Video Article

Assessment of Dendritic Arborization in the Dentate Gyrus of the Hippocampal Region in Mice

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Abstract

Dendritic arborization has been shown to be a reliable marker for examination of structural and functional integrity of neurons. Indeed, the complexity and extent of dendritic arborization correlates well with the synaptic plasticity in these cells. A reliable method for assessment of dendritic arborization is needed to characterize the deleterious effects of neurological disorders on these structures and to determine the effects of therapeutic interventions. However, quantification of these structures has proven to be a formidable task given their complex and dynamic nature. Fortunately, sophisticated imaging techniques can be paired with conventional staining methods to assess the state of dendritic arborization, providing a more reliable and expeditious means of assessment. Below is an example of how these imaging techniques were paired with staining methods to characterize the dendritic arborization in wild type mice. These complementary imaging methods can be used to qualitatively and quantitatively assess dendritic arborization that span a rather wide area within the hippocampal region.

Video Link

The video component of this article can be found at https://www.jove.com/video/52371/

Introduction

Dynamic alterations in the number and structure of synapses are hallmarks of development, aging, and numerous neurodegenerative disorders[1-3]. The ability of neurons to receive and integrate synaptic information depends upon dendritic morphology and dynamic alterations in synaptic connections. Indeed, a positive correlation exists between dendritic spine and synapse number, which both impact cognitive function[4]. Thus, it is not surprising that decrements in dendritic spine number have been associated with cognitive dysfunction in a number of neurological disorders[5-7], prompting great interest in dendritic spine quantification. Nevertheless, the quantification of spine density remains a time-consuming and tedious task that fails to generate useful information regarding the topography and distribution of synapses across the dendritic tree. Fortunately, staining methods (*e.g.*, Golgi-Cox and doublecortin (DCX)) in conjunction with sophisticated imaging techniques can be utilized to overcome current barriers and produce high-resolution images of dendritic arborization in a reliable and expeditious manner. While Golgi-Cox staining method can be deployed to assess the state of dendritic arborization in all neurons[8], DCX can be deployed to label newly-born neurons particularly in the dentate gyrus and subventricular zone[9], an important consideration given that neurogenesis occurs in both these regions throughout the lifespan[10,11].

Following staining, two imaging methods were deployed to assess dendritic characteristics: i) real-time imaging (RTI) and ii) extended depth of field imaging (EDFI). The RTI technique provides a mean to trace and quantify the length and order of arborization along the individual dendritic segments and branches. Thus it enables one to estimate the total area and volume occupied by each dendritic tree**.** More specifically, in the RTI method the user continuously identifies the segments and refocuses iteratively as the neuron tracing software collects the x, y, and z coordinates of the dendritic structure and reconstructs the trajectory of the dendritic structure in 3D. Comparatively, the EDFI method provides a rather simple and expedited means for assessing dendritic density in rather thick tissue specimens by generating a composite image, providing information on the entire z-axis. To do so, the user records high definition video files throughout the thickness of the section and then uses software to search the video frames to identify points wherein a pixel is completely in focus. Subsequently, the focused pixels are merged and integrated into a high-resolution, composite 2D image. This composite image contains all pixels that were in-focus regardless of their position in the z axis. Qualitative and quantitative analysis of these 2D images can be used subsequently to determine the density of dendritic branching in each field.

Lastly, we present a panoramic method for generating extremely high-resolution images for the analysis and assessment of dendrites in an entire region of interest. This technique can be deployed to overcome the lack of access to very high-resolution and expensive digital cameras. Using this method, one captures serial images at different locations along the x- and y-axes and then automatically stitches them together using a

freeware (*e.g.*, Image Composite Editor). Notably, this method can be used for qualitative and quantitative assessment of dendritic arborization in a rather wide area.

Protocol

NOTE: Experiments were conducted in accordance with the ethical standards approved by the Committee on Animal Research at the Veterans Affairs Palo Alto Health Care System.

1. Golgi-Cox Staining

1. Brain extraction and staining
    1. On the 1st day, deeply anesthetize mice with 100 mg/kg ketamine and 10 mg/kg xylazine before euthanizing via exsanguination.
    2. Carefully remove the calvarium and dissect out the brain.
        1. First remove the skin on the top of the skull, placing a curved scissor on the top of the cerebellum and gently cut through the calvarium parallel to the central sulcus with the tip of the scissor pointed outward, moving in the direction toward the olfactory bulb. Repeat this process on both sides.
        2. Use a surgical pincet to lift the calvarium while turning it toward the olfactory bulbs and break it off. Then, flip the skull upside down and use a pick to cut through the optic nerves and loosen the brain. Finally, use the pick to separate the brain from the spinal cord at the level of the foramen magnum.
    3. Immediately immerse the brain in 15 ml of commercially-available, undiluted Golgi-Cox solution. Store the brain in Golgi solution in a capped, 20 ml glass bottle at room temperature in the dark.
    4. On the 2nd day, slowly pour out the solution and replace it with 15 ml fresh Golgi-Cox solution. Recap the bottle containing the brains and leave undisturbed for another 9 days in the dark.
2. Sectioning and embedment
    1. On the 11th day, place the brains in 30% sucrose solution, prepared in ddH_2O for dehydration. Change the solution with freshly prepared 30% sucrose after 12 hours. Incubate the brains in a 30% sucrose solution for 3 days at 4 °C.
    2. On the 14th day, fill the reservoir of the vibratome with a 30% sucrose solution until the blade is covered. Set the speed to 1 mm/s with amplitude of 0.95 mm.
    3. Blot the brains with a Kimwipe to remove excess moisture and remove the cerebellum using a razor blade to cut coronally.
    4. Firmly mount the whole brain onto the vibratome platform using superglue and allow it to set for 2-4 min. Insert the platform with the adhered brain into the reservoir and adjust the blade to the top of the brain.
    5. Using the vibratome, slice the brain into 150 µm thick sections at room temperature, remembering to change the blade after every 3rd brain.
    6. Pick up the sections from the reservoir using a small tipped brush and immerse them into a petri dish containing 0.3% gelatin made in ddH_2O. While the sections remain in the petri dish, add 0.5 ml of 0.3% gelatin on each slide and use a brush to spread and coat the superfrost + slides.
    7. Gently pick up the immersed sections from the petri dish and place them onto the gelatinized slides. Orient the brain sections by touching them only on their sides and not on the tops.
    8. After about ten min, coat the sections with 30% sucrose before the tissue completely dries. Then air dry the prepared slides in a dark place in a slide rack for 3 days.
    9. Dilute commercially-available 10x Developing solution to 1x using ddH_2O. Immerse the slides in developing solution for 7-10 min. Rinse the slides 3 times in ddH_2O.
3. Dehydration
    1. Prepare 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 95% ethanol solutions using distilled ddH_2O.
    2. Soak the slides in increasingly higher ethanol solutions for 10 min each.
    3. Soak the slides in 100% ethanol solution 3 times for 10 min each time.
    4. Soak the slides in 100% xylene 3 consecutive times for 5 min each, keeping the sections in the final solution until they are cover-slipped.
    5. Place a generous amount of DPX (Di-n-butyl phthalate in xylene) mounting medium (about 1-1.5 ml) on each slide using a plastic transfer pipet. Carefully place coverslips to avoid bubbles.
    6. Air dry cover slipped-brain sections horizontally for 3 days.

2. Doublecortin Staining

1. Deeply anesthetize (see step 1.1.1) the animals.
2. Perform cardiac perfusion with freshly prepared 4% paraformaldehyde made in phosphate buffer[12].
3. Extract the brains (see 1.1.2) and store in 45 ml of 4% paraformaldehyde at 4 °C overnight on a rocking shaker.
4. Transfer the brains to a 30% sucrose solution and wait for complete dehydration for 48 hr at 4 °C. Remove the brains from sucrose and place them directly on copper blocks placed on dry ice.
5. Fill the block with optimum cutting temperature (OCT) compound and mark the orientation of the brain using the olfactory bulb as a landmark.
6. Using a cryostat, cut 70 micron-thick sections at -20 ºC and place them in cryoprotectant solution (25% ethylene glycol, 25% glycerol, in 0.05 M sodium phosphate buffer, pH 7.4) and keep at -20 °C until use.
7. Make a solution of 50% cryoprotectant and 50% methanol. To this solution add 0.5% hydrogen peroxide (vol/vol). Incubate floating sections for 30 min at room temperature.

8. Warm sections in 37 ºC in TBS (Tris-buffered saline) for 30-40 min by placing them in a histological oven.
9. Incubate the sections with 0.3% triton and 3% normal horse serum for 45 min at room temperature prior to immunostaining. Incubate the sections at 4 ºC overnight in 3 ml solution of the DCX antibody diluted 1:500 in TBS with 0.3% triton and 1% normal horse serum.
10. The following day wash the sections 3 consecutive times in TBS (10 min each).
11. Incubate sections with a biotinylated horse anti-goat (1:200 in TBS) for two hr at room temperature. Wash the sections 3 consecutive times in TBS (10 min each).
12. Incubate them with ABC Lite (1:1,000) for 1.5 hr at room temperature.
13. Add 5 µl of 30% H_2O_2 to one tablet of 10 mg of DAB (3,3"-Diaminobenzidine) solution dissolved in 15 ml of 1 M Tris-HCl (pH 7.6). Incubate the sections immediately in this solution for 5 min.
14. Terminate the reaction by washing them with ice-cold TBS three times followed by one wash in TBS at room temperature.
15. Dehydrate sections using a series of ethanol solutions (50%, 60%, 70%, 80%, 90%, 95%, 100%, 5 min each), clear in xylene, and then cover slip using DPX as in step 1.3.

3. Visualization

1. Following complete drying of the sections, remove excess DPX on top of the slides with a sharp blade, and place them on the scanning stage of the microscope. The system is composed of a microscope equipped with scanning stage, joystick, and a color 12-bit camera.
2. Start the program. Open a new data file.
3. Place a reference point on the screen by clicking with the mouse pointer at any place. This will activate all the icons from the tool panel of program window.
4. Click on the "Joystick Free" icon on the toolbar and then use the joystick to locate the dentate gyrus of the hippocampus in the first section.
5. In the "Tools" tab of the program window, select "Serial Section Manager". Click on the "New Section" icon on the lower left corner of the window. This will open the "Serial Section Setup" window.
6. Select the Serial Section Setup window and then enter the total number of sections that contain hippocampal regions. Select the evaluation interval. Enter the section cut thickness (70 µm for DCX and 150 µm for Golgi-stained sections).
7. Start tracing the dentate gyrus region by clicking on the "Freehand Contour Drawing" icon in the tool bar. Outline the dentate granular cell layer.
8. Select "100X" from the "Magnification" menu. Add a drop of immersion oil on the section and switch the objective to 100X. Locate the dentate granule cell bodies and dendrites under this objective.
9. Focus on a selected neuron and click on the "Neuron Tracing" icon on the toolbar. Trace the circumference of the dentate granule cell body. When finished tracing, right click to select "Finish Cell Body".
10. Start tracing the dendrites manually in x, y, and z directions and follow each branch using the joystick and z-motor knob. At a bifurcation or trifurcation node, select the respective option from the dropdown menu upon right-click. Trace each of the branches that arise from these nodes. At the end of each branch right click and select "Ending" from the dropdown menu.
11. Using the arrow key icons in the software window randomly select another dentate granule cell and follow the same procedure as described in step 3.9-3.10. Save the entire tracing.
12. Start the neuronal tracing software.
13. Open the first NRX data file from the first mouse of the experimental group. Append the NRX file of the second mouse of the experimental group. Continue until all the NRX files from this group are appended.
14. Under the Analysis tab, select "Fan In-diagram". This opens the Fan In-analysis window. Check mark "dendrites" and click Display, which depicts the lengths and branching patterns of dendrites for this group of mice (**Figure 1**).

4. EDFI

NOTE: This method enables the user to rapidly record a large number of images through the z-axis of each field and generate a 2D image that contains all the focused pixels throughout the z-axis. The result will be a composite image that contains all focused pixels from collected images.

1. Connect the microscope to a digital camera. Place the sections first on the scanning stage connected to the microscope and then switch to a 10X objective. Move the stage to the area of interest.
2. Start a video capturing program.
3. Press the record button. As soon as the record button is pressed, use the macro-focus knob to move from the top to the bottom of the section for a total of 4 sec. Save the resulting video file.
4. Use ImageJ freeware to convert the AVI video files into an uncompressed format by opening the file and saving them in uncompressed file format.
5. Start an image analysis program and open the AVI file. Go to "Process" menu and click on "Extended Depth of Field". Select the corresponding video file. In "Output" options, select "Generate Composite Best-Focus Image".
6. In "Focus Analysis" options, select "Normalized Illumination" and "Max Local Contrast" options. Then, select "Create". The resulting image represents all the focused pixels throughout the z-axis (**Figure 2**).
7. Save the resulting image.

5. Generating Extremely-high Resolution Images

NOTE: This method allows the user to automatically generate low magnification and extremely high-resolution images from high-magnification-high resolution images in an automatized fashion.

1. Place the sections first on the scanning stage connected to the microscope. The microscope is connected to a digital camera. Move the stage to the area of interest.
2. Start an image acquisition program.

3. Use a 10X objective and acquire and save high-resolution (4076 x 3116) images from the region of interest making sure to have at least 10% overlap.
4. Run Image Composite Editor to stitch images.
5. Go to File menu, and click on “New Panorama”. Select the folder in which the images are stored. Select the images that belong to the same region and press “ok”.
6. Press “Export to Disk” button and save the image. This image represents an extremely high-resolution picture of the area of interest that could be used for analysis and/or demonstration (**Figure 3**).

Representative Results

The extent of arborization arising from extant and newly born dentate granule cells was analyzed in wild type mice using either Golgi-Cox or DCX staining (**Figure 1**). Dendritic segments of DCX-positive cells were found to be 13-36 microns long. The normal distribution of dendritic length was tested using Kolmogorov-Smirnov test ($D = 0.1217$, $p < 0.01$, Liliefors $p < 0.001$; **Figures 4** and **5**).

In the analysis of length of segments per order of arborization, the longest segments were found in the first order of arborization (38.38 + 1.45 µm). Similar patterns were found for the surface (111.98 + 3.93 square micron) and volume (27.93 + 1.03 cubic µm). The linear correlation between the length of segments and the surface area and/or volume occupied by these segments were also tested. Both surface ($p = 0.001$, $r^2 = 0.873$) and volume ($p = 0.001$, $r^2 = 0.621$) correlated significantly with the length of each dendritic segment.

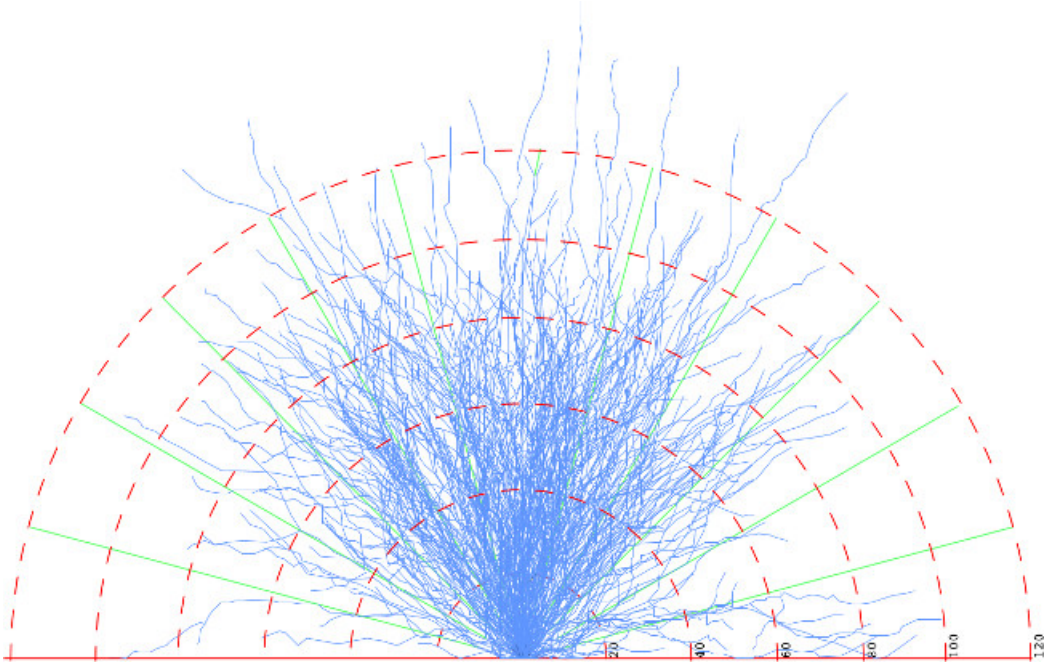

Figure 1: Here Fan diagram was used to assess the dendritic length of dentate granule cells in the hippocampus. This visualization tool can be used to compare the effects of different therapeutic interventions or genotypes on dendritic length. The unit of measurements is µm.

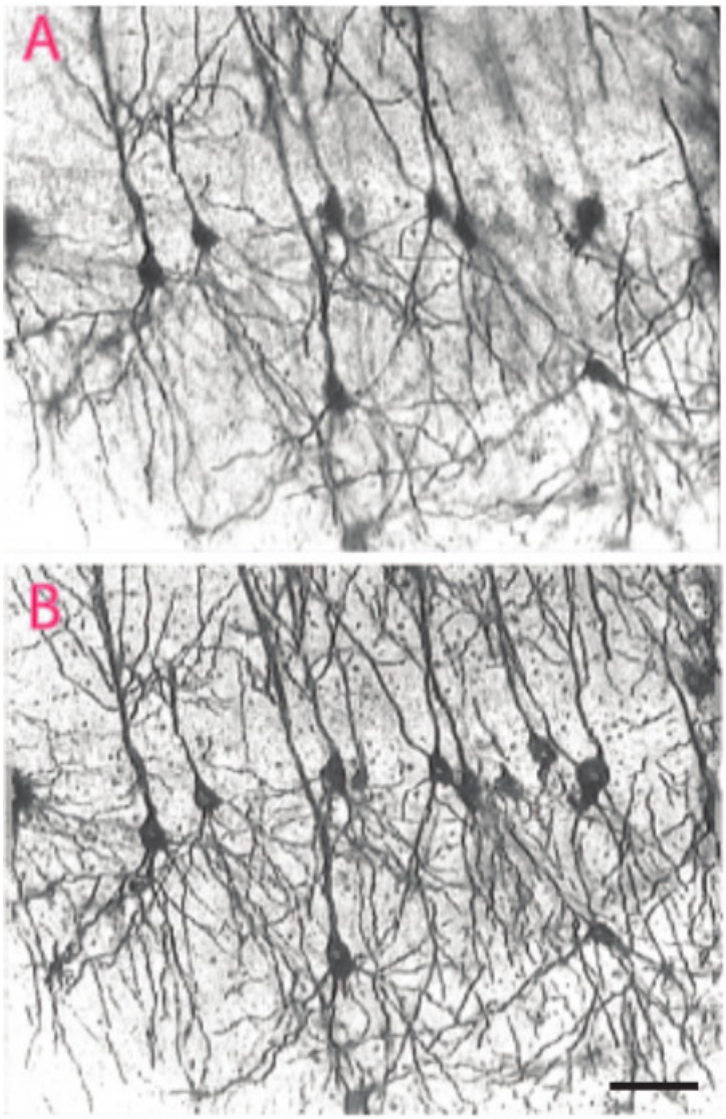

Figure 2: Visualization of dendritic arborization without (**A**) and with EDFI (**B**) methods. The traditional method of finding the best focus plane was compared with EDFI (data not shown) and found significantly higher values of dendritic area using the EDFI method ($p = 0.02$). Scale bar = 100 µm.

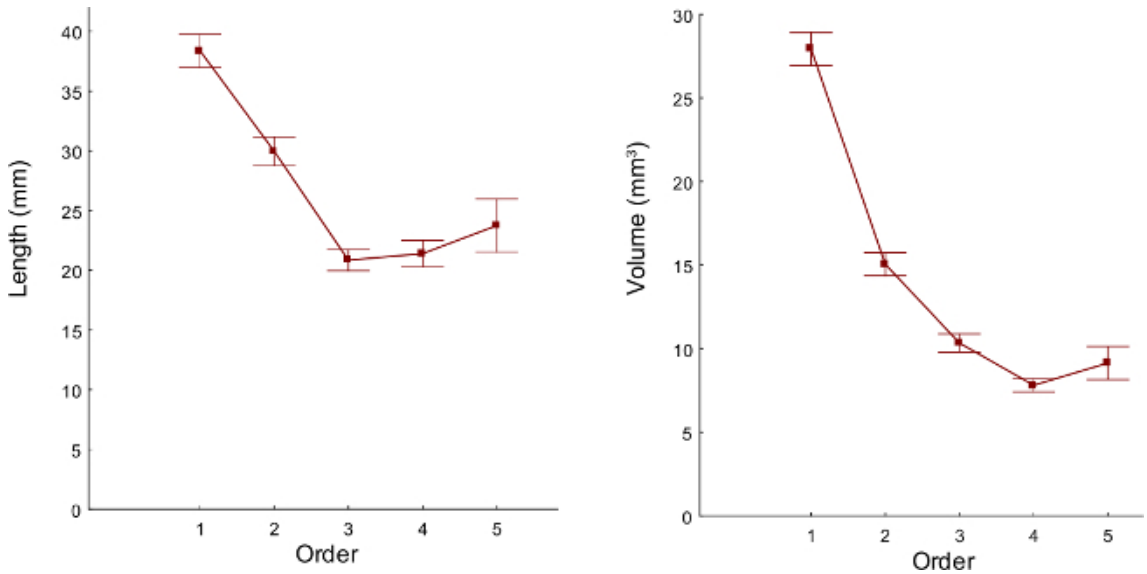

Figure 3: A high-resolution image of the hippocampal region in a mouse. This extremely high resolution image is automatically constructed from stitching 10 (4,076 x 3,116)-pixel images. Scale bare = 100 µm.

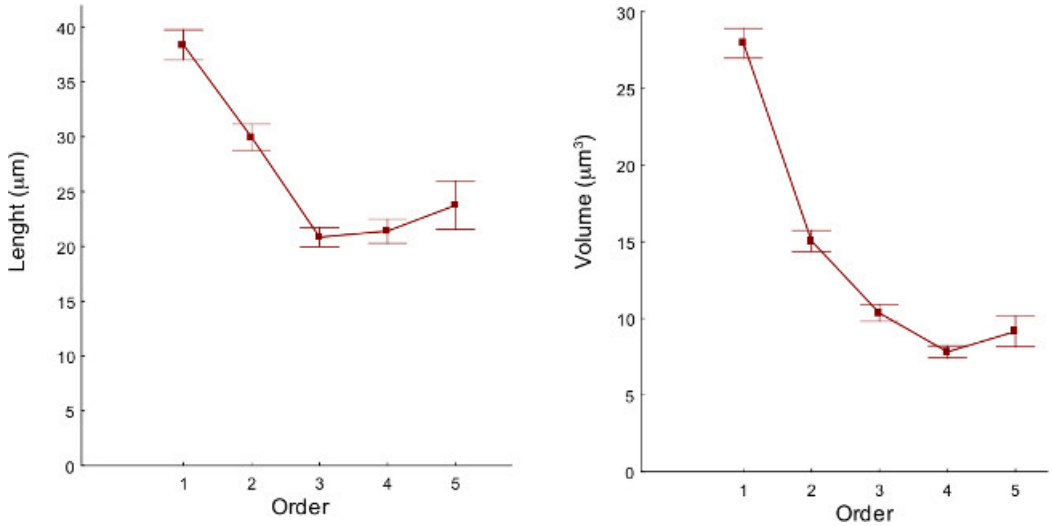

Figure 4: The quantification of length and volume occupied by dendrites in different orders of branching. The maximum length and volume were achieved in the order 1.

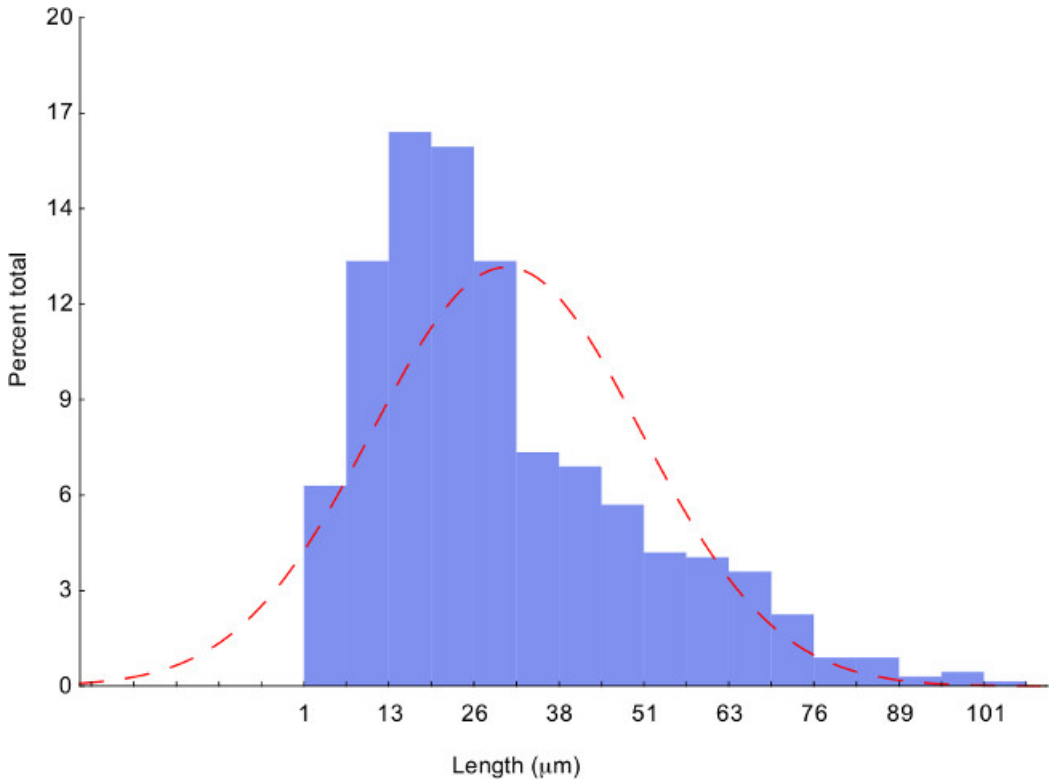

Figure 5: Histogram of dendritic length of dentate granule cells. The majority of dendritic segments of DCX-stained dendrites were 13-26 in length. The red dotted line depicts normal distribution of the data presented.

Discussion

Here, two methods were described to quantify the extent of dendritic arborization in mature and newly-born neurons using conventional staining methods in conjunction with RTI and EDFI. The acquisition of high resolution images of neurons provides an extremely useful method for testing the deleterious effects of neurodegenerative disorders and, in turn, provides a means to assess therapeutic strategies that target hippocampal neurons.

While the RTI method was utilized to capture in-depth data pertaining to the extent of arborization and topography, the EDFI method fails to provide information pertaining to the complexity of individuals segments or trees. Nevertheless, the benefits of the EDFI method lie in the fact that it does not require the purchase of expensive hardware and is less time consuming.

An image analysis program was used to perform EDFI in video files. Having access to high resolution and fast cameras allows the collection of a large number of images throughout the thickness of each field and generates images that truly represent the x, y, and z dimensions. One drawback of the EDFI method pertains to the fact that there could be more than one pixel (same x and y) focused in different planes throughout the z-axis. A method is envisioned to assign different colors to these pixels precisely representing the extent of 3D profiles in 2D images. It should be noted that the EDFI method could also be used for the analysis of cellular profiles (*e.g.*, synapses, microglia, and astrocytes). Moreover, this method can be used to analyze the extent of microglial distribution throughout the thickness of a section[7]. The two methods described here can be used in a complementary fashion. While the RTI method provides precise information on topography of branching pattern, the EDFI gives us a rather simple and expedited method to assess density of dendrites in an area of interest. Notably, the thickness of sections that can be acquired with EDFI are highly variable as the range of z focus on the microscope stage in combination with the optical focus of the microscope objectives dictate the boundaries of EDFI.

The methods described herein have the potential to be deployed in multiple contexts. In this study we provided a means to assess changes in dendritic arborization pre and post intervention, whereas EDFI was used to quantify microglial activation[7]. Thus, this technique is amenable to any application wherein the ultimate goal is to assess changes in small profiles that can be found in multiple layers.

Finally, these techniques can overcome lack of access to very high resolution and expensive digital cameras. In essence, a method was shown on how to capture serial images at different points of locations within a specimen and then stitch them together using a freeware, ultimately yielding high resolution images in a manner that is far more reliable and expeditious than conventional methods.

Critical steps

Golgi staining

Stock quantities of Golgi and Developing solutions are kept at 4 °C for storage and yet the staining and incubation protocols require the solution to be utilized at room temperature. Cooling the Golgi and Developing solutions on ice or in the fridge adversely affects the quality of dendritic staining. Moreover, it should be noted that we used fast-developing Golgi solutions. While the exact composition of the Golgi-Cox solution used

in this study is proprietary, there are multiple sources that list in detail protocol that can be used to perform Golgi-Cox staining[13]. However, the use of other Golgi-solutions will significantly alter staining durations and modifications for these steps should be made accordingly. Also, we along with others have used 150 µm thick sections to analyze DGC neurons[14,15] as it is generally agreed that cutting sections of this thickness coronally and in parallel to the dentate granule cell arborizations minimizes the risk of cutting dendritic branches.

DCX staining

The DCX staining protocol requires explicit attention to temperature. Failure to heat the solution to 37 °C during the preincubation period will significantly impair staining and result in loss of dendritic visualization. Moreover, attention to section thickness is warranted. In this study we used 70 µm thick sections to capture the maximum extent of DGCs dendritic arborization. In our experience, the use of 0.3% triton is a necessity as it significantly facilitates penetration of the antibody throughout the thickness of the floating sections. Indeed, prior studies have used triton to penetrate 70 µm thick[16] or even thicker (120 µm)[17] sections when label DGC dendrites. Please see Hussaini for full details of DCX staining[18].

Finally, it should be noted that alternative open source programs (*e.g.* flNeuronTool or Simple_Neurite_Tracer) exist with the ability to record the movement of the stage in x, y, and z directions. Such programs can be used for dendritic tracing as long as they are enabled to communicate with the scanning stage.

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