OPEN PEER REVIEW REPORT 1

Name of journal: Neural Regeneration Research
Manuscript NO: NRR-D-21-00339
Title: Optical Tissue Clearing Enables Rapid, Precise and Comprehensive Assessment of Three-Dimensional Morphology in Experimental Nerve Regeneration Research
Reviewer's Name: S.W.P. Kemp
Reviewer's country: Canada

COMMENTS TO AUTHORS

The manuscript reports on the use of optical tissue clearing methods and three-dimensional morphological assessment in nerve research. This work follows on previous optical imaging techniques such as 3DISCO, FDISCO, and iDISCO, however the novelty exists in its application to peripheral nerves. Overall, the paper is very well written, and the rationale behind the manuscript is sound. However, there are several points of consideration which must be addressed in a revised manuscript. These are listed below:

1. For the Immunostaining procedure, why do you need to stain with Beta-3 Tubulin when Thy-1 GFP rats were used? Would their nerves not already glow green? It is interesting that the authors injected alpha-bungarotoxin into the tail vein of the rat while it was still alive. The rationale behind this is not completely clear. Furthermore, how do you know how much of this will actually get to the NMJ's of interest? Further clarification should be provided.
2. From the Abstract, it seems like this process takes only a day or so, which is not the case. I'm not sure if this was supposed to be provided in the Supplemental Material, but it is essential to include a protocol detailing how many days each step takes. One of the primary arguments of the authors in favor of this method is that it is time saving as compared to traditional methods (e.g., retrograde labeling).
3. Figure 1 is not referenced at any point in the manuscript. Also, it is unclear why Figure 3 is mentioned first in the text, followed by Figure 2. These should be re-arranged so that they correspond to how they are presented in the manuscript proper.
4. There is a low number of animals included in this study. The authors need to further clarify why these numbers were used. This is particularly important for Figure 3 where the traditional method of counting retrograde labelled cells is compared to the novel method. Given that the n=3, how was their statistical power to see significance?
5. Given that the authors used the common peroneal nerve, it is unclear why they would look at L1 and L2. Even L3 and L6 probably wouldn't be that informative as the majority of the cell bodies would be in L4 and L5.
6. It is unclear exactly how they counted the retrograde neurons in the optically cleared sections. Do you need to rotate the sample in 3D to fully count all of the cell bodies? This is important because the claim in the Discussion is that this method gets rid of the multiple sections needed for the traditional method of retrograde counting.
7. Unless I misunderstood, it seems like the cell bodies were pre-labelled before the clearing process in the optically cleared spinal cords and DRGs. How did this not affect the staining following the administration of the solvents used?
8. I understand that the authors used the hydraulic extrusion method by Richner and colleagues (2017), which is cited, however I believe that further explanation on this method and why they used it is warranted. The primary reason for this is because the method by Richner et al. does not use PFA, which is the common protocol usually.
9. It is unclear what fluorescent antibody was used in order to visualize the vasculature. It is not listed in the Methods.
10. In Figure 4, why are the NMJs not innervated by their axons, i.e., why do they look like they're just floating and not innervated by the Thy-1 GFP axons? Also, why are the NMJs different colors in I and J?
11. The authors claim that they have demonstrated the applicability of optical tissue clearing to experimental nerve regeneration studies, which begs the question as to why an actual injury model was

also not included in this paper. This needs to be clarified and explained further.

OPEN PEER REVIEW REPORT 2

Name of journal: Neural Regeneration Research
Manuscript NO: NRR-D-21-00339
Title: Optical Tissue Clearing Enables Rapid, Precise and Comprehensive Assessment of Three-Dimensional Morphology in Experimental Nerve Regeneration Research
Reviewer's Name: Sami Tuffaha
Reviewer's country: USA

COMMENTS TO AUTHORS
This article describes tissue processing and imaging techniques to enable three-dimensional quantitative evaluation of histologic outcomes relevant to peripheral nerve regeneration research. There is a pressing need within this field not only to reduce the burden of histologic tissue processing and analysis, but also to improve the reliability of quantitative outcome assessments. The latter would be most effectively achieved by eliminating the need to extrapolate total histologic counts from images within a single tissue slice and by reducing opportunities for human error to affect results. The approaches described in this article achieve both of those aims, making the manuscript a valuable addition to existing literature. The article is well constructed, with excellent presentation of figures. I recommend publishing the article following minor revisions.

INTRODUCTION
- Excellent summary of limitations of standard practice using tissue cross sections.

METHODS
- For many protocols it is okay to leave tissue in reagents longer than the specified time as signal is retained a long time after the processing is complete. However, when trying to preserve endogenous fluorescence, as in the author's protocol, this may not be the case. A note about whether it is okay to leave samples in any of the solutions for longer than the specified time in order to spread the processing out over more than one day might be useful for readers.
- Similarly, it would be useful to know if imaging needs to happen immediately after the overnight DBE incubation.

DISCUSSION
- Please include a brief discussion as to why Fluorogold was selected as the retrograde dye? Would other (non-lipophilic) dyes have better/equal/worse performance? Fluorogold has an optimal excitation of 320-370nm depending on pH. The lowest laser on most confocals is 405nm line - an explanation of whether FG would be reliably visible on a confocal without a dedicated UV laser line would be informative.
- Microscopy times: it would be helpful for readers to understand how long they can expect a confocal to confocal image an entire rat peroneus longus muscle, as well as the skin flaps and peroneal nerve samples also harvested in this paper. Access to confocals is for many labs, expensive and time-restricted and this may impact the ability to adopt this protocol for a large scale experiment.
- The discussion provides a well thought-out summary of the benefits of optical clearing with automated whole tissue imaging/analysis relative to conventional tissue processing and manual analysis. However, the discussions of the difficulties associated with the optical clearing and wholemount imaging seems limited. Scattered throughout the article are descriptions of challenges that the authors faced (eg trade-off between imaging depth and spatial aberration, standardizing labelling/tissue processing/imaging procedures). The discussion could be improved by summarizing these challenges

in one paragraph. Other than RI matching, what did the authors spend the most time fine-tuning? What are the other major experimental hurdles a lab might face when attempting to replicate this work?