PURDUE
UNIVERSITY

DEPARTMENT OF BIOLOGICAL SCIENCES

October 15, 2019

To: Dr. Philip Steindel
Editor
JoVE

Re: Manuscript #JoVE60677

Dear Dr. Steindel,

We submit our revisions to the manuscript (#JoVE60677), entitled, "*CryoAPEX method for electron microscopy analysis of membrane protein localization within ultrastructurally-preserved cells"* for publication in JoVE.

We have completed all the requested edits. Additionally, a link to the editorial policy from J. Cell Science that allows re-prints is below:
https://jcs.biologists.org/content/rights-permissions

Our response to your requested revisions is below:

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

We have made the necessary corrections.

2. Please provide email addresses for all authors in the manuscript.
The emails for all authors were previously provided and are currently in the JoVE submission. Additionally, we provide these addresses here:

Elaine M. Mihelc	emihelc@purdue.edu
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Robert V. Stahelin	rstaheli@purdue.edu
Seema Mattoo	smattoo@purdue.edu

Protocol:
1. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
This has been checked and confirmed to be correct.

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Specific Protocol steps:
1. 6.2: How thin, specifically?
We have added this information to Line 302, which indicated the section thickness as 90 nm.

Figures:
1. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.
We have uploaded the Permission file to Editorial Manager

The Figure must be cited appropriately in the Figure Legend (not the figure itself), i.e. "This figure has been modified from [citation]."
Each figure legend has now been edited to include the appropriate citation and above statement.

2. Please remove 'Mihelc et al., Figure 1', etc. from the figures themselves.
This has been done.

3. Please include a space between numbers and their corresponding units, e.g., '5 µm' instead of '5µm'.
This has been done.

4. Figure 4: Please include a more prominent space in 'Plasma membrane'. Also, there do not appear to be white boxes in Figure 4.
Both these corrections have been made.

References:
1. Please do not abbreviate journal titles.
2. Please include journal titles for all applicable references.
Both these edits have been made.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
This has been checked and confirmed to be okay.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This article describes a very useful new technique for localizing specific proteins at high spatial resolution in cells, based on the genetically encoded label, APEX2. In this important adaptation of the standard approach, which is based on conventional preparation, the authors show that they can obtain improved ultrastructure by using mild fixation followed by high-pressure freezing (HPF) and freeze-substitution (FS). Previously, it was not believed that it would be advantageous to use APEX2 with HPF/FS because it was assumed that the artifacts of sample preparation would be due to fixation. However, it appears from the authors' results that the most important cause of artifacts is dehydration rather than fixation. The paper is well written and the description of the method and examples of

applications to localize Golgi, mitochondrial, and plasma-membrane proteins is very clear, and straightforward.

Major Concerns:
None.

Minor Concerns:

The approach has been already described by the authors in a recent paper, which they reference in citation #47 (R. Sengupta et al.). Although there is a lot of redundancy between these papers, in the opinion of this reviewer, it would be beneficial to publish the approach in JoVE, if the editors agree that this is consistent with the journal's policy. However, Figures 3A.3B and 3C are identical to Figures 3A, 3B, and 3C in reference #47 (R. Sengupta et al., J. Cell Sci. 132, jcs222315: 2019), and although it's reasonable to re-publish the method in more visual detail, I'm not sure whether it's allowable to use exactly the same figures.

Line 78, correct typo "ferretin" should read "ferritin".
Corrected.

Line 614, Reference #48, "Elife" should read "eLife".
This reference is now Reference #49, and has been corrected.

Reviewer #2:

Manuscript Summary:
In this manuscript, Mihelc et al. describe an exciting new protocol for visualizing protein localization within biological samples via cryoelectron microscopy. The method relies on generating translational fusions of a protein of interest to APEX2, a robust ascorbate peroxidase that has been used for multiple protein localization analyses. Overall, the manuscript is clearly written and essential protocol steps are outlined. The authors provide an excellent background detailing the experimental advantages of APEX2 labeling techniques over existing protein localization methodologies, as well as convincing figures that demonstrate the high-resolution imaging potential of this system. Incorporation of the following minor changes would help to strengthen the manuscript and clarify the protocol.

Major Concerns:
None noted

Minor Concerns:

1. In the Introduction, it would be beneficial to mention the labeling radius and short half-life associated with APEX2 labeling reactions.
Our methodology focuses on sample preservation, and does not attempt to characterize the APEX2 tag - which has been previously done extensively (references #33 and #34). In addition, reference #35 calculated the radius of a GBP-APEX tag. Based on these papers, we have edited lines 87-88 of our revised manuscript to address the reviewer's concerns.

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2. In the "Chemical Fixation and Peroxidase Reaction" section (lines 178-180), it would be helpful to include a few suggestions regarding optimization of DAB incubation times (perhaps by suggesting a range of times that have worked for specific protein types/subcellular localizations).
We have added this information in lines 180-182.

3. In the "Freeze Substitution" section (lines 245-250), I would again emphasize the potential extreme toxicity of OsO4. There are additional storage considerations for osmium stock solutions due to potential acute toxicity. I would perhaps add to the protocol that only very experienced users should handle and prepare FS Mix 2. I might also add suggested PPE (gloves, lab coat that fully covers exposed arms, optional respirator).
We have added these details in lines 247-252.

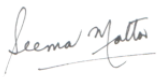
4. In the Discussion, it would be beneficial to include examples of essential positive and negative controls for imaging and labeling [for example, should investigators transfect control cells with an APEX2-only vector in which APEX2 is not fused to the protein of interest to account for potential protein mis-localization patterns that arise due to the translational fusion?] (lines 441-443).
We appreciate the reviewer's suggestion. We have edited our discussion in lines 452-459 to add this information.

We thank the reviewers for their positive feedback and constructive comments. We believe we have addressed all the recommended revisions, and hope our manuscript will be favorably reviewed.

Please let me know if you need anything else.

Thank you for your consideration.

Sincerely,



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