would be nice to include a video showing the 3D reconstruction of the example shown in Figure 4 so as to appreciate better the power of this technique. (Unless such a video will be shown as part of the video of the technique)
Response: We thank the reviewer regarding the positive comments. We are happy to include the 3D model reconstruction as part of the protocol video. However, if the video time is limited, we are also happy to provide a separate video.

Minor Comments:
1. S18, omit "as"
2. S52, "types" instead of type
Response: The above grammar mistakes have been fixed.
3. Section 1.5, specify composition of fix
Response: We have specified the composition of the fixative.
4. Section 1.6, how to select regions of interest?
Response: How to select a region of interest is very research-question dependent, however we have provided details of some general considerations when selecting a region of interest, see Note in 1.6 of the revised manuscript.
5. Section 2.9 is same as section 2.10
Response: Step 2.10 is a repeated step. We have modified the sentence so that it is clearer in the protocol.
6. S218, is Triton used neat?
Response: Apologies, we have now included the concentration of 0.1% Triton X-100 in 3.7 and 3.8 of the revised manuscript protocol section.
7. S448, replace "have" with were
Response: Grammar mistake has been corrected.
8. S449-2450, Sentence starting with "Figure 4E…" is hard to understand. Figure 4, it may work better to make the lines pointing to the contact regions thinner because as it is the small areas of contact are difficult to discern when the fat lines hit them
Response: The corresponding sentence has been rewritten. We have rescaled the lines that annotate the different intermembrane space(s).

Reviewer #3 comments:
Manuscript Summary: The authors describe in detail a protocol to perform Serial Section Transmission Electron Microscopy on liver tissue, to obtain 3D EM information that allows for characterization of ER - organelle contact sites. The structure of the article follows the logical order of the experimental procedures, starting with specimen fixation and preparation, over procedures for sample mounting, image acquisition, image restoration and segmentation, and 3D reconstruction. Each and every step of the workflow has been extremely well described and based on the written text, scientists with prior know-how on electron microscopy techniques will definitely be able to use this documentation as a source of reference to execute the described protocol. The figures that have been provided show essential information and the paragraphs that have been indicated to use for video documentation are spots on because these are steps in the workflow that would require visual demonstration in order to be able to carry out the protocol. I am in absolute favor to publish this work with only very minor suggestions for additions.
Response: We thank the reviewer for the kind comments. We have addressed the minor concerns and have provided more details on other volumetric EM techniques in the text.

Minor Comments:

1. In the discussion the authors elaborate on liver sample preservation by whole liver perfusion or liver wedge needle perfusion. In the protocol, the latter has been described. I would suggest that this procedure would also be included in the video material, because I'm convinced this is valuable to anyone that would like to perform any EM technique on liver tissue.

Response: We agree with the reviewer that for liver researchers a needle perfusion video might be helpful however it will depend on the availability of the mice at the timing of filming. If it is possible, we will endeavour to include it.

2. Please provide info on PO in your table of Materials.

Response: We have now included the ordering details of PO in the table of materials of the revised manuscript.

3. Please check the spelling of Triton X-100. In the current version of the manuscript this is not consistent throughout the text.

Response: We have made the spelling consistent throughout the whole manuscript.

4. Line 423: the symbol for micron is not correctly displayed.

Response: Apologies the typos has been fixed, see Representative results paragraph1

5. Step 6.4: What is the original bit depth? Can the authors elaborate on why they convert images to 8-bit. In view of the processing steps that are used later in the protocol there is no downside to converting images to 8-bit formats. However, if anyone was to apply (semi-)automated segmentation in later steps, it may be beneficial to not alter the original bit depth.

Response: We agree with the reviewer. Our microscope originally produces 16 bit depth images, but are incorrectly read by software packages as 12 bit. We routinely convert for 3D reconstruction to 8 bit as it reduces the data size without compromising resolution to aid data processing and allows easier inter-software handling. We have included the reasons why we recommend converting images to 8-bit as a prerequisite for other data handling steps, see note in protocol 6.4.

Yours sincerely

Jemima Burden