Point-by-point response to editorial and reviewers' comments

JoVE60287, "Time-lapse live imaging and quantification of fast dendritic branch dynamics in developing *Drosophila* neurons" by Sheng et al.

We greatly appreciate the careful evaluation and constructive comments from the editor and reviewers. We modified our text extensively to address all issues raised and to adhere with the standard of the journal. We believe our manuscript has been significantly improved. We hope that the editor and reviewers find this revised version now suitable for publication.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: We proofread the manuscript as suggested by the editor.

*• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.*
1) Examples Not in the imperative voice: steps 6.4, 6.5, 6.6., 6.7.

Response: We modified the text extensively to ensure the statements in the protocol section are written in the imperative tense wherever possible.

*• **Protocol Detail:** 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 add more 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.*

Response: We modified the protocol to include additional information and details as suggested by the editor.

1) 1.1: at what embryonic age?

We specified the stage in the revision to:

> *"1.1 Collect 100-200 eggs within a two-hour window post fertilization"*

2) 1.3: Mention growth conditions.

We modified the text to include the information:

> "*1.3 Culture the larvae at 25 °C under the circadian and humidity controls to the desired developmental stage(s)."*

3) 2.1: Under a dissection microscope? Mention magnification and surgical tools used.

We modified the text to include the information:

> *"2.1 Dissect larval brains in PBS solution under a dissection microscope (4.5X magnification power) with two pairs of #5 standard tip 11 cm dissection forceps. Use one pair of forceps to hold the larval body in place and the other to carefully dissect out the brain. Preserve the eye disks, brain lobes and the ventral nerve cord. Remove attached muscles to minimize sample movements during imaging."*

4) 2.4: How are the larvae handled?

We modified the text to include the information:

> *"2. 4 Transfer dissected larval brains into the chamber on the glass slide using forceps. Adjust the position of the brains under the dissection scope to ensure the dorsal side faces up."*

5) 2.5: What are the units for 22x22-1.5?

We modified the text to include the information:

> *"2.5: Cover the chamber with a glass cover slip (22 x 22 x 0.15mm)."*

• Protocol Numbering: *Please add a one-line space between each protocol step.*

Response: We added the one-line spaces between steps.

• Protocol Highlight: *After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
5) Please bear in mind that calculations and software steps without a graphical user interface/ command line scripting cannot be filmed

Response: We thank the editor for the comments and suggestions. In this revision, we modified the text to include all recommended changes and reduce the length of the protocol section to below 3 pages. We believe steps 2-6 are filmable and highlighted those sections, which is two and half pages.

*• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.*

Response: We greatly appreciate the editor's comments. We reorganized the Discussion (Page 7-8) to 6 paragraphs and included the following information: 1) the limitation of the technique, 2) critical steps, 3) the comparisons to existing methods, 4) modifications and future applications.

*• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Superfrost Plus, Zeiss LSM 780, Coherent Vision II, Huygens professional, Imaris' Surpass, Excel, etc*
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Response: We replaced all commercial sounding language with generic names that are not company-specific and followed by (see table of materials).

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: Fig 3B and **Fig 4C** reused figures from our previous publication. We cited the figure in the figure legend as following:

"Figure 3B has been modified from Sheng, C. et al, 2018[8]."

The copy right clearance form is attached blew. We also included a copy in the resubmission.

SPRINGER NATURE

Title: Experience-dependent structural plasticity targets dynamic filopodia in regulating dendrite maturation and synaptogenesis
Author: Chengyu Sheng et al
Publication: Nature Communications
Publisher: Springer Nature
Date: Aug 22, 2018

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

The authors describe a well-developed protocol for quantifying dendritic terminal dynamics of single labeled Drosophila larval ventral lateral neurons (LNvs) by time-lapse live imaging. They describe the dissection and mounting of live larval brains and how they perform 2-Photon live imaging to visualize LNv dendrite dynamics. They further describe the procedures to optimize the time-lapse image stacks for quantitative analysis of branch dynamics using drift correction, deconvolution and terminal branch annotation.

This method allows to gain quantitative understanding of how developmental and activity-dependent processes regulate dendritic branch dynamics. Overall, the protocol is clearly present and relevant to all researchers working on dendrite dynamics. It will provide a nice resource for scientists working on this or other model systems, as the authors also provide some information how their protocol can be adapted to other neurons. I strongly recommend this methods paper for publication and have only a few minor comments and suggestions that are listed below.

Major Concerns:

None

Minor Concerns:

1) page 3, point 1.3: "designed" should probably be "desired".

Response: We thank the reviewer's suggestion and modified the text accordingly.

2) The authors initially dissect the brains in PBS, but later on switch to physiological external saline solution. Part of the problem with dissection and tissue degradation might be due to the use of PBS, which is not very physiological and might thus cause some tissue degradation. Potentially it might make sense to dissect directly in the given physiological solution.

Response: We thank the reviewer's suggestion and modified our protocol.

"2.1 Dissect larval brains in the physiological external saline solution (120mM NaCl, 4mM $MgCl_2$, 3mM KCl, 10mM $NaHCO_3$, 10mM Glucose, 10mM Sucrose, 5mM TES, 10mM HEPES, 2mM Ca^{2+}, PH 7.2) under a dissection microscope (4.5X magnification power) with two pairs of #5 standard tip dissection forceps (11 cm)."

3) Dissection of larval brains may cause tissue injury and acute responses of neurons. The authors should indicate the maximum time allowed for dissection and mounting before starting with imaging. It would also be helpful to add some trouble shooting, e.g. how to evaluate if the dissected sample is really good (e.g. discard if you see tissue damage, neuronal membrane blebbing, dendrite retraction only etc,). Figure 1 would be a good place for this annotation to also get a sense of the time required for image processing and analysis.

Response: We greatly appreciate the reviewer's suggestions and we agree that brain dissection and sample preparation are critical for the success of the studies on dendrite dynamics. However, because the LNv dendrite is contained within the larval brain lobe, our data indicate that the LNv dendrite morphology and its physiological responses are well-preserved in a carefully prepared brain explant within 30 mins of dissection. The issues with sample preparation are generally associated with the integrity of the brain lobes being compromised during the dissection step, which leads to elongated dendritic arbors that can be easily recognized and excluded. To specifically address the reviewer's comments, we modified the protocol as following:

"1.1 Collect the time lapse image series within 30 minutes of the brain dissection. Data with excessive drift or obvious morphological deterioration should be excluded from post-processing and quantification."

Additionally, we included the following statement in the Discussion:

"High quality raw images of single LNvs are essential for the accurate quantification of dendrite dynamics. Our data indicate that LNv dendrite morphology and its physiological responses are well-preserved in a carefully prepared larval brain explant within 30 mins of dissection. Samples with observable brain tissue deterioration, elongated dendritic arbors and branch breakage should not be used for imaging and quantification"

The reviewer also suggested including a time estimate for image processing and analyses in Figure 1. Although we agree with the reviewer that the information regarding the required time on each step will be helpful, we found it is difficult to provide an estimate with a reasonable range. The complexity of the data set, the skill level of the person performing the analyses and the software tools all significantly impact the processing time.

4) Did the authors try to use poly-L-lysine pre-coated slides to mount the brain samples, which may reduce sample drifting?

Response: We thank the reviewer for the comments. The slides used in our study, Superfrost Plus, are treated to electrostatically adhere tissue or cell samples. The sample drifting in general is correctable by our post-processing procedure.

5) The authors explain and show the time lapse imaging for a total of 10 minutes. Did they try longer imaging? This could be mentioned in the introduction or discussion.

Response: We thank the reviewer for the suggestion. At the initial stage of developing the methods, we tested imaging series with durations ranging from 10 to 30 mins. Due to the high prevalence of the dynamic dendritic filopodia in the developing LNvs, there are sufficient amounts of dynamic events within 10 min, which we used for the subsequent analyses. As suggested by the reviewer, we included this information in the introduction.

6) The authors mention that they developed R scripts for easing the burden on analysis. It will be nice if they are made available and a link is provided to download them.

Response: We developed two batch-processing R scripts to speed up the data analyses. We included those scripts in the revised manuscript.

Reviewer #2:

Manuscript Summary:

In this manuscript, Sheng et al described detailed methods for imaging highly dynamic dendritic branches in live Drosophila larval brains and quantifications for the dynamics of the dendrites. High motility of developing dendritic filopodia is a general phenomenon during the formation of neural circuits, but characterizing the dynamics is challenging. The methods described in this manuscript will be very useful for studying dendrite development not only in Drosophila but probably also in other animal models as long as the dendritic arbor is not extremely complex and individual branches are easily identifiable. The manuscript is very well written. All the steps are clearly described and very easy to follow and all the necessary details are included. Figures presented in the manuscript are all of high quality. I highly recommend publication of the manuscript at JoVE.

Major Concerns:

None

Minor Concerns:

There are only some minor grammatical errors that the authors may want to correct. For example, line 5 from the bottom of the fourth paragraph on page 5, it should read "the LNv dendrite dynamics are developmentally regulated" instead of "the LNv dendrite dynamics were…". It would also be better to spell out what "SNR" represents for in the first paragraph of the "Discussion" part on page 6 although it is easy to guess it represents "signal-to-noise ratio".

Response: We thank the reviewer for the positive comments and made extensive editing to eliminate grammatic errors in the revised manuscript.

Reviewer #3:

Manuscript Summary:

"Time-lapse live imaging and quantification of fast dendritic branch dynamics in developing Drosophila neurons." by Chengyu Sheng, Uzma Javed, Jun Yin, Bo Qin & Quan Yuan

: describes a protocol for live imaging of LNvs neurons in Drosophila larval brain by using two-photon microscopy and Hyugens and Imaris software

Major Concerns:
The sample preparation is very straightforward that anyone who is familiar with sampling Drosophila larval brain would be able to reproduce it with a method section from a research article, which makes the current manuscript dispensable. Moreover, the protocol heavily relies on specific instrumentation and commercial software of Huygens and Imaris, which will limit the application in broad audience - not many labs has access to these expensive software packages - it would have been better if they used Imagej/Fiji. One thing that would have been useful for readers is their R scripts, which was not shared in the manuscript. To increase the usefulness of their protocol, I suggest expanding their sample preparation and imaging protocol - not for microscope setting, but for example the requirement of environmental control chamber and how long the sample preparation can be imaged -- or how long the dissected brain can survive in their protocol, What frequency do they get single neuron labeling from their heat-shock protocol, how many brains do they typically mount on a single slide and why, how much external saline solution need to be added - 10 ul or 100 ul?, does larval brain need to be rinsed with particular solution? Ect. And importantly, their R scripts will need to be shared with detailed instruction.

Response: We thank the reviewer's careful examination of our manuscript and the helpful comments. We address the specific points as following:

1. *The sample preparation is very straightforward that anyone who is familiar with sampling Drosophila larval brain would be able to reproduce it with a method section from a research article, which makes the current manuscript dispensable.*

   There were no previous publications on using the larval brain explants for dendrite dynamic analyses. Therefore, although the main emphasis of our manuscript is not the brain dissection and sample mounting, to illustrate the complete process of data analyses, we included the sample preparation steps and emphasized the importance of maintaining tissue integrity in the dynamic studies.

2. *Moreover, the protocol heavily relies on specific instrumentation and commercial software of Huygens and Imaris, which will limit the application in broad audience - not many labs has access to these expensive software packages - it would have been better if they used Imagej/Fiji.*

   We agree with the reviewer that it would be ideal to perform the analyses using open source software. In the revised manuscript, we provided statements on the features required to support the analyses and introduced possible open source tools that could potentially replace the commercial software used in our protocol. The following statements are included in the revision:

   P4: "4.3 Save the deconvolved image in a file type that is supported by the subsequent image annotation software capable of analyzing 4D data and reporting the spatial coordinates of defined spots in the image "(see table of materials)"."

   P7: "Besides the commercial software used in our studies, ImageJ/Fiji's "Correct 3D drift" ("Plugins" -> "Registration" -> "Correct 3D drift") also works well for drift correction."

   P7: "New developments in open source 3D visualization software could potentially address current technical limitations and make automation of this critical step possible. Several recently

developed software tools provide automatic quantification function for studies on filopodia [16-21] and can be potentially tested and adopted for studies on dendrite branch dynamics."

3. *One thing that would have been useful for readers is their R scripts, which was not shared in the manuscript. To increase the usefulness of their protocol, I suggest expanding their sample preparation and imaging protocol - not for microscope setting, but for example the requirement of environmental control chamber and how long the sample preparation can be imaged -- or how long the dissected brain can survive in their protocol, What frequency do they get single neuron labeling from their heat-shock protocol, how many brains do they typically mount on a single slide and why, how much external saline solution need to be added - 10 ul or 100 ul?, does larval brain need to be rinsed with particular solution? Ect. And importantly, their R scripts will need to be shared with detailed instruction.*

   Following the reviewer's suggestions, we expanded the sample preparation and imaging protocol, **Step 1 and 2 on Page 3**, to include all information mentioned by the reviewer. In addition, we included two R scripts we used for batch processing in this revision.

Minor Concerns:
Some additional questions and comments.
**Replace & to and in author list.*

Response: We made the change as suggested by the reviewer.

**3.3. how does the typical xyz resolution was calculated? The resolution looks too fine to be true by optical microscope - including two-photon microscopy. Was it calculated after deconvolution?*

Response: The x-y-z resolution (0.11 × 0.11 × 0.25µm) is the voxel size provided the image acquisition software (Zen 2012, Zeiss). It is the resolution of the raw image without deconvolution.

**There is a figure that was previously published (Fig 4C). Did authors provide explicit permission from their original publisher?*

Response: Fig 3B and Fig 4C used figures from our previous publication. We cited the figures in the figure legend. The copy right clearance form is included in the resubmission.