Video Article

Analyzing Dendritic Morphology in Columns and Layers

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Abstract

In many regions of the central nervous systems, such as the fly optic lobes and the vertebrate cortex, synaptic circuits are organized in layers and columns to facilitate brain wiring during development and information processing in developed animals. Postsynaptic neurons elaborate dendrites in type-specific patterns in specific layers to synapse with appropriate presynaptic terminals. The fly medulla neuropil is composed of 10 layers and about 750 columns; each column is innervated by dendrites of over 38 types of medulla neurons, which match with the axonal terminals of some 7 types of afferents in a type-specific fashion. This report details the procedures to image and analyze dendrites of medulla neurons. The workflow includes three sections: (i) the dual-view imaging section combines two confocal image stacks collected at orthogonal orientations into a high-resolution 3D image of dendrites; (ii) the dendrite tracing and registration section traces dendritic arbors in 3D and registers dendritic traces to the reference column array; (iii) the dendritic analysis section analyzes dendritic patterns with respect to columns and layers, including layer-specific termination and planar projection direction of dendritic arbors, and derives estimates of dendritic branching and termination frequencies. The protocols utilize custom plugins built on the open-source MIPAV (Medical Imaging Processing, Analysis, and Visualization) platform and custom toolboxes in the matrix laboratory language. Together, these protocols provide a complete workflow to analyze the dendritic routing of *Drosophila* medulla neurons in layers and columns, to identify cell types, and to determine defects in mutants.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55410/>

Introduction

During development, neurons elaborate dendrites in complex but stereotyped branched patterns to form synapses with their presynaptic partners. Dendritic branching patterns correlate with neuronal identity and functions. The locations of dendritic arbors determine the type of presynaptic inputs they receive, while the dendritic branching complexity and field sizes govern the input number. Thus, dendritic morphological properties are critical determinants for synaptic connectivity and neuronal computation. In many regions of complex brains, such as the fly optic lobes and the vertebrate retina, synaptic circuits are organized in columns and layers to facilitate information processing^{1,2}. In such a column and layer organization, presynaptic neurons of a distinct modality project axons to terminate at a specific layer (so-called layer-specific targeting) and to form an orderly two-dimensional array (so-called topographic map), while postsynaptic neurons extend dendrites of appropriate sizes in specific layers to receive presynaptic inputs of the correct types and numbers. While axonal targeting to layers and columns has been well studied^{3,4}, much less is known about how dendrites are routed to specific layers and expand appropriately sized receptive fields to form synaptic connections with the correct presynaptic partners⁵. The difficulty of imaging and quantifying dendritic targeting to layers and columns has hindered the study of dendritic development in columnar and laminated brain structures.

Drosophila medulla neurons are an ideal model for studying dendritic routing and circuit assembly in columns and layers. The fly medulla neuropil is organized as a 3D lattice of 10 layers and approximately 750 columns. Each column is innervated by a set of afferents, including photoreceptors R7/R8 and lamina neurons L1 - L5, whose axonal terminals form topographic maps in a layer-specific fashion⁶. About 38 types of medulla neurons are present in every medulla column and elaborate dendrites in specific layers and with appropriate field sizes to receive inputs from these afferents⁷. The synaptic circuits in the medulla have been reconstructed at the electron microscopic level; thus, the synaptic partnerships are well established^{7,8}. Furthermore, genetic tools for labeling various types of medulla neurons are available^{9,10,11}. By examining three types of transmedulla (Tm) neurons (Tm2, Tm9 and Tm20), we have previously identified two cell-type-specific dendritic attributes: (i) Tm neurons project dendrites in either the anterior or posterior direction (planar projection direction), depending on the cell types and (ii) dendrites of medulla neurons terminate in specific medulla layers in a cell-type-specific fashion (layer-specific termination)¹². Planar projection direction and layer-specific termination are sufficient to differentiate these three types of Tm neurons, while mutations that disrupt Tm responses to layer and column cues affect distinct aspects of these attributes.

Here, we present a complete workflow for examining the dendritic patterning of *Drosophila* medulla neurons in columns and layers (**Figure 1**). First, we show a dual-view imaging method, which uses customized software to combine two confocal image stacks to generate high-quality

isotropic images. This method requires only conventional confocal microscopy to generate high-quality images that allow for the reliable tracing of dendritic branches, without resorting to super-resolution microscopy, such as STED (Stimulated Emission Depletion) or structural illumination. Second, we present a method for tracing dendritic arbors and for registering the resulting neurite traces to a reference column array. Third, we show the computational methods for extracting information on the planar projection direction and layer-specific termination of dendrites, as well as for deriving estimates for dendritic branching and termination frequencies. Together, these methods allow for the characterization of dendritic patterns in 3D, the classification of cell types based on dendritic morphologies, and the identification of potential defects in mutants.

Protocol

Note: The protocol contains three sections: dual-view imaging (sections 1 - 3), dendritic tracing and registration (sections 4 - 6), and dendritic analysis (sections 7 - 9) (**Figure 1**). The codes and example files are provided in **Table of Materials/Equipment**.

1. Dual-image Acquisition

NOTE: This step is designed to acquire two image stacks of the neuron of interest in two orthogonal (horizontal and frontal) orientations.

1. Prepare fly brains that contain sparsely labeled medulla neurons (~10 cells/brain lobe) with a membrane GFP marker (mCD8GFP), as previously described¹². Stain the brain with rabbit anti-GFP (for medulla neuron dendrites) and mouse mAb24B10 (for photoreceptor axons), primary antibodies, and fluorescent secondary antibodies (Alexa 488 anti-rabbit and Alexa 568 anti-mouse antibodies), as described previously¹³. Clear the brain in 70% glycerol in 1x PBS.
2. To mount the brain in the horizontal orientation (**Figures 2A, B**), transfer the glycerol-cleared fly brain to a 20 μ L drop of antifade mounting medium in the center of a slide.
3. Attach small patches of clay at the 4 corners of the coverslip to prevent the coverslip from crushing the brain sample during mounting.
NOTE: The clay patches provide cushioning to prevent the coverslip from crushing the sample. Each clay patch should be about 1 mm in diameter.
4. Under a dissecting microscope, position the brains in the ventral-up position and place the coverslip on top to secure the brain. Use the convex dorsal surface of the brain as a landmark to identify the orientation of the brain sample (**Figure 2A**).
5. Obtain the first image stack (horizontal view) with a confocal microscope. Use a high-NA objective lens (such as a 63X 1.3 N.A. glycerol or oil immersion objective lens) and 2.5X digital zoom (the pixel size is 0.105 μ m per pixel; averaging number is 2). Acquire more than 180 optical sections (512 x 512 pixels) to cover the medulla neuropil with a step size of 0.2 μ m.
6. Remount the brain as described in steps 1.3 - 1.4, but align the brain in the anterior-up position (frontal view).
7. Acquire the second image stack (frontal view) of the same neuron, as described in step 1.5.
NOTE: Finding the same neuron might be challenging when there are numerous neurons labeled in the optic lobe. To identify the same neurons from both orientations, lower-magnification image stacks from both views might be required (for example, use a zoom of 0.7, and a step size of 0.45 μ m to acquire low-resolution image stacks). If the image larger than 512 x 512, the image should be cropped to 512 x 512 before image combination, and the pixel size should keep at 0.105 μ m per pixel while imaging the large field. Loss of signal is a potential problem for deep tissue. If the signal is weak, image the ventral half of the brain. To reduce photobleaching during scanning, use as low a laser power as possible. Use the range indicator to check for overexposure before acquiring image stacks. If possible, use a confocal microscope equipped with GaAsP detectors.
8. Identify and record the location of the neuron of interest with respect to the medulla neuropil (right/left [R/L] and dorsal/ventral [D/V]). Check if the sample moved during image acquisition by examining the image stack.
NOTE: Sample moving is often due to improper mounting. If sample movement occurs, the image stack cannot be used for registration and should be discarded. Re-mount the sample and acquire image stacks from the same neuron.

2. Image Deconvolution

NOTE: The deconvolution step uses image deconvolution software to restore the acquired images that are degraded by blurring and noise. While this step is optional, it significantly improves image quality. It is recommended to use deconvolved image stacks for image registration and combination in section 3.

1. Start the deconvolution program in the interactive mode. Load the image stack (in lsm or specific microscopic format) by choosing Menu:File/Open (or Ctrl-o) in the main window.
2. Click to select the loaded image stack and choose Menu:Ops to open the image operation window. Use the default Classic Maximum Likelihood Estimation (CMLE) algorithm.
3. In the image operation window, click the "Parameters" tab. Enter the appropriate parameters for the lens immersion medium (e.g., oil, glycerin, etc.), embedding medium (e.g., immersion oil, etc.), and numerical aperture (NA; here, 1.3 was used). Check the remaining parameters to make sure that they correctly reflect the imaging conditions. Click the "Set all verified" tab to finalize the parameter settings.
4. In the image operation window, click the "Operation" tab. Assign an output destination (e.g., c). Enter appropriate numbers in "Signal/Noise per channel" (e.g., "12 12 12 12" is a good starting point, while the default setting is "20 20 20 20"). Use default settings for the remaining parameters.
5. Click the "Run Command" tab to start deconvoluting the image stack; this process could take up to tens of minutes to complete, depending on the computer.
6. In the main window, click and select the deconvolved image stack. Choose Menu:Save As to save the deconvolved image in the ICS image file format (.ics and .ids).

NOTE: Each image stack has two files: the ics file contains the header information and the ids file contains the raw image information.

1. Rename the image stack files according to the imaging orientation (e.g., name the horizontal-view image stacks H.ids and H.ics and the frontal-view image stack F.ids and F.ics).

3. Dual-view Image Combination

Note: This step combines two image stacks to generate high-resolution 3D images using the MIPAV software.

1. Generating matrices for image combination.
 1. Start the MIPAV program. Load the H and F image stacks by choosing Menu:File/Open image (A) from Disk (or Ctrl-f) /H.ids and F.ids; the window will show two images.
 2. Select the H image by clicking the image and choose Menu:Utilities/Conversion Tools/RGB/Grays; the window will show GrayG, GrayB, and GrayR images.
 3. Close GrayR and GrayB, only keeping GrayG on the window.
 4. Select the F image by clicking the image and choose Menu:Utilities/Conversion Tools/RGB/Grays. The window will show GrayG1, GrayB1, and GrayR1 images.
 5. Close GrayR1 and GrayB1 and keep only GrayG1 on the window. At this step, only GrayG and GrayG1 are on the window.
 6. Select the GrayG (highlight), choose Menu:Algorithms/Registration/Optimized Automatic Image Registration; the "Optimized Automatic Image Registration 3D" dialog box will pop up.
 1. In the Input Options, change the "Degrees of freedom" from default "Affine-12" to "Specific rescale-9." In "Rotations," key in -105 to 105 in the "Rotation angle sampling range" (default: -30 to 30 degrees), 10 in the "Coarse angle increment" (default: 15 degrees), and 3 in the "Fine angle increment" (default: 6 degrees).
 7. Click OK; the first Matrix "GrayG_To_GrayG1.mtx" will be generated and saved in the image folder. Close all the image windows and proceed to the next step; this step will take at least 15 min.
 8. Load the H and F image stacks by choosing Menu:File/Open image (A) from Disk (or Ctrl-f) /H.ids and F.ids, as in step 3.1.1.
 9. Select the H image by clicking the image and choose Menu:Utilities/Conversion Tools/RGB/Gray; the "RGB->Gray" dialog box will pop up. Click OK; the window will show "HGray" images. Keep HGray and close the H image.
 10. Repeat step 3.1.9 for the F image; the "FGray" images will appear on the window. Keep FGray and close the F image; only HGray and FGray will be left on the window.
 11. Select the HGray image (highlight) and go to Menu:Algorithms/Transformation tools/Transform. The "Transform/Resample Image" dialog box will pop up. Click the "Resample" tab and change the resample to size of "HGray" to "FGray". Next, click the "Transform" tab and load "GrayG_To_GrayG1.mtx" by selecting the "Read matrix from file." Click OK; the window will show the HGray_transform image. Close the HGray image so only HGray_transform and FGray are left on the window.
 12. Select the "HGray_transform" image (highlight) and go to Menu:Algorithms/Registration/Optimized Automatic Image Registration. The "Optimized Automatic Image Registration 3D" dialog box will pop up. In "Rotations," key in -5 to 5 in the "Rotation angle sampling range" (default: -30 to 30 degrees), 3 in the "Coarse angle increment" (default: 15 degrees), and 1 in the "Fine angle increment" (default: 6 degrees). Click OK.
 NOTE: The Affine Matrix (HGray_transform_To_FGray.mtx) will be generated and saved in the image folder and the "HGray_Transform_register" image will be shown on the window. This step will take at least 40 min.
 13. Close the "HGray_transform" image; only "HGray_Transform_register" and "FGray" should be left on the window.
 14. Select the "HGray_Transform_register" image (highlight) and go to Menu:Algorithms/Registration/B-Spline Automatic Registration 2D/3D. The "B-Spline Automatic Registration-3D intensity" dialog box will pop up. Select "Least Squares" in the Cost function (the default is Correlation Ratio).
 1. Click "Perform two-pass registration". In the Pass 1 section, key in 2 into "Gradient Descent Minimize Step Size (sample units)" (the default is 1) and key in 10 into "Maximum Number of Iterations:" (the default is 10). In the Pass 2 section, key in 1 into "Gradient Descent Minimize Step Size (sample units)" (the default is 0.5) and key in 2 into "Maximum Number of Iterations:" (the default is 10).
 NOTE: The NLT matrix, "HGray_transform_register.nlt," will be saved in the image folder and the "HGray_transform_register_registered" image will be shown on the window. This step will take at least 5 min.
 15. Close all the images on the window.
2. Generating the reference image for image combination.

NOTE: This step is meant to generate a registered horizontal image for combination.

 1. Load H and F image stacks by choosing Menu:File/Open image (A) from Disk (or Ctrl-f) /H.ids and F.ids; two images will appear on the window.
 2. Select the H image (highlight) and go to Menu:Algorithms/Transformation tools/Transform; the "Transform/Resample Image" dialog box will pop up. Click the "Resample" tab and change the resample from a size of "H" to "F." Next, click the "Transform" tab and load "GrayG_To_GrayG1.mtx" by selecting "Read matrix from file." Click OK; the H_transform image will appear on the window. Close the H image but keep H_transform and F in the window.
 3. Select the "H_transform" image (highlight) and go to Menu:Algorithms/Transformation tools/Transform; the "Transform/Resample Image" dialog box will pop up. Click the "Resample" tab and change the resample from a size of "H_transform" to "F." Next, click the "Transform" tab and load "HGray_transform_To_FGray.mtx" by selecting "Read matrix from file." Click OK; the "H_transform_transform" image will appear on the window. Close the H_transform image; at this point, only H_transform_transform and F will be left on the window.
 4. Select the "H_transform_transform" image (highlight) and go to Menu:Algorithms/Transformation tools/Transform nonlinear; the "Nonlinear B-Spline Transformation" dialog box will pop up. Next, load "HGray_transform_register.nlt" and click OK; the

"H_transform_transform_registered" image will appear on the window. Save the image as an ics file. Close all the images on the window.

3. Combining image stacks

NOTE: This step is to combine two image stacks acquired in orthogonal orientations (horizontal and frontal) into one high-resolution stack.

- Go to Menu:Plugins/Generic/Drosophila Retinal Registration; the "Drosophila Retinal Registration v2.9" dialog box will pop up. Upload "H.ics" in image H, "H_transform_transform_registered" from step 3.2.4 in Image H-Registered, "F.ics" in Image F, "GrayG_To_GrayG1.mtx" from step 3.1.7 in Transformation 1-Green (optional), "HGray_transform_To_FGray.mtx" from step 3.1.12 in Transformation 2-Affine, and "HGray_transform_register.nlt" from step 3.1.14 in Transformation 3-Nonlinear (optional).
 - Select $\text{SqrT}(\text{Intensity-H} \times \text{Intensity-F})$ and No rescale in "Rescale H to F." Keep the default options for the remaining parameters. Click OK; this step will take about 3 min.
NOTE: After processing, 3 sets of images will be generated: combinedImage_sqrT_trilinear_norescale_ignoreBG.ids (the final recombined image), greenChannelsImage-Gxreg-Gy-Gcomp.IDS (green channel for H, F, and the final recombined image), and redChannelsImage-Rxreg-Ry-Rcomp.ids (red channel for H, F, and the final recombined image); all the output files will be resized to 512 x 512 x 512.
- Open "combinedImage_sqrT_trilinear_norescale_ignoreBG.ids" under the image visualization software. Save the image stack in the ims format and rename the file; this recombined image file will be used for neurite tracing and registration.

4. Neurite Tracing and Reference Point Assignment

NOTE: This step is to trace neurites (4.1) and to assign reference points for registration (4.2) using the image visualization software.

1. Tracing neurites

- Start the image visualization software. Open the recombined image file. Go to Menu:Edit/Show Display Adjustment and turn off the photoreceptor channel (red).
- Visualize the image in "Surpass" mode. Turn on "stereo" and use the "Quad Buffer" mode to visualize 3D images if the computer is equipped with a stereograph system.
- Go to Menu:Surpass/Filaments to add new filaments. Click the "Skip automatic creation, edit manually" tab.
- Click the "Draw" tab and select "AutoDepth."
- Select "Settings," check "Line," and key in an appropriate pixel number for better visualization (a 4-pixel line is used in this protocol). Check "Show Dendrites," "Beginning Point," and "Branching Points". Set "Render Quality" to 100%.
- Select the "Draw" tab and start tracing neurites. Start with the axon and then move to the dendrites (**Figure 2D**). The axon and dendrites of transmedulla neurons are easy to differentiate.
NOTE: A Tm neuron extends its axon from the cell body and projects all the way to the higher visual processing center, the lobula. The system will automatically define the first long filament as an axon and the remaining short filaments as dendrites. Keep the starting point at the beginning of the filament (axon) during tracing and make sure that the traced neurites are connected. Examine the branching points and the beginning point. If the dendrites are not connected, a new beginning point will be defined at the non-connected filament.
- After tracing, go back to "Settings," uncheck "Beginning Point" and "Branching Point," and go to Menu:Surpass/Export selected objects.../. Save the filament as an inventor file (*.iv).

2. Assigning reference points

- Select "Show Display Adjustment" and turn on both imaging channels. In this example, channel 1 is the photoreceptor staining and channel 2 is the Tm20 neuron (GFP).
- Go to Menu:Surpass/Measurement. Select the "Edit" tab and check "specific Channel." (select the photoreceptor channel [red]).
- Assign reference points for the top layer. Go to Menu:/Surpass/Measurement Points to create new measurement points. Mark the beginning of the M1 layer as a top layer. The order of the points is as follows: equatorial, anterior-equatorial, anterior, anterior-ventral, ventral, posterior-ventral, posterior, posterior-equatorial, and center (**Figure 3F**); define the center photoreceptor as the one associated with the most dendritic processes.
- Assign the R8 and R7 layers as in step 4.2.3 (**Figure 3G**). Three individual measurement points should be created in steps 4.2.3 and 4.2.4.
- Export the coordinates of the points for each layer. Click the "Statistics" tab, select "Detailed," "Specific Values," and "Position;" and click "Export Statistics on Tab Display to File." Save as "Comma separated values" (*.csv).
- Open the three csv files (from steps 4.2.3 - 4.2.4) and combine the coordinates of the 27 reference points into a new csv file by copying and pasting (the order is Top, R8, and R7). See the **Materials/Equipment Table** and follow the format of the example file.

5. Rigid-body and TPS Nonlinear Registration

NOTE: This step is to register the neurite traces (in iv format) to the reference column array and to generate a registered swc file using the MIPAV program. This section requires the following files: the recombined image stack (.ids) from step 3.3, the reference point file (.csv) from step 4.2, and the neurite trace filament file (.iv) from step 4.1.

- Go to Menu:Plugins/Generic/DrosophilaStandardColumnRegistration. The "DrosophilaStandardColumnRegistration v6.6.1" window will pop up.
- Load the image files (.ids), the reference points file (.csv), and the filament file (.iv).
- Select 9 points per layer.
- Select the position of the imaged neuron (LV/RD or RV/LD).

5. Select "Rigid Registration and TPS" and "Rigid Registration" to nonlinear and rigid-body registration, respectively.
6. Check "Create SWC file" to generate the following output files: a registered neurite trace file in swc format (see Specific Materials/Equipment for definition), a registered IV file (.iv), coordinates of the standardized neurite (.txt), transformed coordinates (.txt), and the combined image (.ids).
7. Change the name of the swc file. Apply the abbreviation of the location to the end of the file name (step 1.7). For example, use "Tm20_3_RV.swc" for Tm20 neuron #3 located at the ventral half of the right medulla.

6. Standardization to Right-ventral Configuration

NOTE: This step is to convert the neurite traces (in swc format) to standard RV (right-ventral) configuration using the custom script "RV_standardization.m." Here, the script was written in the matrix laboratory language. The names of the input swc files should be in the following format: "NeuronName_Number_Configuration.swc" (e.g., Tm20_3_LV.swc).

1. Open the "RV_standardization.m" script.
2. Edit the following parameters in the "User input" section:
 1. Type the names of the neurons without numbering (e.g., Tm2, Tm20, etc.) in "neuron_names."
NOTE: The default in "file_end_in" is "_*.swc," which looks for files with names containing "_.swc" ("*" is a wildcard matching any number of characters). The default of "swc_file_end_out" is "_f.swc," which will add "_f" to the end of the file name after standardization.
 2. Specify the directory where the swc files are in "directory_in". Specify the directory where the standardized files will go in "directory_swcout".
3. Run the script.
4. Optional: Use Vaa3D¹⁴ to visualize the swc files and validate the conversion.

7. Calculate Dendritic Branching and Terminating Frequencies

NOTE: This step uses rigid-body registered swc files to calculate the Kaplan-Meier estimators for the probability that a dendritic segment will reach a given length without terminating. This script uses two Dendritic_Tree_Toolbox functions: extractDendriticSegmentLengthDistribution and estimateDendriticSegmentLengthProbability.

1. Open the "Branch_term_P.m" script.
2. Edit the following parameters in the "User input" section:
 1. Specify the path to the rigid-body registered swc files in "pathToSWCFiles" (e.g., /Rigid_Registered.swc/). Specify the path that will hold the graphics output in "pathToOutput." Specify the name of the neurons or neural types in "neuron_names" (e.g., Tm2, Tm20, etc.).
3. Run the script; the outputs are the Kaplan-Meier estimate curve for dendritic branching and termination.
NOTE: Optional: Apply the function-fitting method to extract from the Kaplan-Meier estimators the local probability that the dendritic segment will branch or terminate.

8. Plot the Distribution of Layer-specific Termination of Dendritic Arbors

NOTE: This step plots the distribution of dendritic terminals in different medulla layers as a bar graph. This can be applied to one neuron, a group of neurons, or groups of neurons. The script uses the extractDistributionAlongAxis function from Dendritic_Tree_Toolbox.

1. Open the "Layer_term.m" script.
2. Edit the following parameters in the "User input" section:
 1. Specify the directory that contains nonlinear registered swc files in "pathToSWCFiles" (e.g., /Non_linear_Registered.swc/). Specify the directory for graphics output in "pathToOutput." Specify the name of the neurons or neural types in "neuron_names" (e.g., Tm2, Tm20, etc.).
3. Run the tutorial script. The output is a histogram of the proportion of terminal nodes in specific medulla layers.

9. Plot the Planar Projection Direction of Dendrites

NOTE: This step plots the planar projection directions of dendrites as a polar plot. The script uses the extractAngularDistribution function from Dendritic_Tree_Toolbox.

1. Open the script "Planar_proj.m."
2. Edit the following parameters in the "User input" section:
 1. Specify the directory that contains the nonlinear registered swc files in "pathToSWCFiles" (e.g., /Non_linear_Registered.swc/). Specify the directory for graphic output in "pathToOutput." Specify the name of the neurons or neural types in "neuron_names" (e.g., Tm2, Tm20, etc.).
3. Run the script; the output is a polar plot of planar projection directions.

Representative Results

Using the dual-view imaging procedure presented here, a fly brain containing sparsely labeled Tm20 neurons was imaged in two orthogonal directions. Prior to imaging, the brain was stained with appropriate primary and secondary antibodies for visualizing membrane-tethered GFP and photoreceptor axons. For imaging, the brain was first mounted in the horizontal orientation (**Figure 2A, B**). A GFP-labeled Tm20 neuron and the surrounding photoreceptor axons were imaged using a confocal microscope (**Figure 3A**). The brain was then re-aligned (**Figure 2A, B**) and the same Tm20 neuron was re-imaged in the frontal orientation (**Figure 3B**). During the imaging process, the location of the Tm20 neuron was identified as at the ventral half of the right medulla neuropil (RV configuration). These two image stacks were deconvolved using the image deconvolution software and recombined using MIPAV (**Figure 3C**) to generate a recombinant image stack (**Figure 3C'**). The recombinant image stack showed a significant reduction of axial distortion as compared to either input stack (**Figure 3A', B'**).

Using the image visualization software, the axon and dendrites of the Tm20 neuron were traced (**Figure 3D**) and saved as an inventor (iv) file. 27 reference points on photoreceptor axons surrounding the Tm20 neuron were assigned (**Figures 3E - H**) and saved as a comma separated value (CSV) file. The traced dendrite files and reference point file was subsequently applied to the custom plugin in MIPAV to generate rigid-body registered and non-linearly registered dendrite traces in swc file format. The registered dendritic traces were converted to the standard RV configuration using the "RV_standardization.m" script. The registered and standardized dendritic trace files were visualized using Vaa3D to validate the registration and standardization processes (**Figure 3I**).

For dendritic analysis, registered dendritic traces for 15 Tm20 neurons and 15 Tm2 neurons were collected. Using the "Branch_term_P.m" script, the branching and terminating probability of these neurons were analyzed using the rigid-body registered dendritic traces (**Figure 4A, B**). The Kaplan-Meier curves show that both neuronal types have similar branching and terminating frequencies. However, Tm2 has a lower terminating frequency for longer segments ($>4 \mu\text{m}$; **Figure 4A**)¹². For analyzing dendritic properties associated with layers and columns, the dendritic traces registered by the non-linear registration method were used. Using the custom "Layer_term_P.m" script, the distributions of layer-specific dendritic termination for Tm2 and Tm20 neurons were calculated (**Figure 4C, D**). The distinct distributions of layer-specific terminations of Tm2 and Tm20 dendrites are consistent with their specific synaptic partners in different layers: Tm2 dendrites receive inputs from L2 and L4 axonal terminals in M2 and M5, respectively, while Tm20 dendrites receive inputs from R8 and L3 in the M3 layer^{8,15,16}. Using the "Planar_proj.m" script, the planar projection directions of Tm2 and Tm20 dendrites were analyzed and were found to be distinct from one another: Tm2 dendrites project posteriorly while Tm20 dendrites project anteriorly¹² (**Figure 4E, F**).

Figure 1. The Workflow. The central column shows three method sections: dual-view imaging, dendrite tracing and registration, and dendritic analysis. Each method section contains three steps. The left column indicates the software used in each step. The right column shows the input and output files for each step, with the data flow indicated as orange arrows. [Please click here to view a larger version of this figure.](#)

Figure 2. Sample Preparation for Dual-view Imaging and the Symmetry of the *Drosophila* Medulla Column Array. (A, B) Sample mounting for dual-view imaging. (A) The view is shown as if looking at the brain through a coverslip. (B) Schematic diagrams for sample mounting. For imaging horizontal stacks, the immuno-stained brain is mounted first at the ventral-up position. After acquiring the first stack, the brain is re-positioned in the anterior-up position for imaging the frontal stack. A: anterior; P: posterior; D: dorsal; V: ventral; R: right; L: left. (C - E) The symmetry and organization of the medulla neuropil, viewed in the frontal position. (C) Photoreceptor axons, labeled by the 24B10 antibody (red), were used to visualize the organization of the medulla neuropil. The image is shown as if looking from the outside into the brain. Ant: anterior; Pos: posterior; Dor: dorsal; Ven: ventral; Eq: equator. (D) A high-magnification view of (C) at four quadrants of the medulla neuropil at the R7 layer level. The central R7 terminals are marked by yellow dots. The anterior and equatorial R7s are labeled with green and cyan dots, respectively. (E) A schematic representation of the medulla columnar organization. Note that the left and right medulla neuropils are mirror images, and the dorsal and ventral halves of each neuropil are also mirror images, separated by the equator. Red dots indicate equatorial R7s, which are the first reference points. Numbers (1 - 9) and arrows indicate the order of reference point assignments. Scale bar: 15 μ m in C; 5 μ m in D. [Please click here to view a larger version of this figure.](#)

Figure 3. Image Recombination and Reference Point Assignment. (A - C) Confocal images for a single Tm20 neuron acquired in the horizontal (A) and frontal (B) directions. Images are shown as maximum-intensity projections. Photoreceptor axons and a single Tm20 neuron are labeled with Mb24B10 antibody (red) and membrane-tethered GFP (green), respectively. (C) Recombined image of (A, red) and (B, cyan). For clarity, only photoreceptors are shown. (A' - C') Dual-imaging improves axial resolution. The recombined image (A') has a better resolution than those of the horizontal-view (A') and frontal-view (B') images. Lower panels: high-magnification views of the boxed areas in the upper panels. (D) Tracing the axon (white) of a single Tm20 neuron. The beginning point was indicated as a cyan dot. (E) The 27 reference points (white dots) on the 9 photoreceptor axons surrounding the Tm20 neurons are used as landmarks. The reference points are in three layers: Top, R8 and R7. (F) Reference point assignment in each layer. The reference point assignment follows the following order: equator (Eq), anterior-equator (AE), anterior (A), anterior-ventral (AV), ventral (V), posterior-ventral (PV), posterior (P), posterior-equator (PE), and center (C). (G) A schematic representation of the medulla column array used for registration. A Tm20 neuron is shown (green). The medulla layers (M1-6) are as indicated. (H) An example of layer and reference point assignment for a single column. Three layers are used to define each column: Top, R8, and R7 (the junction of M5/6 layers). (I) A registered and standardized neurite trace visualized using Vaa3D. Scale bar: 5 μ m in A for A-C; 5 μ m in A' for A'-C'; 5 μ m in D, E, F, and G. [Please click here to view a larger version of this figure.](#)

Figure 4. Examples of Results from Dendritic Analyses. (A, B) Kaplan-Meier estimates (y-axis) of dendritic branching (A) and termination (B) were plotted in logarithmic scale with respect to segment length (x-axis). Solid and dotted lines denote averages $\pm$ SDs. Tm2 (red) and Tm20 (black) share similar branching and terminating frequencies for segments less than 4 μm . (C, D) Histograms showing the mean proportion of terminal dendritic nodes in each medulla (M) layer. The proportion of terminal nodes in a given medulla layer is first calculated for every neuron of a given type and then averaged over all the neurons of that type. The vertical bars show the standard deviation of the proportion. Note that for Tm2 neurons (C), the distribution of terminal dendritic nodes is concentrated in medulla layer 2, with a minor peak in layer 5, while for Tm20 neurons (D), the distribution is centered around the M2 and M3 layers. (E, F) Polar plots of the planar projection direction of dendrites. The proportion of terminal nodes projected within an angular bin (20°) was first calculated for every neuron of a given type and then averaged over all neurons of that type. The red and blue curves show the mean proportion $\pm$ standard deviation. The corners on the curves are the midpoints of the angular bins. The black circles show the proportion values indicated by the black numeral to their side. Note that, on average, the two classes of neurons are oriented in almost opposite directions. A: anterior; P: posterior; D/V: dorsal/ventral; Eq: equator. [Please click here to view a larger version of this figure.](#)

Discussion

Here, we show how to image and analyze dendritic arbors of *Drosophila* medulla neurons. The first section, dual-view imaging, describes the deconvolution and combination of two image stacks into a high-resolution image stack. The second section, dendrite tracing and registration, describes the tracing and registration of dendrites of medulla neurons to the reference column array. The third section, dendritic analysis, describes the use of custom scripts to analyze dendritic patterns. Together these protocols provide a complete workflow to extract information on dendritic patterns with respect to layers and columns and to determine dendritic branching and terminating frequencies.

The dual-view imaging method presented here is conceptually similar to the multi-view imaging techniques implemented with light-sheet microscopes, in which two or more image stacks collected orthogonally are recombined to achieve a high axial resolution^{17,18}. However, it is technically challenging to orthogonally implement the two high N.A. (numerical aperture) objective lenses that are required for imaging slender dendrites. Thus, the dual-view method requires imaging the same brain sample in two consecutive steps and re-orientating the sample between imaging. Failures in image combination are most likely caused by sample movement during imaging or sample squashing during manipulation. Furthermore, while the image recombination process mitigates the axial distortion problem and results in near-isotropic resolution, the recombined images are still under a diffraction limit. Our dual-view imaging method needs only standard confocal microscopy, which is widely available. However, super-resolution microscopy, such as STED or structure illumination, if available, would be an excellent alternative to the dual-view imaging method present here.

The dendrite tracing and registration section presented here traces dendritic trees and registers dendritic trees to the reference column array. In this protocol, the commercial program Imaris is used for semi-automated dendrite tracing and for assigning reference points. Similar tasks could be carried out using a number of freeware alternatives, such as NeuronJ^{19,20} and Neurite Tracer²¹. Our registration protocol uses a custom plugin implemented in the open source program MIPAV to register dendrite traces to the reference column array. To our knowledge, this registration method is the only method that registers neurite traces to columns and layers. This procedure uses regularly arranged photoreceptor axons as landmarks for registration. The dendritic traces generated by the non-linear registration method are ideal for extracting information on dendritic patterns relating to columns and layers. On the other hand, the rigid-body registration method aligns the Cartesian axes of dendritic traces to the cardinal axes of the neuropil without changing the dendritic lengths or other local geometrical properties. Therefore, the rigid-body registered dendritic traces are suitable for standard morphometric analyses.

Traditional dendritic morphometric analysis, such as L-measure²², characterizes local and global dendritic properties without referencing surrounding tissue. We have previously shown that many medulla neurons share dendritic geometrical properties and that such metrics are insufficient to differentiate medulla neuron types¹². Instead, type-specific dendritic attributes are directly associated with the layers and columns where dendrites reside. In particular, different types of medulla neurons project dendrites in distinct planar directions to terminate in specific medulla layers. These two dendritic attributes reflect the dendritic function of medulla neurons to receive retinotopically directed afferents organized in layers and columns. To facilitate the extraction of these properties, we developed the dendritic tree toolbox that contains a series of

custom functions for calculating dendritic properties. In the dendritic analysis section, we show how these functions can be applied to registered dendritic traces to calculate the distribution of layer-specific termination and planar projection directions of dendrites.

The dendritic tree toolbox provides a platform for adding other custom functions for dendritic analysis. In the dendritic analysis section, we also describe the process to derive estimates for apparent dendritic terminating and branching frequencies. In particular, these functions calculate the branching and terminating frequencies based on a Kaplan-Meier nonparametric estimator for the probability that a dendritic segment will extend to a certain length without branching or terminating^{12,23}. It is important to note that the rigid-body registered (but not the nonlinearly registered) dendritic traces should be used for this calculation because the non-linear registration process alters dendritic segment lengths. The dendritic branching and termination frequencies are the first derivative (the slope) of the KM curves and can be calculated using the function-fitting method. Furthermore, the accurate calculation of the branching and terminating frequencies often requires a sizable data set (>10 neurons for Tm neurons) because single neurons have only a relatively small number of dendritic segments. In the provided examples, Tm2 and Tm20 have similar branching and terminating frequencies, but the Tm2 branching frequency is not homogenous across branching length, a characteristic feature of Tm2 dendrites¹².

While our protocols are designed for *Drosophila* medulla neurons, they can be adapted to analyze other neurons that elaborate dendrites in layers and columns, such as other *Drosophila* optic lobes and the vertebrate retina and cortex. Such adaptations would require building a new reference column array based on the system of interest¹², as well as minor modifications of the MIPAV plugin. We provide all our programs as open source software to promote collaboration and sharing.

Disclosures

The authors have nothing to disclose.

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