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Quantitative Analysis of Neuronal Dendritic Arborization Complexity in Drosophila

--Manuscript Draft--

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	Rudolph E. Tanzi
Author Comments:	57139_R2 Rebuttal Letter Dear Editors: Thank you for considering our manuscript titled "Quantitative Analysis of Neuronal Dendritic Arborization Complexity in Drosophila" for publication in Journal of Visualized Experiments (JoVE). We would like to thank you for a prompt and comprehensive review. We would also like to thank you for very helpful comments on our manuscript. Below is a point-by-point summary of how we have addressed the Editorial s concerns and revised the manuscript, accordingly. The JoVE Scientific Review Editor's Comments: Comment [A1]: Please thoroughly revise your manuscript for grammar. Several sentences cannot be understood. Revisions: We have checked grammar and corrected errors accordingly. Comment [A2]: Which tissues? In 3.5 the body was flattened but no tissues were extracted. Presumably the brain is extracted and then sectioned. Please describe all tissue processing steps to ensure proper imaging. Revisions: "tissue" is removed. Changed to "larva body wall". Comment [A3]: Mention all the necessary microscopy settings, e.g. excitation and emission wavelengths. How are the various da neuron classes identified? Revisions: Follow instructions for imaging of confocal microscope according to the laboratory's specific system. We deleted subtitle 4 due to the set-ups of confocal microscope vary in different labs. Please see the change in 4 (deleted). Imaging processing. We used the da-neuron specific GAL4 lines. The GFP was co- expressed in class IV da neurons specifically, which can be visualized directly by microscope. Comment [A4]: Are the images processed at all before this step? Is any window and leveling performed? How do you determine the ends of the dendrites? Revisions: These were the Z-projection images from confocal microscope and can be directly analyzed by tracing neurites. There might be some short branches cut off because of the limited number of Z-projection images. This method was the relative measurement of dendrites length compare to the controls. So we chose the endpoints of the selected path in the image visuals. Comment [A5]: Unclear what this means. Revisions: The sentence was reworded to "the dendrite path was traced until the

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Question	**Response**

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One Alewife Center, Suite 200
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Dear Editors:

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Best regards

Sincerely,

Airong Li, MD PhD
Assistant Professor of Neurology

TITLE:

Quantitative Analysis of Neuronal Dendritic Arborization Complexity in *Drosophila*

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KEYWORDS:

Drosophila, Dendritic Arborization, Neuron, Dendrite Complexity, Development, Quantitative Analysis

SHORT ABSTRACT:

This protocol focuses on quantitative analysis of neuronal dendritic arborization complexity (NDAC) in *Drosophila*, which can be used for studies of dendritic morphogenesis.

LONG ABSTRACT:

Dendrites are the branched projections of a neuron, and dendritic morphology reflects synaptic organization during the development of the nervous system. *Drosophila* larval neuronal dendritic arborization (da) is an ideal model for studying morphogenesis of neural dendrites and gene function in the development of nervous system. There are four classes of da neurons. Class IV is the most complex with a branching pattern that covers almost the entire area of the larval body wall. We have previously characterized the effect of silencing the *Drosophila* ortholog of *SOX5* on class IV neuronal dendritic arborization complexity (NDAC) using four parameters: the length of dendrites, the surface area of dendrite coverage, the total number of branches, and the branching structure. This protocol presents the workflow of NDAC quantitative analysis, consisting of larval dissection, confocal microscopy, and image analysis procedures using ImageJ software. Further insight into da neuronal development and its underlying mechanisms will

improve the understanding of neuronal function and provide clues about the fundamental causes of neurological and neurodevelopmental disorders.

INTRODUCTION:

Dendrites, which are the branched projections of a neuron, cover the field that encompasses the neuron's sensory and synaptic inputs from other neurons[1,2]. Dendrites are an important component of synapse formation and play a critical role in integrating synaptic inputs, as well as propagating the electrochemical stimulation in a neuron. Dendritic arborization (da) is a process by which neurons form new dendritic trees and branches to create new synapses. The development and morphology of da, such as branch density and grouping patterns, result from multi-step biological processes and are highly correlated to neuronal function. The goal of this protocol is to provide a method for quantitative analysis of neuronal dendritric arborization complexity in *Drosophila.*

The complexity of dendrites determines the synaptic types, connectivity, and inputs from partner neurons. Branching patterns and the density of dendrites are involved in processing the signals that converge onto the dendritic field[3,4]. Dendrites have the flexibility for adjustment in development. For instance, synaptic signaling has an effect on dendrite organization in the somatosensory neuron during the developmental phase and in the mature nervous system[5]. The establishment of neuronal connectivity relies on the morphogenesis and maturation of dendrites. Malformation of dendrites is associated with impaired neuronal function. Studies have shown that the abnormality of da neuron morphogenesis might contribute to the etiologies of multiple neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Lou Gehrig's disease/Amyotrophic lateral sclerosis (ALS)[6-8]. Synaptic alterations appear in the early stage of AD, in concert with the decline and impairment of neuron function[7,8]. However, the specifics of how dendrite pathology contributes to pathogenesis in these neurodegenerative diseases remains elusive.

The development of dendrites is regulated by genes that encode a complex network of regulators, such as the Wnt family of proteins[9,10], transcription factors, and ligands on cell surface receptors[11,12]. *Drosophila* da neurons consist of four classes (Class I, II III, IV), of which class IV da neurons have the most complex branching patterns and have been employed as a powerful experimental system for better understanding morphogenesis[13,14]. During early morphogenesis, overexpression and/or RNAi silencing of genes in class IV da neurons result in changes in branching patterns and dendrite pruning[13]. It is important to develop a practical method for quantitative analysis of the neuronal dendritic arborization.

We have previously shown that silencing of the *Drosophila* ortholog of *SOX5*, *Sox102F,* led to shorter dendrites of da neurons and reduced complexity in class IV da neurons[15]. Here, we present the procedure of quantitative analysis for the neuronal dendritic arborization complexity (NDAC) in *Drosophila*. This protocol, adapted from the previous described methodology, provides a brief method to assay the development of da sensory neurons. It illustrates the robust image

labeling and the da neuron in the third instar larval body wall[16-19]. It is a valuable protocol for researchers who wish to investigate the NDAC and developmental differences *in vivo*.

PROTOCOL:

1. Experimental Preparation

1.1. Prepare the following reagents: Dulbecco's phosphate buffered saline (PBS); Triton X-100; 0.2% PBST (PBS + 0.2% Triton X-100); 32% paraformaldehyde (PFA), diluted into 4% before use; silicone elastomer base and curing agent; antifade mounting medium (*e.g.*, ProLong Gold); and fingernail polish.

1.2. Prepare the following equipment: dissection microscope, two sharp forceps and a pair of scissors for microdissection, a number of pins for microdissection, a Petri dish for making the dissection dish, microscope slides and coverslips, a confocal microscope, and a computer with Fiji ImageJ software installed.

2. Larvae Collection

2.1. Mate UAS-Sox102F-RNAi flies with UAS-GFP, ppk-GAL4, or W^{1118} wild type flies, respectively. Culture flies in standard conditions at 25 °C.

2.2. In ~5 - 6 days, collect the third instar larvae of UAS-Sox102F-RNAi/ ppk-GAL4, UAS-GFP or UAS-GFP, and ppk-GAL4 /+ control carefully with forceps for dissection.

3. Dissection of Larvae

Note: All procedures in this section are operated under a microdissection microscope. The magnification is up to the investigators. Try to adjust to the optimal sight view. ~4 - 6X magnification is recommended.

3.1. Place a larva on a dissection dish made of silicone elastomer base and a tissue culture Petri dish.

3.2. Pin the larva mouth hook to allow the dorsal side to face up, and then pin the tail of larva. Place the dorsal side in the middle as much as possible to expose the midline, which will be the open-up marker.

3.3. Add 200 µL of PBS to the larva to maintain body moisture.

3.4. Make a cut with a pair of scissors along the dorsal midline between the two tracheas, from caudal to rostral. Make a small cut at each of the four corners of the larva body.

3.5. Place a pin at each of the four corners of the body so the body lies flat.

3.6. Fix the larva body wall in 4% PFA for 25 min at room temperature.

3.7. Wash in PBST for 5 min. Repeat twice more.

3.8. Remove the pins from the tissue. Then transfer the tissue onto a glass slide, cover with antifade mounting medium, and mount with a cover slip. Air dry for 1 h and seal the edges with fingernail polish.

4. Imaging Processing

Note: Images were taken using an inverted confocal microscope system. The user can photograph the sample using a 20X objective (recommended).

4.1. Capture Z-series images. Open the "Capture Z-series" dialog box in the confocal microscope software. Determine the range for capturing the Z series images.

4.1.1. Click the "Top and Bottom" button. Determine the top and bottom position and input the values to "Bottom:" and "Top:" boxes. Set the "Step" in "Capture Z-series" dialog box as 0.5 µm. Then click the "Run now" button to acquire the Z-series images.

4.2. Save the obtained images as *.tiff or *.nd2 files.

4.3. Store the slides in a dark holder at 4 °C after imaging.

5. Dendrite Evaluation

Note: GFP protein was co-overexpressed in UAS-GFP and ppk-GAL4 flies in the da neurons for GFP fluorescence imaging analysis. The length, branching, and structure of da neurons in the third instar larvae were quantified. Analysis parameters include length of dendrites (µm), surface area (μm^2), total number of branches, and branching structure (%). **Figure 1** shows the analysis parameters in detail.

5.1. Length of Dendrites

Note: The length of dendrites is the sum of all dendrites labeled in tracer plugin.

5.1.1. Setup and run Fiji ImageJ (https://fiji.sc/)[20] software. Then split images into separate channels, if there are several channels of images.

Note: The image in this protocol only has one channel of GFP.

5.1.2. Run “Image | Stacks | Z project” to get a Z projection; a new window will appear with the name ‘Z-projection’. Click "max intensity" for projection type, and organize numbers between start slice and stop slice depending upon individual preferences. Click “OK”. A Z-projection image will appear presenting the dendrite projection clearly.

5.1.3. Trace the neurites.

5.1.3.1. Select “Plugins | Segmentation | Simple Neurites Tracer” in the window to trace the dendrites. Find the neuron soma, and then click one point at a dendrite starting from the cell body, and another point at the tip of this dendrite.

Note: The program will connect the two points with a blue line.

5.1.3.2. Press “Y” in the plug-in window if the path is clear; the dendrite path is traced until the endpoints of the selected path in the visible images. Click “complete path” on the same plugin window; the segment is completed (**Figure 2**).

Note: Some dendrites ended farther from the starting points, in which the pathway was divided into smaller segments. The segments were combined to provide a complete path for the tracer.

5.1.4. After a path is completed, go back to a new point where the branches are. The new path can be built on; the “All paths” window box will show the length values of all the paths (**Figure 3**). Save the tracing file, and continue with unfinished traces as shown in **Figure 2**.

5.1.5. Export the dendrite length data by clicking, “File | Export as *.cvs” in the ‘Plugin’ window. Then sum up all the path lengths, and export the data to a data analysis/spreadsheet software. (**Figure 3**).

5.2. Surface Area of the da Neurons

5.2.1. Select the Freehand drawing tool in the ‘Fiji ImageJ’ window. Trace the path, and then connect the endpoints. From the ‘Analyze’ menu, select “Set Measurements | Area”. Click “measure” from the ‘Analyze’ menu. The result appears in a new box with the value for the area selected (**Figure 4**). Copy it to the data analysis software.

5.3. Total Number of Branches and Branching Structure of the da Neurons

5.3.1. Calculate the total number of branches by opening “Analysis” and then “Render | Analyze Skeletonized Paths” in the plugin window of tracing (**Figure 5**).

Note: The structure of the arbor is the sum of the levels of branches. The path was defined between the cell body and the dendrite tips, and one path can include multiple branches, such as the primary arbors are the dendrites from the neuron cell body. The secondary arbors are

branches from the primary and so on with the tertiary, quaternary, and quinary arbors. The structure of dendrite branching is separated depending on the levels of branches. For instance, the primary % is the number of branches divided by total number of branching, and so on.

REPRESENTATIVE RESULTS:

The dendrites of da neurons were labeled by co-overexpressing GFP (UAS-GFP; ppk-GAL4) in the da neural soma and dendritic arbors for GFP fluorescence imaging analysis. The morphology of da neuron dendrites was imaged by an inverted confocal microscope (**Figure 2**).

The dendrites of da neurons were traced using Fiji ImageJ software. The file was used to estimate the dendrite length (**Figure 3**). Silencing of *Sox102F* in da neurons (N=21) (UAS-Sox102F-RNAi/ppk-GAL4; UAS-GFP) led to a significant reduction in the number of dendrites and a shorter branch length with a simpler structure compared to controls (N = 20) (UAS-GFP; ppk-GAL4 /+) in the third instar larva (**Figure 6**). Specifically, flies in which *Sox102F* was silenced exhibited a reduction in average dendrite length to 127 µm compared to 249 µm in controls ($p = 0.02$), and had a smaller average arbor surface area of 552,476 μm^2 compared to 847,571 μm^2 in controls ($p = 0.04$). In addition, silencing of *Sox102F* resulted in a smaller number of branches and a simpler branching structure of arbors with total branches of 17 (17.3 ± 6.7), compared with 28 (28.4 ± 9.5) in controls ($p = 0.04$). Student t-tests were performed to compare the differences between the groups, and the significance level was set to 0.05.

FIGURE LEGENDS:

Figure 1: **Schematics of dendrite analysis parameters of *Drosophila* da neuron.** Panel **(A)** is the original image of a da neuron. **(B)** Dendrite length was the average of all dendrite lengths in all measured da neurons. **(C)** The surface area of da neuron dendrite was manually defined by the ImageJ freehand tool. **(D)** This schematic shows how to count the total number of branches and analyze branching structure of a da neuron. 1: Primary arbor. 2: Secondary arbor. 3: Tertiary arbor. 4: Quaternary arbor.

Figure 2: Representative tracing of the dendrites of a da neuron. A da neuron was imaged with a confocal fluorescence microscope system. **(A)** The first tracing was created with a start point from the cell body and the end point of a dendrite by using the plugin/segmentation tool. The blue line (white arrow) represents the path defined. After clicking “Yes” (red arrow), the line changes color from blue to red. **(B)** Clicking on “Finish Path” it will appear purple **(C)**. **(A-C)** Shows the process of tracing a single dendrite; **(D)** The complete traced image.

Figure 3: Measurement of traced paths. The image on the left shows how to measure the trace paths after running the “Analysis |Measure Paths”. Export path length values into a spreadsheet file, and calculate the sum of the length.

Figure 4: An example of defining the dendrite surface area manually. A defined image was obtained by using the freehand tool in the ImageJ window menu (shown by red arrow). Set the

measurements by selecting Area. Run the "Measure" function to obtain the area shown in the red box.

Figure 5: An example of analysis of branching. Run "Analysis_Render | Analyze Skeletonized Paths" in the "Segmentation" plugin window. The rendered paths and their number were obtained by selecting "Render all paths | Obtain summary". The path was defined between the cell body and the dendrite tips, and one path can include multiple branches; for example, the primary arbors were the dendrites from the neuron cell body; the secondary arbors were branches from the primary and so on with the tertiary, quaternary, and quinary arbors. The structure of dendrite branching was separated depending on the levels of branches. For instance, primary % was the number of branches divided by total number of branching, and so on.

Figure 6: Representative results of silencing of *Sox102F* in da neurons. Silencing of *Sox102F* in da neurons (UAS-Sox102F-RNAi/UAS-GFP; ppk-GAL4) led to significantly reduced dendrite length and shorter branching with simpler structure in the third instar larva compared to controls. The differences between Sox102F-RNAi flies (UAS-Sox102F-RNAi/UAS-GFP; ppk-GAL4, N = 21) and control (UAS-GFP;ppk-GAL4/+, N = 20) flies were on (**A**) dendrite length (**B**) arbor surface area (**C**) total number of branches, and (**D, E**) branching structure of the da neurons. Student T-test was perfomed for statistical analysis. * indicates statistical significance ($p < 0.05$). The error bars are mean ± standard deviation.

DISCUSSION:

Dendrites that innervate the epidermis are the input regions of neurons, and their morphologies determine how information is received and processed by individual neurons. Development dendrite morphology reflects gene modulation of dendrite organization. The *Drosophila* larval da neuron of the peripheral nervous system is an important model for studying dendrite development because of: 1) the functional similarity with mammals[11,12]; 2) four class distinctions based on dendrite structure[11,12]; and 3) the genetic factors that regulate morphogenesis. In this protocol, we present the workflow from larvae preparation to image analysis of *Drosophila* da neurons. The methods describe the four important parameters for analyzing dendrites in da neurons in detail, which are the length of dendrites, the surface area, the total numbers of branches, and the branching structure. The critical step was to remove as many tissues from the larva body wall as possible to fully expose the da neurons for imaging and analyses. There might be some short branches cut off because of the limited number of Z-projection images. As this method is the relative measurement of dendrites length in comparison with the controls, a large number of images for each group of da neurons should be taken to increase the coverage areas of Z-projection images.

Drosophila class IV da neurons have been the focus of research concerning dendrite arbor morphology[15,21-23]. Morphogenesis of dendritic arbor development might be disrupted by loss or gain of gene function, demonstrating that da neuron development is sensitive to genetic changes[24]. For genetic studies, it is important to analyze morphology accurately in order to understand its impact on da neurons. Class IV da neurons are complex but still accessible for high-

quality imaging because they are located just beneath the semi-transparent body wall and branch, almost entirely in two dimensional space. The dynamic GFP fluorescence labeled da neurons (ppk-GAL4; UAS-GFP) provide a readily visualized model to investigate neuronal development. The direction of morphology change varies, arbors can become overbranched or simplified. Here, we categorized these morphological changes by quantitive parameters, *e.g.* the dendrite length, the surface area, the total numbers of branches, and the branching structure of da neurons. The results reflect the response in *Sox102F* expression regulating neuronal development, as shown in this study.

Our protocol was used to show the morphological changes of class IV da neurons in flies in which the *Drosophila* ortholog of *SOX5*, *Sox102F,* was silenced, indicating an important functional role of *SOX5* in dendrite development and morphogenesis. The Wnt proteins are a family of secreted glycoproteins that have been implicated in regulating dendrite morphogeneis. Wnt2 and Wnt7b promote da and neuronal complexity[9,10]. In the Wnt canonical pathway, this activation is followed by activation of GSK3β, a serine-threonine kinase, which in turn activates β-catenin mediated transcription[10,25]. We have previously found that silencing of *SOX5* in human SH-SY5Y neuroblastoma cells resulted in a significant repression of Wnt signaling activity and regulated the expression of a number of Wnt genes including an overexpression of *GSK3*β[15]. Increased expression of *GSK3*β has been associated with the hallmark characteristics of AD, including memory loss, β-amyloid plagues, and abnormal hyperphosphorylation of tau[26]. Silencing of *SOX5* increased *GSK3*β expression*,* which might indicate a functional link between *SOX5* and *GSK3*β. The Class IV da neurons have the most highly complex dendrites of all sensory neurons.

In this protocol, we have developed a method to analyze the complexity of arbor and branches to abstract the properties of class IV da neurons in *Drosophila* based on conventionally available plugins and software. Images that were taken from a confocal microscope were analyzed, and the plugin segmentation of a simple neurite tracer provided an ideal tool to trace the dendrite branches[27,28]. In addition, this quantification method allows for the detailed analysis of dendrite complexity by presenting the ratios of various levels of branching from the soma to the more distant dendrite. Moreover, this method might be used to analyze other classes of da neurons. For instance, class I da neurons extend secondary dendrites to one side of the body wall; class II has bifurcating branches symmetrically; and class III da neurons have more complexity of branching and spikes. In summary, we have provided a protocol of imaging and analysis of class IV da neurons for neuronal dendrite analyses in *Drosophila*.

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DISCLOSURES:
The authors have nothing to disclose.

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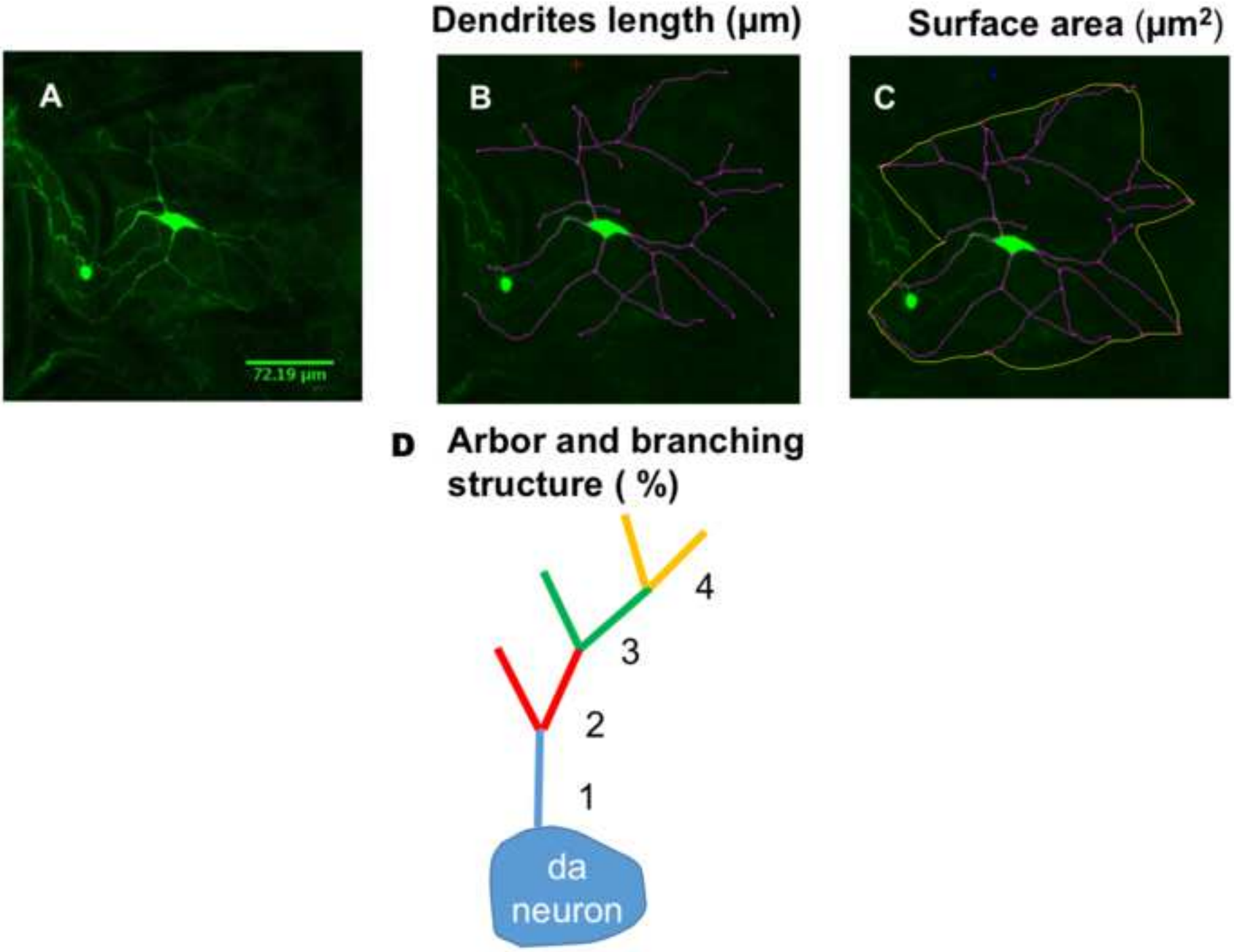

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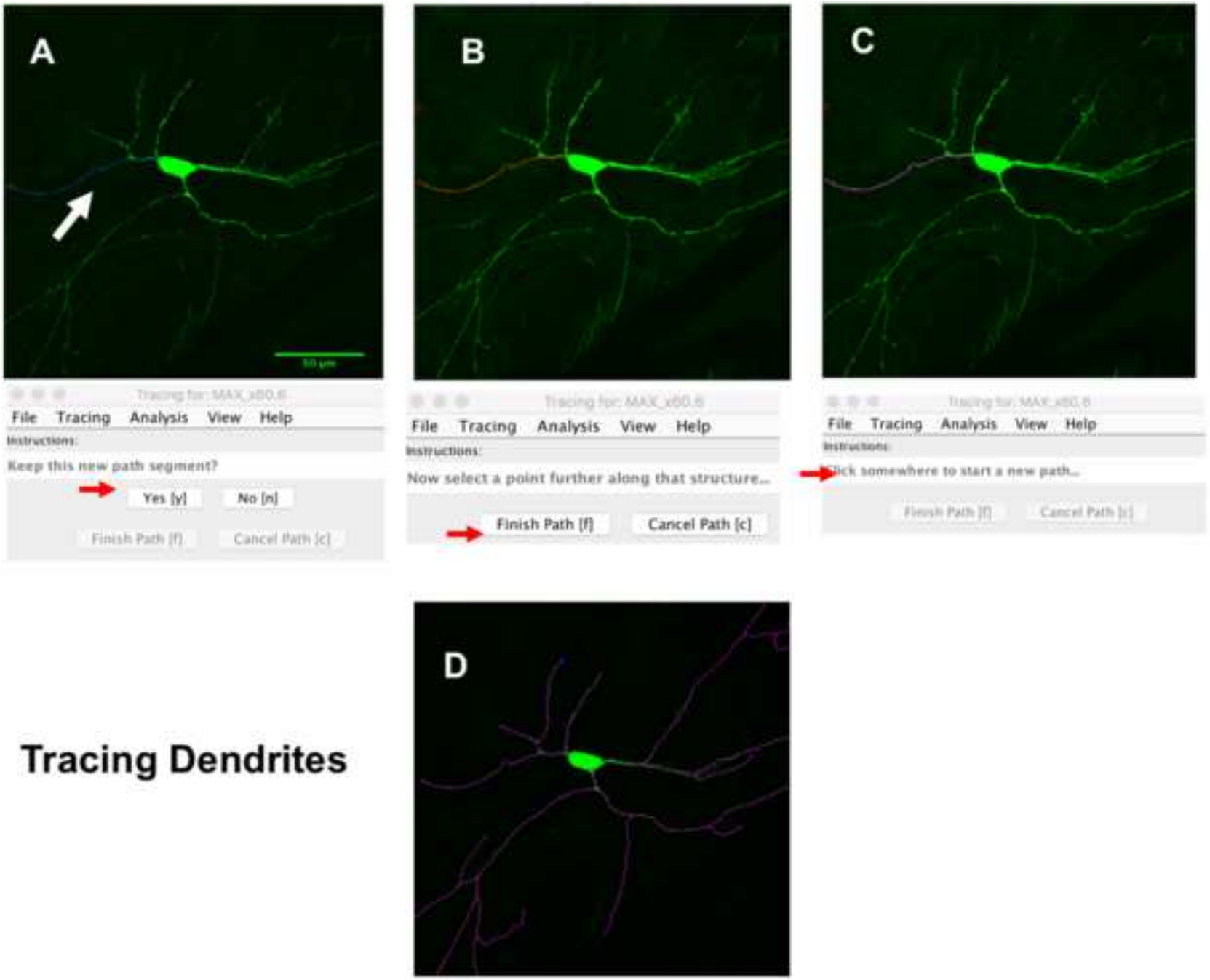

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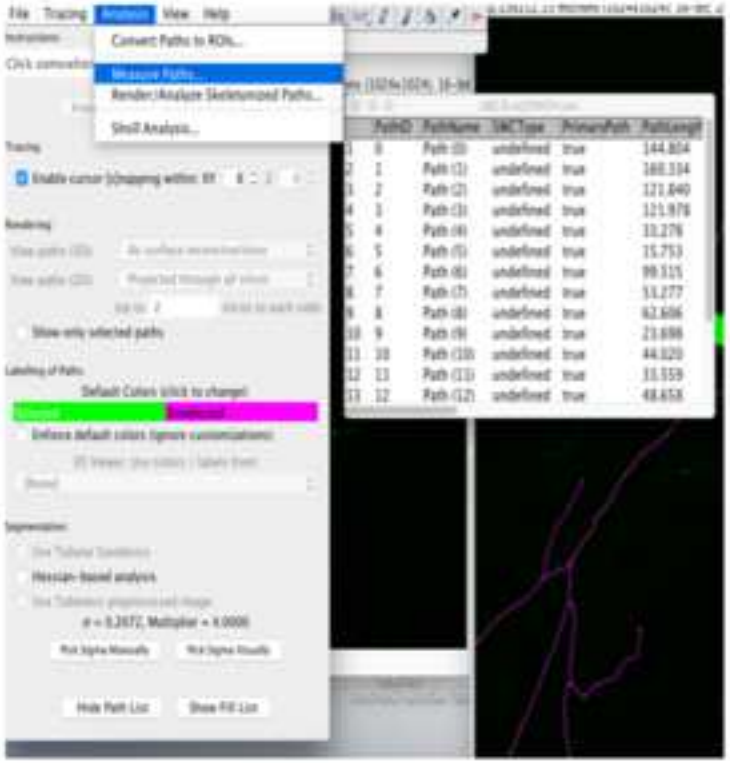
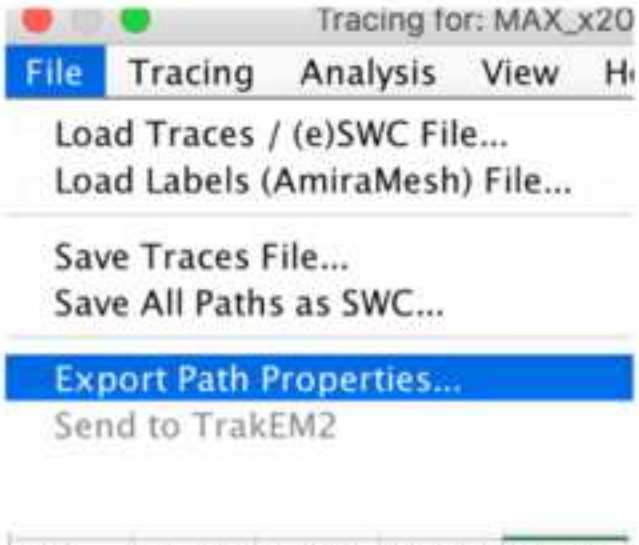
- **Caculate Average length**





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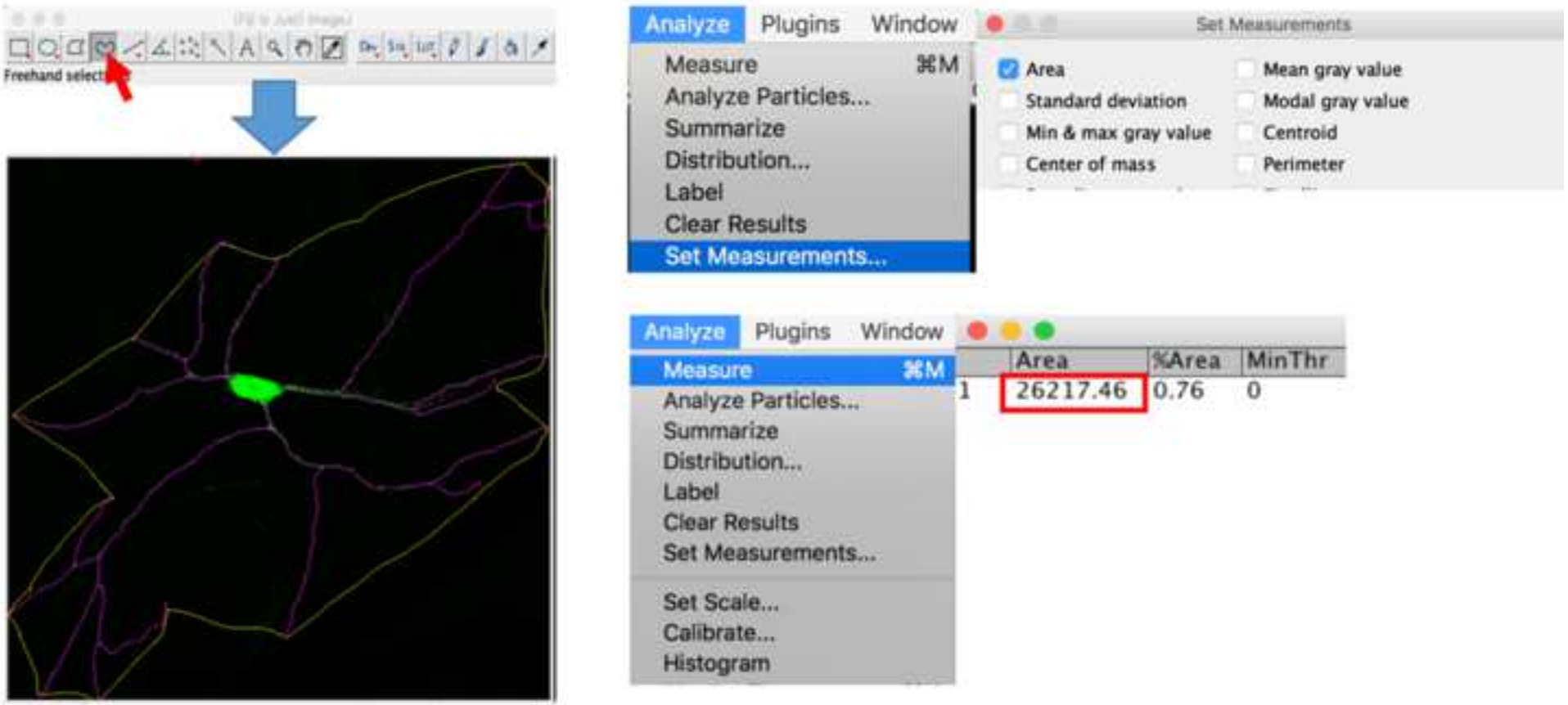

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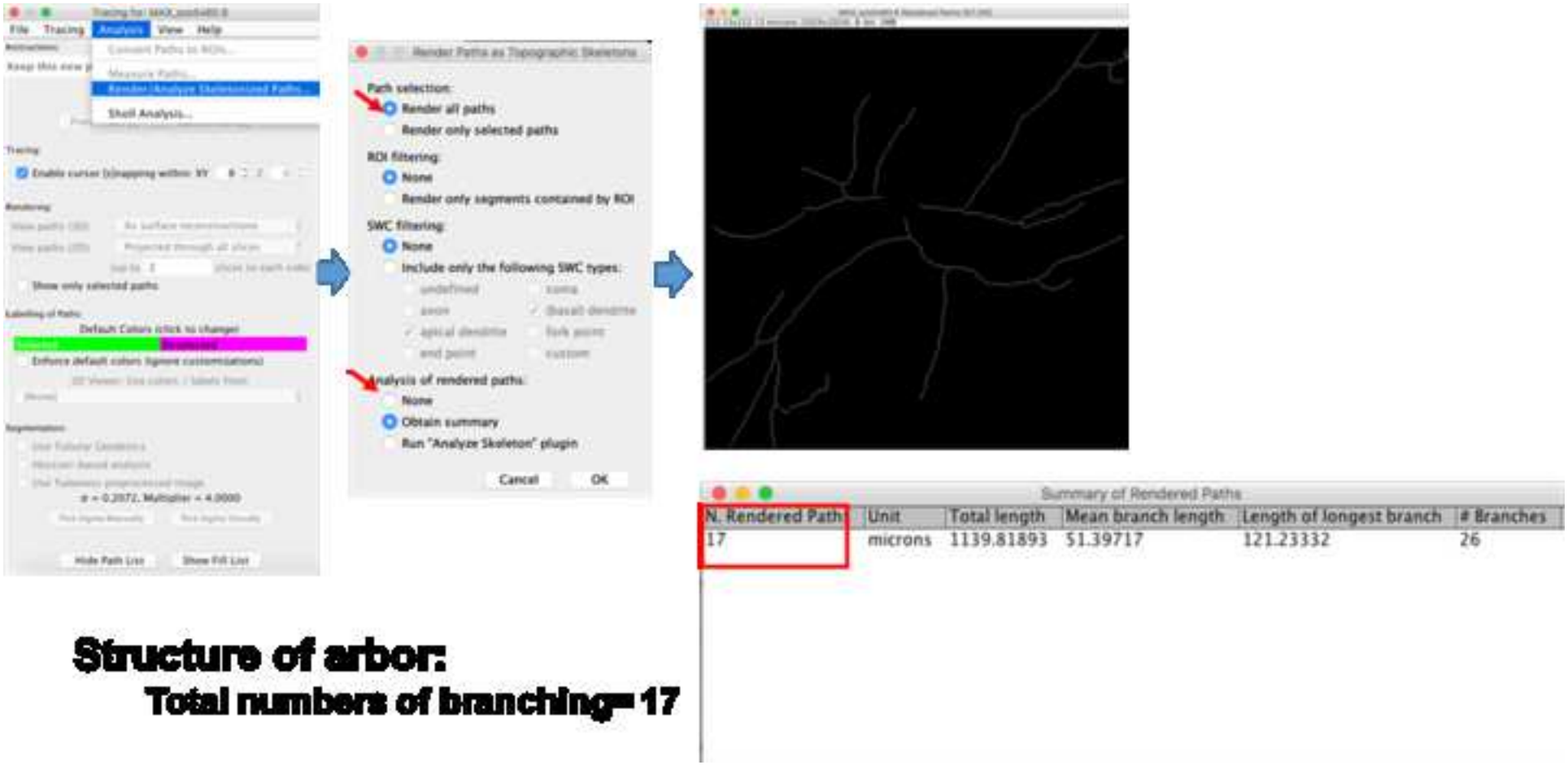

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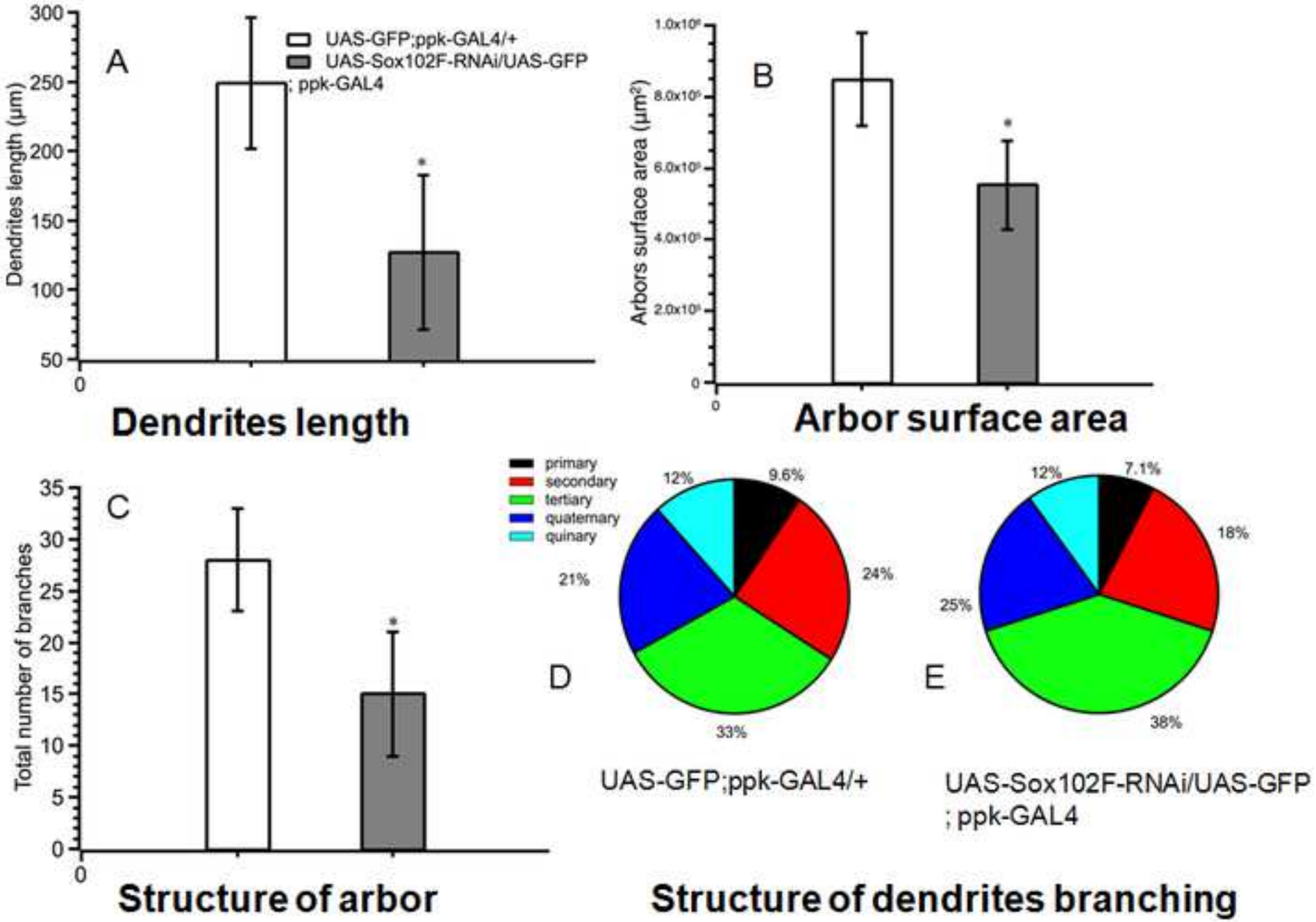

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TritonX-100	Fisher Scientific	9002-93-1
Paraformaldehyde(PFA)	Electron Microscopy Sciences	15714-S
Sylgard 184 silicone elastomer base and curing agent	Dow Corning Corportation	3097366-0516;3097358-1004
ProLong Gold Antifade Mountant	Thermo Fisher Scientific	P36931
Fingernail polish	CVS	72180
Stereo microscope	Nikon	SMZ800
Confocal microscope	Nikon	Eclipse Ti-E
Petri dish	Falcon	353001
Forceps	Dumont	11255-20
Scissors	Roboz Surgical Instrument Co	RS-5611
Insect Pins	Roboz Surgical Instrument Co	RS-6082-25
Microscope slides and cover slips	Fisher Scientific	15-188-52

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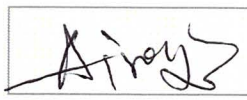
Institution: Massachusetts General Hospital, MassGeneral Institute of Neurodegenerative Disease

Article Title: Quantitative Analysis of Neuronal Dendritic Arborization Complexity in Drosophila

Signature:  Date: 8/3/17

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57139_R2 Rebuttal Letter

Dear Editors:

Thank you for considering our manuscript titled "Quantitative Analysis of Neuronal Dendritric Arborization Complexity in Drosophila" for publication in *Journal of Visualized Experiments (JoVE).* We would like to thank you for a prompt and comprehensive review. We would also like to thank you for very helpful comments on our manuscript.

Below is a point-by-point summary of how we have addressed the Editorial s concerns and revised the manuscript, accordingly.

The JoVE Scientific Review Editor's Comments:

Comment [A1]: Please thoroughly revise your manuscript for grammar. Several sentences cannot be understood.
Revisions: We have checked grammar and corrected errors accordingly.

Comment [A2]: Which tissues? In 3.5 the body was flattened but no tissues were extracted. Presumably the brain is extracted and then sectioned. Please describe all tissue processing steps to ensure proper imaging.
Revisions: "tissue" is removed. Changed to "larva body wall".

Comment [A3]: Mention all the necessary microscopy settings, e.g. excitation and emission wavelengths. How are the various da neuron classes identified?
Revisions: Follow instructions for imaging of confocal microscope according to the laboratory's specific system.
We deleted subtitle 4 due to the set-ups of confocal microscope vary in different labs. Please see the change in 4 (deleted). Imaging processing.
We used the da-neuron specific GAL4 lines. The GFP was co- expressed in class IV da neurons specifically, which can be visualized directly by microscope.

Comment [A4]: Are the images processed at all before this step? Is any window and leveling performed? How do you determine the ends of the dendrites?
Revisions: These were the Z-projection images from confocal microscope and can be directly analyzed by tracing neurites. There might be some short branches cut off because of the limited number of Z-projection images. This method was the relative measurement of dendrites length compare to the controls. So we chose the endpoints of the selected path in the image visuals.

Comment [A5]: Unclear what this means.
Revisions: The sentence was reworded to "the dendrite path was traced until the endpoints of the selected path in the visible images". Added a new sentence "Some dendrites were ended farther from the starting points, in which the pathway was divided into smaller segments. The

segments were combined to provide a completed path for the tracer".

Comment [A6]: How do you determine the tip ends consistently?
Revisions: The dendrite path was traced until the endpoints of the selected path in the visible image. Some dendrites were ended farther from the starting points, in which the pathway was divided into smaller segments. The segments were combined to provide a completed path for the tracer. After tracing path, the endpoints were connected by the Freehand selections in Fiji ImageJ program window.

Comment [A7]: How is a path defined? Is it the length between two nodes? OR is it the path between the cell body and the dendrite tips meaning that 1 path can include multiple branches?
Revisions: The path was defined between the cell body and the dendrite tips and one path can include multiple branches, such as the primary arbors are the dendrites from the neuron cell body; the secondary arbors were branches from the primary and so on with the tertiary, quaternary and quinary arbors. The structure of dendrite branching was separated depending on the levels of branches. For instance, primary% was the number of branches divided by total number of branching, and so on.

Comment [A8]: Please mention all statistical test performed and sample sizes. Revisions: Added statistical test performed and sample sizes.

Comment [A9]: Please mention all statistical test performed and sample sizes. Revisions: Added statistical test performed and sample sizes.

Comment [A10]: Were all estimates based on just one sample per cohort? This study is unacceptable if that is the case and given the major concerns expressed by the peer reviewers we will not be able to accept this. If more than one samples were used please report the standard deviations.
Revisions: Group samples were tested. Sample size and the standard deviations were added.

Comment [A11]: Were all estimates based on just one sample per cohort? This study is unacceptable if that is the case and given the major concerns expressed by the peer reviewers we will not be able to accept this. If more than one samples were used please report the standard deviations.
Revisions: Group samples were tested. Sample size and the standard deviations were added.

Comment [A12]: Please mention all statistical test performed and sample sizes. Revisions: added statistical test performed and sample sizes.

Comment [A13]: Unclear what this means.
Revisions: Dendrite length was the average of all dendrite lengths in all measured da neuron.

Comment [A14]: Per neuron?

Revisions: Yes, per neuron.

Comment [A15]: Dendrite length per neuron?
Revisions: No. Dendrite length was the average of all dendrite lengths in all measured da neurons.

Comment [A16]: Define 1,2,3,4 here.
Revisions: 1. Primary arbor. 2. Secondary arbor. 3. Tertiary arbor. 4. Quaternary arbor.

Comment [A17]: Please use an arrow to point to it. The low line weight makes it hard to see.
Revisions: An arrow is added.

Comment [A18]: Please remove the tradename Excel from the figure.
Revisions: "Excel" is removed.

Comment [A19]: How is a path defined? Is it the length between two nodes? OR is it the path between the cell body and the dendrite tips meaning that 1 path can include multiple branches?
Revisions: The path was defined between the cell body and the dendrite tips and one path can include multiple branches, such as the primary arbors were the dendrites from the neuron cell body; the secondary arbors were branches from the primary and so on with the tertiary, quaternary and quinary arbors. The structure of dendrite branching was separated depending on the levels of branches. For instance, primary% was the number of branches divided by total number of branching, and so on.

Comment [A20]: This isn't really a schematic. The information can simply be presented in the text somewhere. The only graphical component is already present in figure 1. Please consider deleting this figure.
Revisions: Fig 6 is deleted.

Comment [A21]: Please mention the sample sizes for each fly cohort.
Revisions: Sample size is added.

Comment [A22]: Define * here and mention the significance levels.
Revisions: added "$p<0.05$ was defined as the statistically significant".

Comment [A23]: Please thoroughly discuss the critical steps, troubleshooting, and limitations.
Revisions: The critical step was to remove as many tissues from larva body as possible to fully expose the da neurons for imaging and analyses. There might be some short branches cut off because of the limited number of Z-projection images. As this method is the relative measurement of dendrites length in comparison with the controls, a large number of images for each group of da neurons should be taken to increase coverage areas of Z-projection images.

Comment [A24]: Please cite a reference.

Revisions: References are added.

Comment [A25]: Please cite a reference.
Revisions: References are added.

Comment [A26]: Please define this.
Revisions: Confusing text was removed.

Comment [A27]: Unclear.
Revisions: Confusing text was removed.

Comment [A28]: needs a reference.
Revisions: Added references[15].

Comment [A29]: Unclear why the method was not applied to other classes of neurons and presented here along with results if you can claim that it "can" be applied. Revisions: Revised the sentence to remove unsupported claim.

Comment [A30]: Reference?
Revisions: Revised the sentence to remove unsupported claim.

We have uploaded the revised manuscript file in word format and the high quality versions of revised figures in tiff file format to ManuscriptCentral in accordance with the JoVE publication requirements during the resubmission process.

Thank you once again for a very helpful and comprehensive review. We believe that our revised manuscript has been improved with the Editors' suggestions and sincerely hope that it is now appropriate for publication in *JoVE.*