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29th November 2021

Vidhya Iyer, PhD
Review Editor,
JoVE

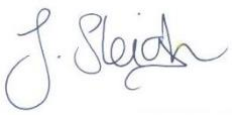
Dear Dr Iyer,

We are pleased to resubmit to JoVE our revised manuscript entitled "Expanding the toolkit for in vivo imaging of axonal transport (JoVE63471)" for your consideration.

The manuscript has been revised in response to all of the helpful comments and suggestions from yourself and both Reviewers.

Thank you for considering our manuscript.

Yours sincerely,



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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have given the manuscript several more proofreads.

2. Please provide an email address for each author.

We now include email addresses for all authors

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

We have removed all pronouns from the protocol section.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (TM), 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.For example: MMRRC Stock Number: 000296-UCD; Jackson Laboratory Stock Number: 005029; Jackson Laboratory Stock Number: 007967; Jackson Laboratory Stock Number: 006410; Zeiss Zen;

We have done as asked.

5. 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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have amended text to use only the imperitive tense in the protocol section.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have done as asked.

7. 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 and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

We have done as asked

8. Please move the legends section to appear after the representative results section.

We have done as asked

9. Please add all items (plasticware, glassware, buffers, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

We have done as asked

Reviewer #1:

Manuscript Summary:

In the present manuscript Tosolini and colleagues describe in detail a method for in vivo imaging of axonal transport, and especially of signaling endosomes and mitochondria. Interestingly, by utilizing a mouse genetic approach, the authors could visualize organelles and cargoes while differentiating between motor and sensory axons.

Since axonal transport in motor and sensory neurons is known to be differentially impaired in neurodegenerative and neurodevelopmental disorders, in my opinion this method is of interest for the field.

Major Concerns:

There are no major concerns.

Minor Concerns:

-Line 267, also recommended numerical aperture for the objectives should be stated.

We have now included these details.

Lines 263-265 – “Either the 40x, 1.3 NA DIC Plan-Apochromat or 63x, 1.4 NA DIC Plan-Apochromat oil-immersion objectives can be used to image *in vivo* transport in the sciatic nerve.”

-The images shown in figure 1 are supposedly taken at the same magnification, however, the length and the thickness of the axons seem different. Is there a reason for it?

This distinction arises due to the age/size of the animals use to generate the two images. Therefore, the following text has been added to the legend of Figure 1:

Line 372: “Distinctions in axon calibre result from differences in mouse age and size.”

-In figure 3, motor axons visualized utilizing the Hb9GFP reporter look fragmented and not continuous (especially when compared to the ChatGFP and the ChatTdt shown in the previous images). Is this reporter dependent?

This is an interesting observation and one we cannot currently explain. However, we have included the following text (lines 616-617) to highlight this:

Lines 338-339: “Note that, unlike ChAT-driven expression, GFP has a more punctate/granular pattern in HB9.GFP axons – the reason for this is unclear.”

Reviewer #2:

Manuscript Summary:

Tosolini et al described a method to image axonal transport in vivo using transgenic animals and organelle markers. The focus on axonal transport, a critical cellular process for neuronal health and disease, is interesting to the field. While many assays exist to measure axonal transport in vitro, it is still challenging to quantify specific cargos in live animals. Therefore, this manuscript addresses an important knowledge gap. By using ChAT.tdTomato mice crossed with mito-CFP mice and fluorescent HcT, they were able to perform multiplexed imaging to track mito and signaling endosomes in motor vs. sensory neurons. Altogether, they have provided a useful platform to distinguish motor from sensor neurons by cell types specific reporter proteins; to measure transport of signaling endosomes in mice under anesthesia; to measure mito transport in motor neurons in vivo; and to co-measure signaling endosomes and mito in the same axon. While the assays are well described and details, like limitations, application, and critical steps, are well discussed, quantitative analysis should be better described and results be discussed in more detail. Finally, anesthesia has effects on axonal transport, though anesthesia is inevitable for this set of experiments, this needs to be discussed. However, these are minor details and don't dampen the enthusiasm for the manuscript, which is both interesting and useful to the neuroscience field.

Major Concerns:

1. It is important to describe the camera settings, particularly the imaging speed, as different cargoes can move at various speeds, undersampling using low fps has caused artefacts and misinterpretations of the transport velocity in the axonal transport field. It will be interesting to compare the speed of cargoes measured in vivo with that in vitro.

We thank the reviewer for this excellent comment and for highlighting this omission from our protocol. We agree that the frame rate should vary dependent upon the cargo being imaged. For example, signalling endosomes undergo fast axonal retrograde transport and have mean velocities of ~2.6 µm/s *in vivo* (Sleigh et al., 2020 *Cell Rep.* 30, 3655–3662.e2), and, as such, imaging should be performed at fast acquisition rates (e.g., 0.33-2 frame/s). Importantly, the mean velocities of signalling endosomes *in vivo* from wild-type mice were equivalent whether the frame acquisition rate was 0.5 s (Fellows et al., 2020 *EMBO Reports* 21 (3), e49129) or 3 s (Sleigh et al., 2020 *Cell Rep.* 30 (11), 3655–3662.e2). On the other hand, *in vivo* mitochondrial transport is much slower with mean velocities of ~0.5 µm/s (Bilsland et al., 2010 *PNAS* **107** (47), 20523–20528), and, as such, will require slower acquisition rates (e.g., 0.12-0.33 frame/s). What is critical is that the frame acquisition rate remains consistent across the entire axonal transport experiment/project as fluctuations in frame rates will likely cause biases within the data. We have therefore included the following relevant details in the manuscript.

Lines 297-299: “It is essential that the frame acquisition rate remains consistent across comparable datasets, because imaging at different frequencies may cause inconsistencies.”

We have previously compared in vitro vs. in vivo axonal transport of endosomes (Gibbs et al. 2016 *J. Neurosci Meth* **257**, 26–33) and have discussed in detail why

distinctions may arise (Sleigh et al. 2017 *F1000 Res* **6**, 200). We refer to this at the end of the fourth Introduction paragraph (i.e., lines 83-94).

2. Protein expression levels and fluorescent marker concentrations should be noted.
We thank the reviewer for allowing us to clarify this point. The only signalling endosome marker we used was fluorescent H_CT. We have successfully observed typical motile signalling endosomes after injecting 2.5-10 µg/µl of H_CT intramuscularly. As described in *1.1* #2 – the volume injected per muscle needs to be considered as smaller muscles cannot handle the larger volumes that are necessary to access the majority of pre-synaptic terminals at NMJs. We have also provided the fluorescent marker concentrations of the failed probes in the "Limitations of the technique" paragraph in the discussion – (lines 480-485; lines 491-497).

Minor Concerns:
1. Why certain organelle markers (e.g. lysosome) are not successful should be discussed. Is that just a concentration problem as briefly mentioned? If so, do other concentrations work? If not, are there other ways to measure those cargoes, using transgenic animals or other optogenetic tools for example?

We thank the reviewer for addressing this issue and agree that it requires a longer discussion. We used Lysotracker following Sasaki et al., (2014 PLoS Genet 26;10:e1004409), which labelled whole zebrafish embryos, BODIPY-FL-pepstatin A following Baltazar et al., (2012, 7:e49635), which labelled ARPE-19 cells and Magic Red following the manufacturer's instructions, with TMRE concentrations as reference for a probe that successfully labels organelles in the sciatic nerve *in vivo*. The probes labelled the nerve, but mainly in Schwann cells, therefore the lack of signal in vesicular compartments may also be due to the inability of the probes to reach the neurons. We cannot rule out that different concentrations might result in a better delivery of the probes. We have added this possibility to the "limitations of the technique" section.

Currently, most studies tracking lysosomes in neurons use *in vitro* approaches (Ferguson 2018, *Curr Opin Neurobiol* 51:45-51), however it would be possible to study their intravital axonal transport with mouse models carrying fluorescently tagged lysosomal markers, such as LAMP-1. We have also added this alternative and its caveat to the "other potential applications" section.

Lines 497-500: "The probes accumulated around axons, likely being retained in Schwann cells, hence the unsuccessful labelling of lysosomes may arise from deficient probe delivery into neurons, although the existence of more suitable concentrations cannot be ruled out."

Lines 535-537: "Furthermore, the lysosomal labelling difficulties can be surpassed using mice expressing LAMP1-GFP, with the caveat that LAMP1 is also present in endocytic organelles different from lysosomes [74]."

2. The effects of anesthesia should be acknowledged and discussed
This is another excellent point that we have now addressed. The effects of anesthesia on axonal transport of signalling endosomes and mitochondria are largely unknown. The only study to investigate this revealed that the axonal transport dynamics of dense core vesicles from the superficial cortical layers are similar in anesthesited and awake

mice (Knabbe et al., 2018 *J Phys* 596, 3759–3773). However, whether all methods of anesthesia elicit the same lack of influence on transport dynamics is unknown, especially as certain physiological readouts (e.g., cochlea function (Cederholm et al., 2012, *Hearing Res.* 292, 71–79) and cortical electrophysiology (Michaelson and Kozai, 2018, *J Neurophys* 120, 2232–2245) differ depending upon the method used (e.g., isoflurane vs ketamine/xylazine/acepromazine). We have included these points in the “Limitations of the technique’ section in the discussion.

Lines 506-515: “Methods of anesthesia can alter distinct physiological readouts (e.g., cochlea function [63] and cortical electrophysiology [64]); however, whether anesthesia influences axonal transport in the sciatic nerve is currently unknown. Given the reduced neuromuscular activity under isoflurane-induced anesthesia, it is possible that transport kinetics differ compared to the wakeful state. However, the only *in vivo* study that has directly investigated this revealed that transport of dense core vesicles in thalamocortical projections does not differ between anesthetized and awake mice [65]. Furthermore, since distinctions in transport between wild-type and disease model mice are detectable under anaesthesia [35,43], it is clear that isoflurane exposure does not prevent identification of perturbances in signalling endosome or mitochondrial trafficking.”