Dr. Xue-Jun Li, Associate Professor
Department of Biomedical Sciences
Regenerative Medicine & Disability Research Lab
College of Medicine at Rockford
Department of Bioengineering
University of Illinois at Chicago
Phone: 815-395-5882
Email: xjli23@uic.edu

September 29, 2019

Dear Dr. Bajaj,

We would like to thank you and the reviewers for the positive feedback and constructive suggestions, which were helpful for improving our manuscript. We have addressed the editorial comments and reviewers' concerns in the revised manuscript, as detailed in the following section and the manuscript file (major changes are labelled in blue color in the manuscript file).

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- The spelling and grammars have been checked and corrected in the text.

2. Please provide an email address for each author.

- The email address has been added.

3. Please provide at least 6 keywords or phrases.

- 6 Keywords have been provided.

4. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

- Formatted.

5. The current Long Abstract is over the 150-300-word limit. Please rephrase the Long Abstract to more clearly state the goal of the protocol

- The goal of the protocol has been added. We have 212 total words for the long abstract.

6. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

- Yes, it is within the word limit.

7. 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.

- These have been checked.

8. Please revise the Introduction to include all of the following parts:

- The introduction has been modified.

9. Please move the hyperlinked text to the reference section or materials table.

- Corrected.

10. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

- This has been adjusted.

11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.”

- These have been modified.

12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

- Modified

13. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

- The related info has been added.

14. 2: please include how the steps are performed. Please include all the button clicks in the software, all the knob turns etc. How do you distinguish the cell body?

- The cell bodies were distinguished by the corresponding phase image that was taken under a microscope.

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

- Revised.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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.

- These have been highlighted.

17. 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. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

- Per the policy of the Journal (Human Molecular Genetics), “Copyright of any article published in HMG will belong to the author or their designee”. “As part of the licence agreement, authors may use their own material in other publications provided that the Journal is acknowledged as the

original place of publication and Oxford University Press as the Publisher". We have attached the related policy and also acknowledged the Journal and the publisher in the manuscript.

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- This has been revised.

19. Please do not abbreviate the journal titles in the references section.

- Corrected.

20. Please sort the materials table in alphabetical order.

- We have updated the table.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The article describes a method to analyze mitochondrial axonal transport and morphology using Fiji software with several available plugins including MultiKymograph, Bioformat importer and Macros. This method can make it easy to analyze mitochondrial transport and morphology in an automatic way, and will be useful for studying mitochondrial deficits in neurodegenerative diseases.

Major Concerns:
The method is described in detail, however, **the efficacy of the method is not well demonstrated**. In figure 1, **the results of mitochondrial movement and velocity are missing**, eg **the percentage of motile mitochondria versus static mitochondria**, and **the frequency histogram of mitochondrial velocity**. It is not shown whether there is any difference of mitochondrial transport between WT and HSP iPSC-derived neuron that can be detected using this method. In both figure 1 and figure 2, to show the efficacy of this method, the results should be compared to manual analysis and other automatic method for example metamorph.

- We thank the reviewer for bringing up this critical point. Using MetaMorph analysis, our previous work showed a significant reduction in the percentage of motile mitochondrial in SPG3A neurons compared with normal neurons. The velocity did not change in SPG3A neurons, suggesting that the transport machinery may not be changed. Therefore, to evaluate the ImageJ software, we examined the percentage of motile mitochondria and found a similar reduction in SPG3A neurons, confirming the effectiveness of this method. As for the analysis of mitochondrial morphology, our published paper used the ImageJ for analysis and found a significant alteration in mitochondrial length and aspect ratio in SPG3A neurons compared with control neurons (Fig.2), confirming the effectiveness of the ImageJ method.

Minor Concerns:
It would be better to test this analysis on live imaging of mitochondrial tagged fluorescent protein and living imaging of mitochondrial dye, and see whether it works well in both cases and how is the difference.

-This is a good suggestion. Though we have not used other probes, they have been used by other studies. We have cited the related references in Table 1.

Reviewer #2:
Manuscript Summary:
How to visualize and quantify mitochondrial transport in hiPSC-derived neurons

Major Concerns:
None

Minor Concerns:
Almost nothing is said about the pros and cons of different mitotrackers or other fluorescent dyes for mitochondria such as TMRE or tagged protein expression. Each has advantages and disadvantages. Some are specific for healthy mitochondria, for example, and are sensitive to pH. I would insist that the authors add a substantial paragraph and a table on pros and cons of the various choices. It would be so helpful to have that coalesced in one place.

-Thanks for the great suggestion. We have discussed this and added a table to the revised manuscript.

Reviewer #3:
The authors, Drs. Mou Y. et al., described a protocol for analyzing mitochondrial transport and mitochondrial morphology in human induced pluripotent stem cells (iPSCs) derived forebrain neuron axons. This protocol can characterize mitochondrial trafficking along axons and analyze their morphology to facilitate the study of neurodegenerative diseases.

The paper contains interesting novel findings, although it is too long and redundant in some parts. Peripheral material should be deleted. Finally, the quality of written English should be meliorated.

- We thank the reviewer for these good points. We have re-organized the introduction and discussion to make them more concise. The English has also been checked.