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Diolistic Labeling and Analysis of Dendritic Spines

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Abstract

Dendritic spines are highly diverse and plastic components of the neuronal cell apparatus and are highly responsive to trophic factors during both development and adulthood. Diolistic labeling of neurons with lipophilic fluorescent dyes, coupled with advanced high resolution microscopy methods, provides robust labeling of dendritic spines for assessment of their density and morphology. Here we describe a method for labeling of dendritic spines using diolistic labeling in ex vivo brain slices, visualization using confocal laser scanning microscopy, deconvolution, and analysis using the Surpass module of Bitplane Imaris software.

Keywords

Dendritic spine; Morphology; Lipophilic dye; Diolistic; Confocal imaging; Deconvolution; 3-D reconstruction

Introduction

Dendritic spines are highly dynamic, multifunctional and specialized elements of neuronal cell connectivity and signaling within the nervous system. Growth factors largely regulate dendritic spine dynamics, and underlie neurodevelopmental and other pathologies. Spines are comprised of actin cytoskeleton, an underlying structural architecture that is highly dynamic and changes in accordance with synaptic activity. Regulation of spine morphology involves several actin binding proteins, including drebrin which is itself regulated by an early growth factor, early growth response-1 (1). Interestingly, brain-derived neurotrophic factor and its receptor TrkB are both heavily involved in processes of synaptic plasticity (2,3). Recent findings indicate that individual spines contain autocrine signaling mechanisms that involve brain-derived neurotrophic factor release when stimulated. Additionally, individual spines are activated by TrkB, which controls structural and functional plasticity (4). Glial cell line-derived neurotrophic factor and its receptor GFR α 1 play an important role in dendritic growth and spine formation in the hippocampus (3,5). In several neurodevelopmental disorders, growth factors are mutated and lead to intellectual disability and stereotypies such as those that occur in Rett syndrome (6). Specifically, mutations in the CDKL5 gene, which codes for a putative kinase, have been shown to underlie

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neurodevelopmental pathologies (7). Importantly, dendritic spine stabilization is dependent on CDKL5; thus, loss of CDKL5 leads to a dramatic reduction in spine density. Insulin-like growth factor-1 rescued the loss of spine density and thus may be an important therapeutic avenue in patients with CDKL5 mutations (8). Additionally, neuregulin-1 is a pleiotropic growth factor that heavily influences synaptic plasticity in that optimal neuregulin-1 signaling is needed for spine morphological growth (9). Thus, advancing methods to examine dynamic changes in dendritic spine morphology is imperative to further understand the impact of neurotrophic factors on dendritic spine formation, growth, pruning, and differentiation.

Historically, many investigators impregnated neurons using a modified Golgi-Cox stain and analyzed under brightfield microscopy by means of manual or semi-automatic analysis computer-based algorithms (10). However, this approach is generally less amenable to 3-D reconstruction of neuronal cell morphology, and is difficult to use in conjunction with other labeling methods. In this chapter, we describe methods for diolistic labeling of neurons using the lipophilic 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) to impregnate cells for the analysis of dendritic spine morphology. The methods described below have been used by various laboratories (11-17), including our own (18-20). This method utilizes tungsten particles as a carrier mechanism for diolistic delivery of DiI; however, lipophilic dyes with different absorption and emission spectra such as 3,3'-dioctadecyloxacarbocyanine (DiO), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine (DiR), 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD) can also be used. In addition, when carrier particles are coated with a mixture of these lipophilic dyes, cells within the same brain area can be labeled and viewed using different excitation wavelengths (e.g., 488, 568, and 647 nm) to distinguish between different labeled cells. Although the emission spectra of DiI does have some “bleed-through” into wavelengths that are typically in the upper end of common green emission filters, in our experience, its emission spectra does not have significant overlap with wavelengths in the far red range, thus potentially allowing for multi-color labeling with other fluorophores.

Though the diolistic labeling technique allows for 3-D high resolution analysis of dendritic spines, there are some drawbacks to this methodology. For example, DiI does not dissociate between cell types or subtypes (e.g., dopamine D1 and D2-containing medium spiny neurons, neurons vs. glia, etc.), thus making such distinctions difficult without additional labeling techniques or the use of transgenic animals expressing fluorescent reporter proteins. Other dendritic spine labeling approaches allow for more ultra-precise cell labeling. For example, 6-amino-2,3-dihydro-1,3-dioxo-2-hydrazinocarbonylamino-1H-benz[d,e]isoquinoline-5,8-disulfonic acid (Lucifer Yellow) is introduced into cells via intracellular injection, which allows for cell-specific dye application and examination of neuronal cell structure following electrophysiological recordings (21). In contrast to DiI, an additional benefit to Lucifer Yellow is that fewer cells are labeled per brain region, and thus analysis is improved due to less interference from overlapping cellular processes. For all fluorescent labeling methods, cells are typically visualized using confocal microscopy and analyzed in 3-D using automated software programs including Imaris (Bitplane Solutions),

NeuronStudio, or MatLab. Such methods allow for unbiased classification of spines into subtypes, including stubby, thin, and mushroom, among others, or for precise quantitative measures of specific parameters such as dendritic branching, dendritic spine density and head/neck diameter, dendrite/spine volume, and many others. Here we provide a step-by-step protocol for DiI labeling and analysis, inclusive of materials and methodology for tissue preparation, DiI delivery, confocal imaging, and analysis.

2. Materials

Prepare all solutions using ultrapure water (18 M Ω -cm) and analytical grade reagents, and store at room temperature unless otherwise noted. Due to the carcinogenicity of paraformaldehyde, wear appropriate personal protective equipment and follow all appropriate chemical handling and waste disposal regulations.

2.1. DiI-containing “bullets”

1. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI; see **Note 1**)
2. polyvinylpyrrolidone (PVP, binding agent): add 200 mg PVP to 20 mL water

2.2. Tissue fixation

1. Paraformaldehyde (PFA, 1.5% w/v): dissolve 15 g PFA (granular, histology grade) in 900 mL water and heat to 55-60°C while stirring. Add 1-2 sodium hydroxide pellets and allow solution to clear. Add 100 mL of 1 M phosphate-buffered saline (PBS, pH=7.4). Allow solution to cool to room temperature and verify pH=7.2-7.4. Adjust pH with drops of 85% phosphoric acid as needed. Store solution at 4°C overnight prior to use. The amount of 1.5% PFA solution needed per animal is ~25 mL for mice and ~250 mL for rats.
2. PFA (4% w/v): prepare as described above for 1.5%, except utilize 90 mL water, 10 mL of 1 M PBS, and 4 g PFA.
3. Heparinized PBS: add 100 mL of 1 M PBS to 900 mL water. Add 20 mL of 1000 U/mL heparin to make a final concentration of 50 U/mL. The amount of heparinized saline solution needed per animal is ~10 mL for mice and ~100 mL for rats.
4. Anesthetic: 100 mg/mL sodium pentobarbital or ketamine/xylazine (see **Note 2**)

2.3. Diolistic labeling

1. Helium-pressurized diolistic labeling “gene gun” (e.g., Bio-Rad Helios® system, see **Note 3**) with modified 40 mm barrel (16).
2. Nylon mesh cell strainer filters (70 μ m pore size)
3. 22 $\times$ 22 mm microscope slide cover glasses (No. 1.5 thickness, see **Note 4**)
4. Anti-fade mounting medium (see **Note 5**)

2.4. Image acquisition and analysis

1. Confocal laser scanning microscopy system (methods provided here are specific to a Leica TCS SP5 spectral confocal system, but other systems can be used)
2. Deconvolution software (methods provided here are specific to AutoQuant X3 (Media Cybernetics) but other platforms can be used).
3. Image analysis software (methods provided here are specific to Bitplane Solutions Imaris v.7.4., but other platforms such as MatLab can be used).

3. Methods

Carry out all procedures at room temperature unless otherwise noted.

3.1. Preparation of DiI-coated “bullets”

1. Cut Tefzel tubing (2.36 mm ID × 3.17 mm OD) into 3 segments, each ~2 feet long, and place into tubing preparation station (see Fig. 1A).
2. Place one end of tubing into the PVP solution, and using a 30 mL syringe, pull ~2 mL of the solution into each piece of Tefzel tubing. Triturate 5-10 times to ensure coating of the entire inner surface of the tubing (**Fig. 1B**).
3. Apply nitrogen to tubing at a flow rate of ~0.4 L/minute for ~20 minutes.
4. Add 3.5 mg DiI to a 1.5 mL microcentrifuge tube.
5. Under a fume hood, add 100 µL of anhydrous dichloromethane to the tube and place on an orbital platform shaker.
6. Add 0.1 mg of 1.3 µm diameter tungsten particles onto a glass slide (Fig. 2A), and using a single-edged razor blade, “chop” and spread the tungsten particles evenly across the glass slide until the tungsten particles appear to be a fine powder
7. Pipette the DiI/dichloromethane mixture in 10- to 15-µL aliquots to the tungsten particles (Fig. 2B) and wait for the dichloromethane to evaporate and turn gray (~5 minutes).
8. Using a razor blade, scrape the DiI-coated tungsten particles onto a new glass slide and chop the particles until they appear to be a fine powder.
9. Carefully add all of the DiI-coated tungsten particles to 10 mL of the PVP solution and sonicate for 10 minutes with intermittent vortexing (solution will appear pink and foamy when it is ready).
10. Attach one end of a piece of the Tefzel tubing to a 30-mL syringe and draw the DiI/PVP into the tubing enough to fill the length.
11. Allow particles to settle for 15 minutes then rotate the tubing over to ensure coating of both sides of the tube, and allow particles to settle for another 15 minutes.

12. Slowly withdraw the PVP solution from the tubing and discard, making certain not to disturb the DiI-coated tungsten particles.
13. Slowly rotate the Tefzel tubing 360° to evenly coat the inside with the DiI-coated tungsten particles.
14. Dry the DiI-coated tubing for 20 minutes under 0.4 L/minute nitrogen gas.
15. Insert coated tubing into tubing cutter (Fig. 3A) and cut into 1.3 cm segments (“bullets”, Fig. 3B). Important: store bullets with a desiccant up to 1 month at 4°C in a light-proof container.

3.2. Perfusion, fixation, and sectioning of tissue

1. Anesthetize the animal (e.g., with intraperitoneal injection of sodium pentobarbital), and following loss of righting reflex, affix animal to dissection tray. Ketamine and other anesthetics can be used to deeply anesthetize the animal; however, ketamine is an N-methyl-D-aspartate-type glutamate receptor antagonist and thus may impact spine dynamics. Although this is a potential caveat to using ketamine, many studies have used this as an anesthetic and have found dynamic changes in spine morphology relevant to behavior (see *17-21*).
2. Perform tail or paw pinches to until no motor responses are observed.
3. Open the chest cavity and insert gavage needle into the left ventricle of the heart. Continue to insert the needle until the ball tip is visible at the base of the aorta.
4. Secure the gavage needle to the base of the left ventricle with hemostats and initiate perfusion of heparinized saline.
5. Clip the right atria with fine-tipped scissors to exsanguinate animal. Successful exsanguination is characterized by clearing of the liver to a light brown color. Perfuse ~10 mL for mice and ~100 mL for rats.
6. Commence perfusion of 1.5% PFA. Perfuse ~25 mL for mice and ~250 mL for rats.
7. Remove the brain from the skull and post-fix for 1 hour in 1.5% PFA on ice.
8. Using a razor blade, cut a flat surface for mounting the brain on a vibratome mounting block.
9. Use cyanoacrylate glue to secure the brain to the mounting block and allow it to dry for a few minutes.
10. Fill the microtome specimen bath and bath reservoir with ice-cold PBS.
11. Section the brain into 150-µm serial sections for each area of interest (Fig. 4). Using a paintbrush, carefully handle sectioned tissue. Do not use plastic-bristled paintbrushes, as these will puncture the lightly fixed tissue.
12. Place each section individually into a well of a 12-well tissue culture dish containing 2 mL PBS.

3.3. DiIolistic labeling of tissue sections

1. Secure 70- μ m nylon mesh filter at the head of the gene gun barrel to prevent large clusters of tungsten penetrating the tissue (Fig. 5).
2. Load the gene gun cartridge with DiI “bullets”.
3. Turn on helium tank and adjust pressure to 85 psi.
4. Using a paint brush, remove the section from the well and place it on a clean microscope slide.
5. At ~4 cm distance between the filter tip and the tissue section (Fig. 6), depress the trigger to activate helium pulse to propel the DiI-coated tungsten particles into the tissue. Change bullet after each labeled section.
6. Immediately following DiI delivery, return the section to the well containing PBS. The side of the tissue that received the DiI **MUST** be placed “face-up” on the slide for successful imaging.
7. Cover wells of tissue culture plate with lid, seal edges with Parafilm, and cover plate with aluminum foil.
8. Allow DiI to diffuse through the tissue for 24 hours at room temperature.
9. Aspirate PBS from each well and add 2 mL 4% PFA per well to fix DiI in place. Ensuring that each tissue section is fully submerged.
10. Wash sections 2×2 minutes in PBS.
11. Mount sections (1-2 per slide) on microscope slides using anti-fade mounting medium (*see Note 5*). Some investigators may wish to utilize square rubber spacers (120-140 μ m thickness, Electronic Microscopy Sciences) to surround the tissue section to avoid compression of the tissue section by the cover class.
12. Place individual cover glass (No. 1.5 thickness, *see Note 4*) on each slice while minimizing the presence of air bubbles. Rubber spacers
13. Lay slides flat and allow to dry overnight at 4°C.
14. Seal the edges of the cover glasses with clear nail polish, and store slides at 4°C until imaging (*see Note 5*).

3.4. Image acquisition

Neurons to be imaged should be spatially isolated from labeled neighboring neurons or other structures, and devoid of crossed fibers. For acquisition of images, we routinely utilize a Leica TCS SP5 spectral confocal system. Due to the variability in laser power, number of laser lines, objective numerical apertures, operating software, etc., the following information should be utilized as a general guide. System-specific imaging parameters should be determined empirically by the end user.

1. Acquire a z-series of images of the entire dendritic branch using a 10x objective. The following imaging parameters are recommended: excitation wavelength 488

nm, acquisition mode xy, no immersion oil, laser power 15%, resolution 512×512 pixels, zoom factor 1, frame average 1, scan speed 400 Hz, z-step size 2 µm.

2. Acquire a z-series of images using a 63x objective. Of particular importance are the numerical aperture of the objective (*see Note 4*), refractive index (RI) of the immersion medium (discussed in **Notes 5** and **6**), pixel size (determined by scan resolution and zoom factor, *see Note 7*) and optical thickness (determined by objective magnification and numerical aperture, as well as pinhole size, *see Note 7*). We typically find the following imaging parameters to be adequate for imaging dendritic spines in DiI labeled neurons: excitation wavelength 488 nm, acquisition mode xy, immersion oil RI 1.515 (*see Note 5*), laser power 25-30%, resolution 512×512 pixels, zoom factor ~3, frame average 4, scan speed 400 Hz, z-step size 0.21 µm. However, pixel saturation detection tools in various software packages can be utilized to guide the adjustment of gain, offset, and laser power.
3. Save z-series and image metadata in .lsm file format.

3.5. Image deconvolution

A number of software packages can be utilized for image deconvolution and manual or semi-automated analysis of dendritic spines. The method provided here utilizes AutoQuant X3 (Media Cybernetics) for deconvolution and the Filament Tracer module of Imaris v.7.4 (Bitplane Solutions). Since this software is capable of numerous types of cellular analyses that extend beyond analysis of dendritic spines, we include various screenshots to guide the user through the process.

1. Drag and drop the .lif file into the main window of Imaris. If the file contains numerous sets of images, select the one of interest.
2. Go the Edit menu, select Crop 3D. In the following window that appears, use the yellow box (denoted by arrows) to crop the image in the x, y and z dimensions to contain only the dendrite of interest (Fig. 7).
3. Go to File menu and save file in .icd/.ics format.
4. Open AutoQuant X3, and drag and drop file in .icd/.ics format into Data Manager window.
5. Click on the file name, and below the Data Manager window, the Summary tab will appear (Fig. 8).
6. The Spacings settings will be filled in automatically based on the resolution of the acquired images as well as the thickness of the z-step size. For the rest of the settings use Ch. 1= DiI (565 nm), Modality = Laser Scanning Confocal, Objective Lens = 60×1.4 NA, Num. Aperture = 1.4, Imm. Medium = Oil (1.51), and click Apply.
7. Open the View Task Batch window and drag the file from the Data Manager window to the Staging window under Batch Viewer. Select 3D Convolution from

the Task pull-down menu. If the image parameters have been properly set, a green check mark will appear under the “Valid?” column.

8. Use the arrows to move the selected files to the Pending window, which will then automatically transfer them to the Launched window.
9. When image processing is completed, the File will automatically be saved in .icd/.ics format, but the file name will have a “10” at the beginning (indicating that it has been deconvolved). The file size should be about twice that of the original cropped image.
10. For assessment and potential correction of spherical aberration, see **Notes 5 and 6**.

3.6. Dendritic spine analysis

1. Drag and drop the cropped and deconvolved image file into Imaris.
2. The image may need adjustments to the color balance if it is too dim. To adjust the color balance, go to the Edit menu and select Show Display Adjustment. Move the arrows as needed to adjust the brightness/contrast.
3. Go to the File menu and save as .ims (Imaris) format.
4. Set View mode to Surpass (Fig. 9). Utilize the Camera Pointer in Navigate mode to rotate the dendrite in 3D or use the mouse wheel to zoom in or out, and find the area of interest. Utilize the Camera Pointer in Select to select individual points on the dendrite. It will be necessary to toggle back and forth between Navigate and Select at various points in the analysis process.
5. On the left side of the screen, click on the leaf icon to Add New Filament (Fig. 10).
6. In the window that appears, Click Skip Automatic Creation, Edit Manually. Under Algorithm Settings, select Calculate Diameter of Filaments from Image. In the Methods window, select Autopath and Dendrite, which allows for automatic following the path of the dendrite segment (Fig. 11).
7. Change Camera Pointer to Navigate and zoom in to region of interest.
8. At the starting point of the dendritic segment of interest, right-click while depressing the Shift key (Fig. 12). A gray circle will appear. Next, move cursor to the end of the segment of interest. A yellow line will form. At the desired endpoint left-click while depressing the Shift key. Using the scale bar at the bottom of the window, verify that the yellow line spans a sufficient length (typically 40-80 μm). Also, ensure dendrite is free of crossovers of other dendrites or fibers.
9. Click Selection as Starting Point, then click on Edit (pencil icon) tab (Fig. 13).
10. In the Process Selection box (Fig. 14), click Center. This will center the line in 3-D within the selected dendritic segment.

11. On the Style menu (Fig. 15), click on Settings (leaf icon) tab and then select Cone. Next, click on the Edit (pencil icon) and under Mouse Selects, choose the Filament option. Then click on the line in the dendrite (when selected it will be yellow in color, when not selected it will be gray).
12. In Process Selection menu (Fig. 16), select Diameter...
13. A dialogue box will appear (Fig. 17) which allows for adjustment the diameter of the yellow line. Set the parameters as follows: source channel = 1, small diameter = 0.3, large diameter = 3.7, contrast threshold = 1.3.
14. The yellow line will now have a varied diameter. In the menu above, adjust the small and large diameter settings (which are the minimum and maximum diameters of the dendrite) until the yellow line fills the dendrite but does not overflow into the spines (see Fig. 18 for correct and incorrect line diameter adjustments). Also verify filling of only the dendrite shaft in Navigate mode by zooming and rotating image, and adjust as necessary.
15. In the Style menu (Fig. 19), select the Draw (paintbrush icon) tab, and change Filament Type from Dendrite to Spine. Also change method to AutoDepth. This will allow to selection and measurement of individual dendritic spines.
16. Using the Camera Pointer, choose Select. Move cursor to the base of spine, and while depressing the Shift key, left click at the base of an individual spine, hold mouse button down, and drag cursor to the tip of the spine (Fig. 20). When the mouse button is released, the spine should appear as a blue filled object. It may be necessary to toggle between Select and Navigate in order to closely view the dendrite and spines from various angles. To delete an erroneously drawn spine, simply click on the blue object and press the Delete key. Repeat this process for all spines visible on the dendritic segment. Toggle between Navigate and Select modes to visualize dendrite and spines from various angles to ensure all spines have been highlighted.
17. In the Styles menu (Fig. 21), select the Edit (pencil icon) tab, change Mouse Selects to Filament. Click anywhere on dendrite and it will turn yellow. Click Invert. This will highlight all spines in yellow for adjustment. Note that spines are overfilled; this will be adjusted in the next steps.
18. Under Process Selection (see Fig. 16), click Center to center the filling of dendritic spines, then click Diameter to adjust fill parameters.
19. In the dialogue box that appears (Fig. 22), adjust the following settings until spines are appropriately filled: Channel (always set at 1), small diameter (minimum 0.143), large diameter (maximum 10.5), and contrast (always set at 3)
20. In the Styles menu, under the Edit (pencil icon) tab, set Mouse Selects to Point (see Fig. 21A).
21. Left click on the end of dendritic segment and it will turn yellow (Fig. 23A). In the Process Selection menu, select Assign as Dendrite Beginning Point (Fig. 23B), and the end of the dendritic segment will turn into a blue sphere. This

establishes the end point of the dendritic segment being analyzed, which is necessary in order for calculation of dendrite spine count and other quantitative variables.

22. In the main Surpass mode menu (Fig. 24), select the Filaments 1 folder to designate it as the data to be exported. Below, select the Settings (leaf icon) tab and ensure that Cone is selected (Fig. 15A).
23. On the main analysis window, select the Graph tab and below, click multiple disk icon to save all data into a single Excel file (Fig. 25).
24. Save file as Excel format (.xls). Each parameter dendrite spine density, spine terminal point (head) diameter, spine diameter, spine length, spine volume, spine terminal point (head) volume, etc. will be stored in a separate tab.

4. Notes

1. Our laboratory typically utilizes DiI which has peak excitation/emission wavelengths at approximately 549 and 565 nm, respectively. This allows for viewing neurons in the “red” channel of most microscopes which typically contains reduced artifact from lipofuscin-based autofluorescence. However, the contrast of red-emitting fluorophores may be more difficult to visualize for some investigators, in which case green-emitting fluorophores such as DiO can be utilized. When multiple labeling is needed, fluorophores such as DiD and DiR, which emit in the far red and infrared ranges, can be used to minimize spectral overlap.
2. Sodium pentobarbital is a Schedule IV controlled substance and thus necessitates appropriate licensing and recordkeeping as mandated by local and federal authorities. Non-controlled substances such as volatile anesthetic can be used as alternatives but require proper ventilation and a deep plane of anesthesia to be reached prior to initiation of perfusion procedures.
3. The BioRad Helios® “gene gun” is a commercially available hand-held instrument designed for pressurized helium-based delivery of fluorescent dye-coated particles. It should be noted that recently several investigators reported on the successful design and implementation of a laboratory-built diolistic labeling apparatus (22), which offers more flexibility for customization of individual labeling methods as well as a more economical option for some laboratories.
4. We find that cover glasses with No. 1.5 thickness (0.17 mm) are ideal when utilizing high magnification (e.g., 63x) objectives with a high numerical aperture (e.g., NA=1.4). It is also important to note that cover glasses with thickness-restricted tolerances (e.g., ± 0.005 mm, Carl Zeiss Microscopy) offer more optimal resolution and consistency than cover glasses with less restrictive tolerances from common scientific vendors (e.g., ± 0.2 -0.3 mm). Finally, it is important to be aware of the RI of the cover glass, which should closely match that of the mounting medium and immersion oil utilized when imaging (*see Note 5*).

5. One of the most common causes of blurring and spherical aberration during serial confocal image acquisition is a mismatch between the RI of the mounting medium and that of immersion oil used during image acquisition. This can result in distortion of neuronal cell elements that are imaged, resulting in unreliable measurements of dendritic spine head/neck diameter, spine counts, etc. Recently, Petersen et al. (23) systematically investigated different combinations of various mounting media and immersion oils when acquiring z-series images of fluorescent microspheres and DiI-labeled dendrites and spines. Calculated point-spread function (PSF) reports of full width and half maximum values in the lateral (x-y) and axial (z) directions were observed to be the lowest when an immersion oil with an RI of 1.515 (e.g., Type LDF, Cargille Laboratories) was used in conjunction with FluorGlo mounting medium (Spectra Services). FluorGlo has an RI of 1.42 in its liquid state. However, as with other anti-fade mounting media (e.g., ProLong Gold, ThermoFisher), applied FluorGlo cures and hardens over days or weeks, causing a gradual rise in the RI. Thus, optimal image acquisition may be delayed for several days or weeks until RI mismatch is minimized. Recently, Fouquet and colleagues observed reduced axial blurring with the use of CFM3 (Citifluor Ltd.), a mounting medium with a refractive RI of 1.518 upon initial application (24). Overall, proper RI matching should be tailored to the needs and resources available in each laboratory. However, some axial blurring can be corrected during image deconvolution, as described in **Note 6**.
6. Autoquant allows for some correction of spherical aberration by adjustment of PSF settings. This is typically accomplished by conducting Z-scans of fluorescent nanobeads (100-120 μm diameter, available from various commercial vendors), utilizing the identical laser power, laser power and excitation/emission wavelength ranges, objective, mounting and immersion media, cover glasses, z-step size, image resolution, etc. as used when acquiring z-series images from labeled tissue. Then, deconvolution of tissue images is performed and deviation from the theoretical PSF parameters can be viewed and adjusted. For a detailed description of the theoretical basis, approaches, and limitations to spherical aberration correction, see (25).
7. Although the capacity of axial scanning depth of most confocal microscope systems is typically up to 100-200 μm , the image quality is often not preserved at focal points deep within the tissue. If considerable axial blurring occurs when dendritic segments are imaged deep within the tissue section, it may be more optimal to acquire images from segments located more superficially within the tissue section.
8. In addition to the issues noted above, image quality is dependent on numerous other factors, including line and frame averaging, zoom factor, objective characteristics, pinhole size, pixel and voxel resolution, and sampling frequency based on Nyquist and other theorems. The biophysical basis of these issues are technically complex and beyond the scope of this chapter for adequate discussion. Fortunately, Dumitriu and colleagues (26) recently provided excellent

recommendations on optimization of imaging parameters for dendritic spines. Using a 100× 1.4 NA objective, these investigators recommend cubic voxel sizes of 50 nm³, which is equivalent to a z step size of 0.05 μm. However, in our experience imaging dendritic spines with a 63× 1.4 NA objective, this results in significant photobleaching, and we find optimal image quality using a frame average of 4, pinhole setting of 0.65, 512 × 512 xy pixel resolution, and a Z-step size of 0.21 μm.

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Figure 1.
(A) Tubing preparation station (BioRad). (B) Filling of Tefzel tubing with PVP solution via withdrawing it from the microcentrifuge tube with a syringe.

Figure 2.

(A) Use of a razor blade to finely chop tungsten particles on a glass microscope slide. (B) Appearance of tungsten particles after addition of dichloromethane.

Figure 3.
(A) Placement of coated tubing into tubing cutter (BioRad). (B) Appearance of bullets, which should be stored in a light-proof container with a dessicant at room temperature.

Figure 4. Sectioning of 1.5% PFA-fixed brain tissue on a vibratome. Coronal brain sections through the dorsal and ventral striatum can be observed at the bottom of the photograph, one of which overlying the bristles of a paintbrush which is used to manipulate the sections.

Figure 5.
BioRad Helios gene gun with modified barrel and attached nylon mesh filter.

Figure 6.
Proper placement of gene gun barrel with filter tip for labeling of tissue section.

Figure 7.
Cropping of the acquired dendrite image in the xy (upper arrow) and z (lower arrow) axes.

Volume	288 x 319 x 38
Time Points	1
Spacings	0.14996 x 0.14996 x 0.20981 (µm)
Ch 1:	Dil (565 nm) ▼
Modality	Laser Scanning Confocal
Objective Lens	60x 1.4 NA Oil
Num. Aperture	1.4
Imm. Medium (RI)	Oil (1.515)
Sample Med. (RI)	Glycerol (1.47)
Dist. From Coverslip	0

Figure 8.
Example of image parameters in Summary tab of Data Manager window in AutoQuant.

Figure 9. Selection of Surpass mode and option for using the mouse pointer in Select mode (for individual points on dendrites) or Navigate mode (for 3-D rotation of image).

Figure 10.

Add New Filament icon for creation of selected dendritic segment.

Figure 11.

Manual selection of dendritic segment, selection of Autopath mode of detection, and assignment of selection as a dendrite.

Figure 12.
Selection of start and end points of dendritic segments for analysis.

Figure 13.
Assignment of selected starting point as a segment.

Figure 14.
Centering the line within the selected dendritic segment.

Figure 15.
Style and Edit menus for filament selection.

Figure 16.
Process selection menu for selection of spine diameter settings.

Figure 17.
Spine diameter settings (minimum, maximum, and threshold).

Figure 18.
Correct and incorrect filling of dendritic shaft with the analysis line.

Figure 19.
Style menu for initiating spine analysis using Autodepth method.

Figure 20.

A. Selection of the terminal point of an individual dendritic spine. B. Filling of individual spine (blue color). C. Filling of all spines.

Figure 21.

A. Inversion of dendritic selection color from yellow to gray. **B.** Selection of all dendritic spines as indicated by a change from blue to yellow fill color.

Figure 22.
Appropriately filled dendritic spines. Not the variation in thickness of spine heads, necks, and dendrite attachment points.

Figure 23.

A. Selection (A) and assignment (B) of dendrite segment end point. C. Marked dendritic segment endpoint.

Figure 24.
Selection of filament data set for export.

Figure 25.

A. Selection of data analysis tool. **B.** Exporting all dendritic spine data to Excel.