Journal of Visualized Experiments

Time-lapse live imaging and quantification of fast dendritic branch dynamics in developing Drosophila neurons

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60287R1
Full Title:	Time-lapse live imaging and quantification of fast dendritic branch dynamics in developing Drosophila neurons
Keywords:	Dendrite development; dendritic filopodia; branch dynamics; Drosophila; ventral lateral neurons; time-lapse imaging
Corresponding Author:	Quan Yuan National institutes of Health BETHESDA, MD UNITED STATES
Corresponding Author's Institution:	National institutes of Health
Corresponding Author E-Mail:	quan.yuan@nih.gov
Order of Authors:	Chengyu Sheng
	Justin Rosenthal
	Uzma Javed
	Jun Yin
	Bo Qin
	Quan Yuan
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bethesda, Maryland, USA

TITLE:

Time-Lapse Live Imaging and Quantification of Fast Dendritic Branch Dynamics in Developing *Drosophila* Neurons

AUTHORS & AFFILIATIONS:

Chengyu Sheng[1], Justin Rosenthal[1], Uzma Javed[1], Jun Yin[1], Bo Qin[1], Quan Yuan[1]

[1]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

Corresponding Author:
Quan Yuan
Email Address: quan.yuan@nih.gov

KEYWORDS:

dendrite development, dendritic filopodia, branch dynamics, *Drosophila*, ventral lateral neurons, time-lapse imaging

SHORT ABSTRACT:

Here, we describe the method we employed to image highly motile dendritic filopodia in a live preparation of the *Drosophila* larval brain, and the protocol we developed to quantify time-lapse 3D imaging datasets for quantitative assessments of dendrite dynamics in developing neurons.

LONG ABSTRACT:

Highly motile dendritic filopodia are widely present in neurons at early developmental stages. These exploratory dynamic branches sample the surrounding environment and initiate contacts with potential synaptic partners. Although the connection between dendritic branch dynamics and synaptogenesis is well established, how developmental and activity-dependent processes regulate dendritic branch dynamics is not well understood. This is partly due to the technical difficulties associated with the live imaging and quantitative analyses of these fine structures using an in vivo system. We established a method to study dendrite dynamics using *Drosophila* larval ventral lateral neurons (LNvs), which can be individually labeled using genetic approaches and are accessible for live imaging. Taking advantage of this system, we developed protocols to capture branch dynamics of the whole dendritic arbor of a single labeled LNv through time-lapse live imaging. We then performed post-processing to improve image quality through drift correction and deconvolution, followed by analyzing branch dynamics at the single-branch level by annotating spatial positions of all branch terminals. Lastly, we developed R scripts (**Supplementary File**) and specific parameters to quantify branch dynamics using the coordinate information generated by the terminal tracing. Collectively, this protocol allows us to achieve a detailed quantitative description of branch dynamics of the neuronal dendritic arbor with high temporal and spatial resolution. The methods we developed are generally applicable to sparsely labeled neurons in both in vitro and in vivo conditions.

INTRODUCTION:

Dendrites are specialized neuronal compartments that receive and process sensory and synaptic

input. The complex and stereotyped structure of dendritic arbors has been under intense investigation since their discovery. A number of model systems, including *Xenopus* optic tectal neurons, chick retinal ganglion cells, and dendritic arborization (da) neurons in the *Drosophila* system, have been established to study the development, remodeling and plasticity of neuronal dendrites[1-4]. *Drosophila* ventral lateral neurons (LNvs) are a group of visual projection neurons initially identified for their important functions in circadian regulation of fly behaviors[5]. Studies also revealed the role of larval LNvs as the direct postsynaptic target of the larval photoreceptors (PRs)[6,7]. Importantly, culturing developing larvae in different light regimes strongly affects the size of LNvs' dendritic arbors, demonstrating the suitability of LNvs as a new model for studying dendritic plasticity[7]. Recent work from our group further indicates that both the size of the LNv dendrite and the dynamic behavior of the dendritic branches display experience-dependent plasticity[8,9]. As part of this work, we developed a new live imaging and quantification protocol to perform analysis on the dendrite dynamics of LNvs from 2nd or 3rd instar larvae.

The transparent nature of the *Drosophila* larval brain makes it ideal for live imaging. However, the dendritic arbors of LNvs are situated in the densely innervated larval optic neuropil (LON) in the center of the larval brain lobe[6]. To capture images of fine dendrite branches and filopodia in the intact brain tissue, we utilize two-photon microscopy, which increases the depth of light penetration and reduces the phototoxicity during live imaging experiments[10]. Using this setup, we successfully performed live imaging experiments on LNvs for over 30 min without observing obvious morphological deterioration of the neuron. In addition, genetic manipulations using the Flip-out technique enabled us to label the individual LNvs with a membrane tagged GFP, which is also critical for monitoring the movements of individual branches[11-13].

To capture the dynamic behavior of all branches on the LNv dendritic arbor with optimal optic resolution, we performed time-lapse 3D imaging on freshly dissected larval brain explants with a high spatial resolution at 1 min per frame for 10 to 30 min. Developing LNv dendrites are highly dynamic, with a large percentage of the branches displaying observable changes within the 10 min window. This leads to one of the main technical challenges in studying dendrite dynamics, quantifying branch behavior based on the 4D image data sets. Previously established methods have various limitations, including lack of accuracy and excessive time requirement. Therefore, we developed a semi-automatic method that combines image post-processing, manual marking of the branch terminals, and automatic 4D spot tracing using an image annotation software. We calculate the movements of branch terminals at different time points based on the 3D coordinates of the spots. The data are then exported and analyzed to produce quantitative measurements of the branch dynamics. This method accurately assesses the duration and extent of extension and retraction events of existing branches, as well as the formation of new branches, allowing us to monitor dendrite dynamics in a large number of neurons.

PROTOCOL:

CAUTION: This protocol involves the use of class IV lasers and will require proper training and safety guidelines to be followed. Avoid eye or skin exposure to direct or scattered laser light.

NOTE: The protocol includes six steps. The workflow is shown in **Figure 1A**.

1. Labeling individual neurons using the Flip-out technique

NOTE: The single labeling of LNvs is achieved by expressing mCD8::GFP in single LNvs using flippase -mediated stochastic labeling. The genotype of the fly line is: *hs-flp; Pdf-Gal4; UAS-FRT-CD2-stop-FRT-mCD8::GFP*[11-13]. The frequency of obtaining a single labeled LNv is around 10%. Fly stocks are maintained in standard medium in circadian- and humidity-controlled 25 °C incubators.

1.1 Collect 100-200 eggs within a 2 h window post fertilization on a grape juice plate with yeast supplement. Incubate the embryos at 25 °C for 24 h and collect newly hatched first instar larvae for the next step.

1.2 Heat shock the newly hatched first instar larvae at 37.5 °C for 40 min two times, with a 40 min recovery period in between.

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

2. Dissecting and mounting larval brain explants

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

2.2 Prepare a glass slide (25 x 75 x 1.0 mm) and use a syringe to draw a square chamber with vacuum grease.

2.3 Add 20 µL of external saline solution to the square chamber with grease barriers.

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.

2.5 Cover the chamber with a glass cover slip (22 x 22 x 0.15 mm). The larval brain is now mounted on the slide within a chamber filled with the external saline solution (**Figure 1B**).

Note: Pressing gently on the coverslip confines the brains and reduces sample drifting in the subsequent imaging session.

3. Time-lapse live imaging

NOTE: We perform time-lapse imaging experiments using a confocal microscope equipped with a multiphoton laser. The acquisition parameters need to be adjusted for other imaging setups.

3.1 Identify brain explants containing individually labeled neurons using a 40X water immersion objective (NA 1.3) and an epifluorescent light source. For image collection, use a two-photon laser tuned to 920 nm and a non-descanned (NDD) detector.

3.2 Collect images at 512 x 512 pixels per frame and 1 min per Z-stack for 10 min. Adjust the optical and digital zoom to achieve a sufficient x-y-z resolution while ensuring the coverage of the whole dendritic arbor within 1 min (**Figure 2, Supplementary Video 1**). The settings generate images with a typical x-y-z resolution of 0.11 × 0.11 × 0.25 µm.

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

4. Drift correction and deconvolution

NOTE: Depending on the image quality, both steps are optional but strongly recommended.

4.1 Open an acquired image file with a drift correction software. Edit microscopic parameters to match those used for the experiment. For the software (see **Table of materials**), click **Edit** | **Edit microscopic parameters**. Set microscope type as widefield for two-photon images if there is no two-photon option and follow the workflow defined by the software. For example, open the tab **Deconvolution** | **Object Stabilizer**, and choose **Stabilize time frames**.

4.2 Open the drift-corrected image in the deconvolution software and follow its workflow. For the software (see **Table of materials**), select the stabilized image and then click **Deconvolution** | **Deconvolution Express**. To get a better result, fine-tune the parameters using **Deconvolution Wizard**.

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**).

5. Image annotation

5.1 Open the deconvolved image in the image annotation software. Examine and mark branch tips at all time points in 3D. The image annotation software reports and stores the spatial and temporal coordinates of the marked branch tips. Export the coordinate information as a .csv file for subsequent calculations.

NOTE: The following two steps are specific to the annotation software we used (see **Table of materials**). The workflow may be different for other software.

5.2 Within the Spots module, click "**Skip automatic creation, edit manually**" and check the bottom "**Auto-connect to selected Spot**" checkbox (**Figure 3A**). Go over the frames in the time series and select a branch for annotation. Hold the Shift key and click the branch terminal tip to add a spot. Click through all time points.

NOTE: The image annotation software connects the spots between frames and generates a trajectory automatically. The spatial and temporal information of the branch tips is now associated with marked spots. Repeat these steps until all branch tips are annotated (**Figure 3B**).

5.3 Within the Spots module, click the **Statistics** tab, choose **Detailed** and select **Specific Values** | **Position**. The recorded spatial and temporal coordinate information will be on display. Click the **Save** bottom to export that information as a .csv file (**Figure 3C**).

6. Calculating dendritic branch dynamics

NOTE: Using the coordinate information, displacement of the branch tips in 3D can be easily calculated. In our study, all dendritic branch movements are categorized as **Extension** or Retraction. The steps below describe how to process the .csv files using custom-written R scripts (**Supplementary File**) and by adding the directional information through manual editing.

6.1 Open the .csv file as a spreadsheet. Select the **Track ID** column, click the **Sort Smallest to Largest** option and accept **Expand the selection**. After sorting, Track ID identifies each unique dendritic branch, and spots from the same branch share the same Track ID. The **Time** column stores the information of different time frames.

6.2 In the spreadsheet, add a **Distance** column. Calculate the distance of every two temporally adjacent spots using their coordinates and put the values in the **Distance** column (**Figure 4A**).

6.3 Minor movements at the single voxel level. Based on the imaging settings, 0.3 µm are usually artifacts from uncorrected drifting or imperfect image annotation. Filter these movements out by resetting all **Distance** values smaller than 0.3 µm to 0. Process multiple .csv files manually or use our R script **batch column filtering.R (Supplementary File)**.

6.4 Manually generate a new column named **Displacement** in the spreadsheet. Copy the values from **Distance** column to **Displacement** column. Manually assign the extension and retraction events for each branch tip. If it is an extension, leave the **Displacement** value unchanged, which is a positive value. If it is a retraction, change the corresponding **Displacement** value to a negative value (e.g., 0.35 to -0.35) (**Figure 4B**).

6.5 Generate a new column named **Event**. In this column, manually sum the **Displacement**

values for individual extension and retraction events (**Figure 4B**).

6.6 Process the modified spreadsheet and quantify the extension and retraction events based on their displacement values using R script **batch column sum.R (Supplementary File)**. The output parameters include **Number of extension events**, **Number of retraction events**, **Cumulative length extended**, **Cumulative length retracted**, **Net length changed**, and **Total length traveled**.

REPRESENTATIVE RESULTS:

Using the live imaging protocol described above, we capture high resolution image stacks for the subsequent analyses and quantification. **Supplementary Video 1** shows the maximum intensity projected (MIP) image series collected from a representative individually labeled LNv. **Figure 2B** shows the corresponding montage of eight frames of the image series. In both panels, arrowheads mark retraction events and arrows mark extension events.

Next, we perform semi-automated 4D tracking of the branch terminals using an image annotation software (**Table of materials**). **Figure 3B** shows annotations of the dynamic branch terminals from a representative individually labeled LNv. Using this software, we visualize the image stacks in 3D, manually mark all branch terminals and use the Spots module to track the terminals' movements through time. This software generates time-stamped trajectories for every annotated branch terminal, serving as visual representations of the dynamic events on the dendritic arbor.

We then use the coordinate information from annotated branch tips to calculate the direction and distance of branch movements at each time point. Screenshots in **Figure 4A,B** show how this is achieved. The processed spreadsheets generate output files that we use to quantify dendrite dynamics with four parameters, including percentage of dynamic branches, number of new branches, cumulative distance traveled, and number of branch movement events. **Figure 4C** shows comparisons of dendritic branch dynamics for individually labeled LNvs from different developmental stages. Consistent with the findings in mammalian neurons and zebrafish tectal cells, the LNv dendrite dynamics are developmentally regulated[14,15]. LNv dendrites in younger larvae, 48-72 h after egg laying (AEL), are significantly more dynamic compared to those in older larvae, 96-120 h AEL, as measured by all four parameters, and the developmental transition from the dynamic to stable state occur between 72 to 96 h AEL[8].

FIGURE AND TABLE LEGENDS:

Figure 1. Workflow of the dendrite dynamics imaging and quantification protocol. (A) The protocol contains six steps covering sample preparation, image collection, image processing and semi-automated quantification of dendrite dynamics. **(B)** A schematic diagram illustrating an imaging chamber containing a larval brain explant mounted with the dorsal side up. The brain explant has an individually labeled LNv in each lobe and is immersed in the external saline solution. The vacuum grease barriers support the weight of the coverslip and forms a small chamber that prevents the brain from moving.

Figure 2. Time-lapse imaging of dendritic branch dynamics in 3D. (A) The dendritic arbor of an individually labeled LNv from a 3rd instar larva. **(B)** The corresponding montage image of **(A)** illustrating the representative branch movements. Arrowheads (Green) mark retraction events; arrows (Red) mark extension events.

Figure 3. Manual annotation and automatic tracking of dendritic branch terminals in an image annotation software. (A) A screenshot shows the settings for branch terminal tracking using the Spots module. The red oval outlines the **Manual Tracking** option. **(B)** Representative time-stamped trajectories of annotated branch terminals (yellow spots) generated by the image annotation software in an individually labeled LNv. Scale bar, 5 µm. **(C)** A screenshot shows the interface for viewing and exporting the coordinate information from the Spots module. **Figure 3B** has been modified from Sheng, C. et al, 2018[8].

Figure 4. Quantification of dendritic branch dynamics using the coordinate information of the branch terminals. (A) A screenshot shows the spreadsheet containing the X, Y and Z coordinates, Track IDs and formula used for calculating displacement of spots in the adjacent frames. **(B)** A screenshot shows representative results obtained from tracking one branch. The minor movements (< 0.3 µm) were filtered out. The retractions are marked by assigning their value to negative (Distance column). By summing up adjacent positive/negative values, the displacement of each biological extension/retraction is computed (Events column). **(C)** Representative quantification of results showing the developmental regulation of dendrite dynamics. Data are presented as a box plot (box, 25–75%; center line, median) overlaid with a dot plot (individual data points). **Figure 4C** has been modified from Sheng, C. et al, 2018[8].

Supplementary Video 1. Ten maximum-intensity-projection (MIP) images played at a speed of 2 frames per second. The whole dendritic arbor was imaged at 1 min per Z-stack.

Supplementary File. R scripts.

DISCUSSION:

Here, we describe a protocol we developed to record and quantify the dynamic behavior of dendritic branches in individually labeled neurons in *Drosophila* larval brains. Notably, our live imaging protocol contains specific parameters that enable us to capture the whole dendritic arbor of a larval LNv and provide a global view of the dynamic state of its dendrite branches. However, because our quantification methods heavily rely on the annotation of branch terminals, neurons with complex dendritic structures and condensed arborizations are not suitable subjects for this analysis.

Sample preparation, image acquisition and post-processing are the critical steps in this protocol. 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 min of dissection. Samples with observable brain tissue deterioration, elongated dendritic arbors and branch breakage should not be used for imaging and quantification. Although optional for datasets with low

background and no observable movements, we strongly recommend including the drift correction and deconvolution steps to improve the image quality, which is important for the annotation of branch terminals and automatic spot tracking. 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.

One of the major differences between our protocol and existing methods is that we track the movements of individual branch tips but not the whole dendritic arbor. Reconstruction of whole dendritic arbors, although useful for measuring the total dendritic length and architecture, is not ideal for dynamic studies using 3D imaging datasets. For consecutive Z-stack slices, automatic tracing programs often generate different combinations of "branches" and reconstruct the dendritic arbor differently, making the comparison at the single branch level between different time points particularly difficult. In addition, comparing to tracing the whole dendritic arbor, terminal tracking significantly reduces the processing time and the requirements on computation power.

Several modifications can potentially improve the efficiency and analytical features of our method. To extract quantitative positional information from the time-lapse image series, accurate annotation of branch tips at all time points is critically important. This step is currently performed manually, which not only is time consuming, but also introduces variability. 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.

Another step that is currently performed manually is the identification of extension vs. retraction events. Automation of this step requires the 3D coordinates of branch bifurcation sites. By comparing distances to the branching site at two time points, a custom-written script (**Supplementary File**) can be developed to assign the directionality of the terminal displacements. Following the same logic, additional 3D coordinates along the branch may also be used for analyzing the extension and retraction events occurring within the branch. These add-ons will not only optimize the workflow of our protocol, but also increase the information contents regarding the structural changes of the dendritic arbors at different temporal scales.

In summary, to support our studies on experience-dependent dendrite plasticity, we developed time-lapse live imaging protocols and semi-automated quantification methods to perform assessments of fast branch dynamics in developing neurons. Based on the results generated by the methods described here, our study identified highly motile dendritic filopodia in *Drosophila* central neurons and revealed a developmental coordination between heightened dendrite dynamics and synaptogenesis. Future improvements at the image acquisition and automation level will greatly enhance the potential of this protocol and facilitate studies on dendrite dynamics and structural plasticity.

ACKNOWLEDGMENTS:

This work is supported by the Intramural Research Program of National Institute of Neurological Disorders and Stroke, National Institutes of Health. Project number 1ZIANS003137.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

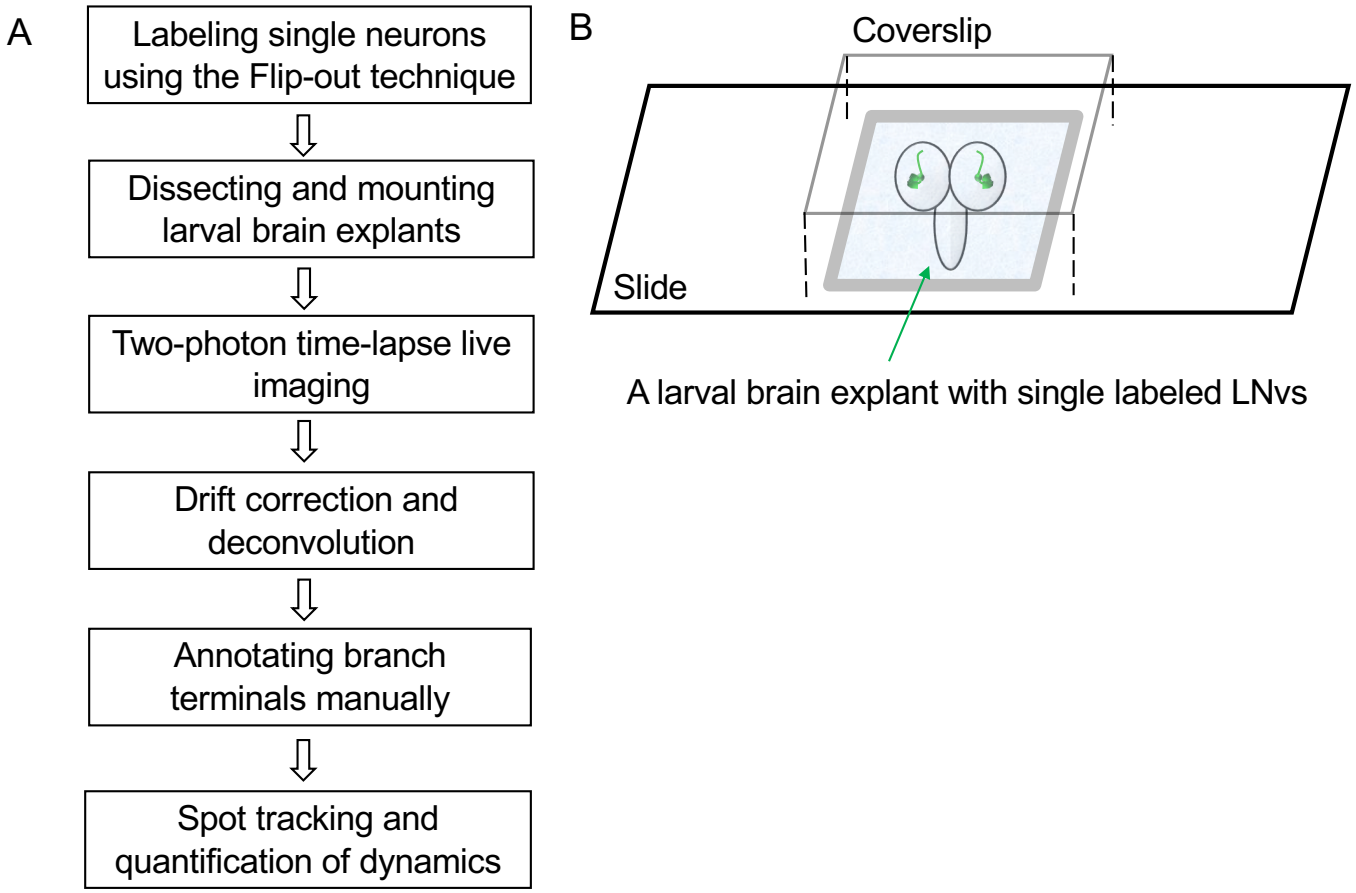


Figure 2

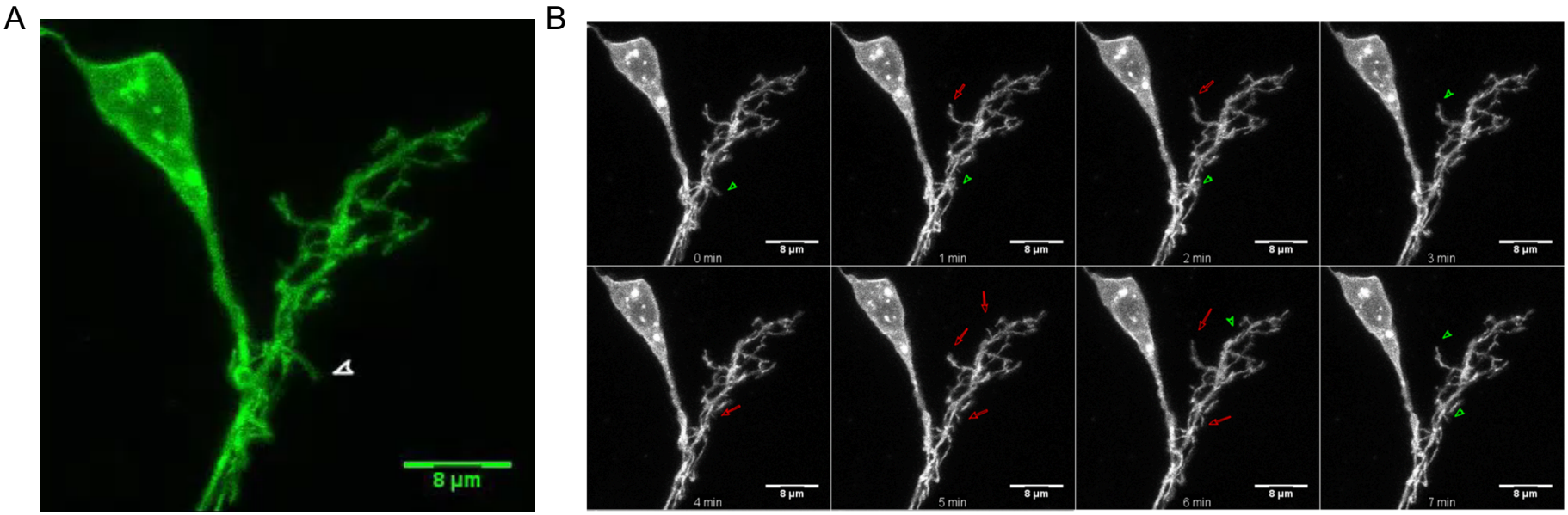
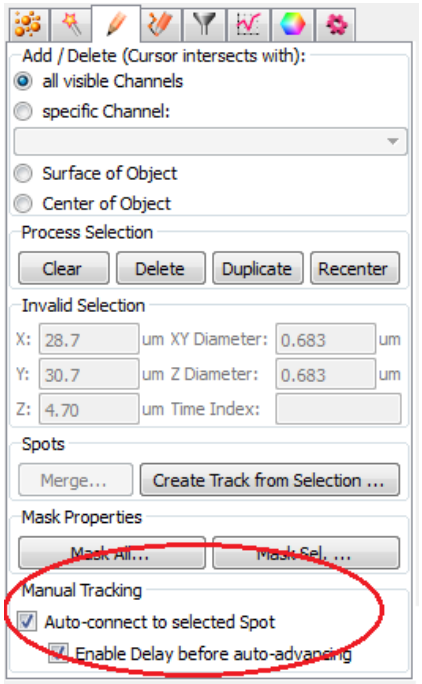


Figure 3

A



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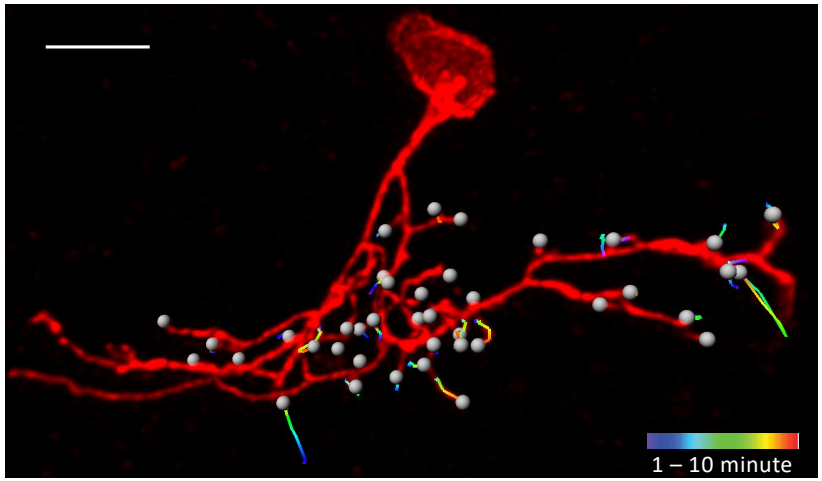

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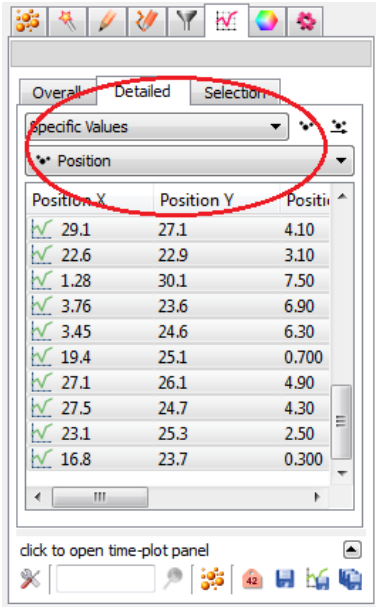


Figure 4

A

$$d(P_1, P_2) = (\sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2}$$

F3 =SQRT((A3-A2)^2+(B3-B2)^2+(C3-C2)^2)

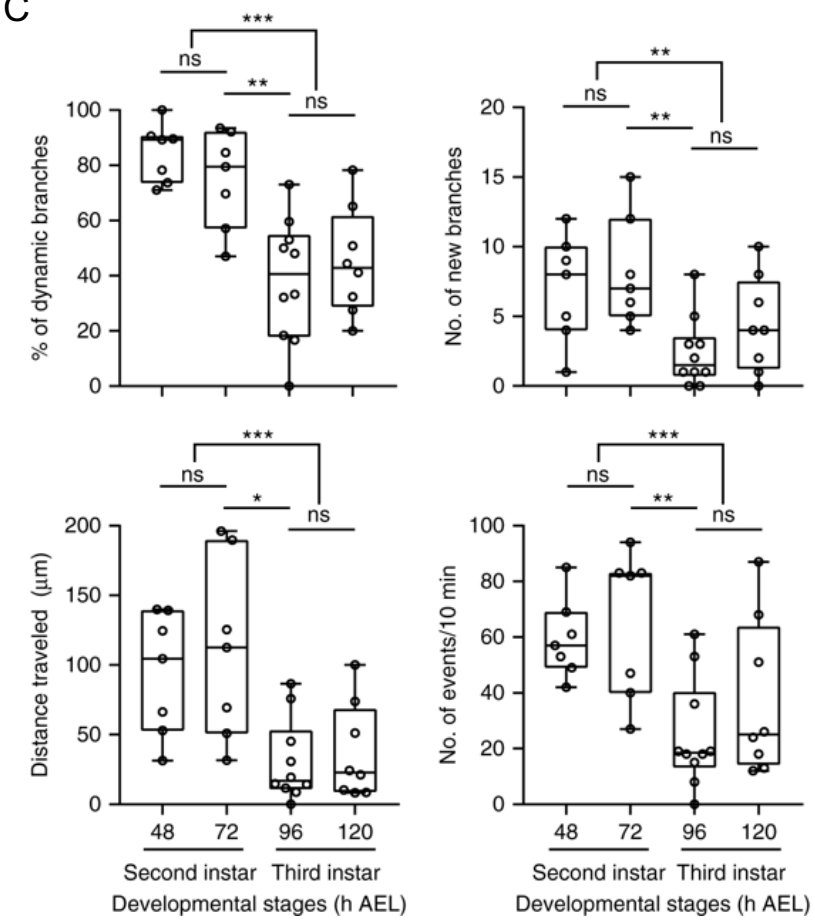
	A	B	C	D	E	F	G
1	Position.X	Position.Y	Position.Z	Time	TrackID	Distance	
2	20.7892	18.8049	4.3	1	1000000001		
3	20.7536	18.8001	4.1	2	1000000001	=SQRT((A3-A2)^2+(B3	

B

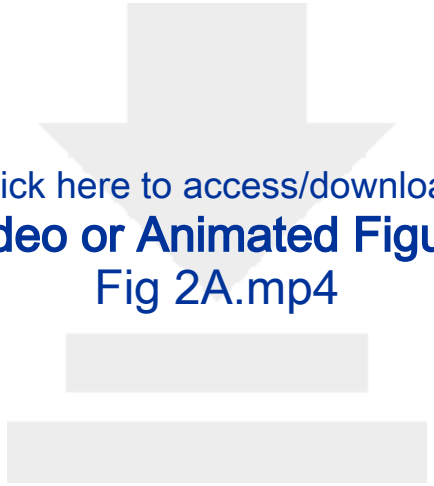
G13

	A	B	C	D	E	F	G
1	Position.X	Position.Y	Position.Z	Time	TrackID	Distance	Events
2	20.7892	18.8049	4.3	1	1000000001		
3	20.7536	18.8001	4.1	2	1000000001	0.00	
4	20.581	19.3623	4.1	3	1000000001	-0.59	
5	20.2981	19.9302	3.7	4	1000000001	-0.75	
6	20.1055	20.4558	3.7	5	1000000001	-0.56	
7	19.9979	20.8998	3.5	6	1000000001	-0.50	-2.40
8	19.9612	21.062	3.5	7	1000000001	0.00	
9	19.9001	21.3582	3.3	8	1000000001	-0.36	-0.36
10	19.9005	21.4975	3.3	9	1000000001	0.00	
11	19.7631	21.7145	3.1	10	1000000001	-0.33	-0.33

C



Supplementary video



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Video or Animated Figure

Fig 2A.mp4

Name of Material/ Equipment	Company
Chameleon Vision II multiphoton laser	Coherent
high vacuum grease	Dow Corning
LSM 780 two-photon laser scanning confocal microscope	Carl Zeiss
Microscope Cover Glass	Fisher Scientific
Superfrost Plus Microscope Slides	Fisher Scientific
Software	
Excel	Microsoft
Huygens Professional	Scientific Volume Imaging
Imaris	Oxford Instruments
Reagents	
Glucose	
HEPES	
KCl	
$MgCl_2$	
NaCl	
$NaHCO_3$	
PBS	
Sucrose	
TES	

Catalog Number	Comments/Description
79751-30	
	upright configuration
12-544-E	
12-550-15	
	for processing .csv files
	for drift correction and deconvolution
	for 3D visualization and image annotatior

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tel. 617.945.9051
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Title of Article:	Time-lapse live imaging and quantification of fast dendritic branch dynamics in developing Drosophila neurons.
Author(s):	Chengyu Sheng, Uzma Javed, Jun Yin, Bo Qin & Quan Yuan

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CORRESPONDING AUTHOR

Name:	Quan Yuan
Department:	Dendrite Morphogenesis and Plasticity Unit
Institution:	National Institute of Neurological Disorders and Stroke, NIH
Title:	Investigator
Signature:	Quan Yuan
Date:	5/17/2019

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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.

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

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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.

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Supplemental Coding Files

batch column filtering.R

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