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Sparse labeling-enabled confocal imaging of dendritic spines followed by analysis

 Forked from [Perfusion and fixation of brain tissue for fresh frozen sections followed by immunofluorescence staining](#)

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We use this protocol and it's working

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Abstract

This protocol describes all the procedures starting from sparsely labelling neurons to analyzing their dendritic spines, including stereotaxic viral injection, brain perfusion and fixation, brain sectioning and mounting, confocal imaging and spine reconstruction using Imaris software.

Materials

Intracranial surgery

- Anesthetic: isoflurane
- Anesthesia machine (Smiths Medical) with connector tubing, induction chamber and filter canisters for isoflurane waste
- Scales to weigh the animals
- Stereotaxic surgery frame and scope (David Kopf Instruments)
- Light source to illuminate the surgery area (e.g., Fiber-Lite Mi-150 or Mi-LED_{A2})
- Sterile surgery tools (forceps, fine scissors, needle holder as needed)
- Sterile drape
- Heating pad and temperature probe
- Hair clipper
- Ophthalmic ointment
- Non-steroidal analgesic (e.g. Metacam)
- Sterile 0.9% saline
- Syringes with needle (0.3-1 ml)
- EMLA cream or bupivacaine line block
- Antiseptics: povidone-iodine swabs and 70% ethanol swabs
- Sterile cotton swabs
- Non-toxic food dye
- Drill with foot pedal and sterilized drill bit
- Viral stock solution
- Sterile PBS as needed
- Ice bucket
- Parafilm
- 1-3 ml syringes without needle and with connectors/tubing that can be connected to glass pipettes
- Suture material
- Sutures
- Antibiotic ointment
- Glass micropipettes (Drummond Scientific) pulled with P-97 glass puller (Sutter Instruments). It is recommended to add some volumetric references on the pipettes based on their specifics.
- Blade
- Microforge (e.g., Narishige MF-830)
- Post-surgery care: clean empty mouse cage on heating pad for recovery; clean mouse cage with extra gel food/treats/protein drink for post-surgery holding.
- Biohazard bin
- Sharps container

Perfusion and fixation

- Anesthetic (ketamine 50 mg/Kg and xylazine 4.5 mg/Kg – varies according to institutional protocols)

- Peristaltic pump (e.g., Gilson) with tubing and connectors
- Dissection tools (scissors, fine scissors, spring scissors, tweezers, spatula, according to preferences)
- Dissection tray
- 50ml falcon tubes
- 15ml falcon tubes
- PFA stock solution (e.g., 16% PFA solution, Electron Microscopy)
- 10X PBS
- Perfusion needle (preferred: 27 gauge)
- Solid PFA waste collection bin
- Liquid PFA waste collection bin
- Water wash bottle
- Carcass bag
- Platform shaker (e.g. Heidolph Unimax 1010)

Tissue sectioning

- Vibratome (e.g., Leica VT1200S with accessories)
- Blades that fit with the vibratome
- Tools (tweezers, spatula, petri dish, according to preferences)
- Glue
- PBS
- PBS with 0.1% sodium azide
- 24-well plates
- Glass pipette with smooth end (burned with fire) or fine brush
- Parafilm
- Aluminum foil

Sections mounting

- Reusable petri dish (recommended PYREXTM Reusable Petri Dishes: Bottoms Only, Cat# 08-748D, Fisher Scientific)
- Microscopy slides
- Glass coverslips
- PBS
- Glass pipette with smooth end (burned with fire) or fine brush
- Hard-drying mounting medium (recommended ProLong Diamond Cat# P36961, ThermoFisher Scientific)
- Designated container for storage of microscopy slides

Confocal imaging

- Confocal microscope (e.g., Nikon AXR)
- Immersion oil that works with the specific objective lens



Spine reconstruction

- Nikon NIS-Elements software (e.g., version AR 5.41.02)
- Imaris software (e.g., version 10.0.0)

Safety warnings

Intracranial viral injection

Recommended PPE:

- Lab gown/disposable gown
- Face mask
- Examination gloves (sterile gloves)
- Bouffant Caps
- Shoe cover

During the surgery, it is very important to keep the isoflurane level low while the animal is being deeply anesthetized, to minimize human exposure to the isoflurane which can have acute and chronic health effects.

Perfusion and fixation

Recommended PPE:

- Performing the procedure and handling PFA under a fume hood is strongly recommended, and mandatory in some cases - varies according to institutional protocols.
- Lab gown/disposable gown
- Face mask
- Face shield/goggles
- Examination gloves (cut-resistant gloves are recommended)

Brain sectioning and mounting

Recommended PPE:

- Lab gown/disposable gown
- Face mask
- Safety goggles
- Examination gloves and cut-resistant gloves

Confocal imaging

Recommended PPE:

- Examination gloves

Ethics statement

The protocols.io team notes that research involving animals and humans must be conducted according to internationally-accepted standards and should always have prior approval from an Institutional Ethics Committee or Board.

Intracranial Viral Injection - Before the Procedure

- 1 Pull glass pipettes to be used for delivering virus or marking bregma, lambda and injection sites.
 - 1.1 For pipettes to be used for viral injections, cut the tip with a blade or another pipette so that the inner diameter is within ~2-4 scales (~5-10 μm) under the 40x objective with a 15x eyepiece of a Microforge and the tip is relatively smooth and blunt. For pipettes to be used for marking, the tip diameter can be much bigger.
 - 1.2 Mark the injection pipette with volumetric references according to the calibration of the pipette type.
- 2 Prepare virus. Get an aliquot of virus out of freezer and put it on ice in a bucket. For sparsely labeling neurons, dilute the virus (e.g., Cre-dependent eGFP AAV) to a titer of $\sim 5 \times 10^{10}$ viral genomes/ml with sterile PBS and vortex the mixture. The titer can be varied depending on the virus and the sparsity needed.
- 3 Prepare a clean empty mouse cage on a heating pad and a clean mouse cage with gel food, treats or protein drink for post-op care.
- 4 Turn on equipments (anesthesia machine, stereotaxic reader, light source, drill, heater). Set up sterile working area including stereotaxic frame. Set up surgery tools on a sterile drape.
- 5 Weigh mouse.

A2a-Cre driver line can be used with diluted Cre-dependent eGFP AAV to sparsely label indirect-pathway striatal projection neurons.
- 6 Anesthetize mouse in induction chamber (recommended: 5% isoflurane, 200ml/min flow rate).
 - 6.1 Once mouse breathes very slowly or gasps, stop isoflurane and flux oxygen or air to the chamber to expel remaining isoflurane before opening the chamber and taking the mouse out.
- 7 Hair over surgery area can be quickly clipped before transferring the mouse onto the stereotaxic frame. If mouse breathes fast, can put mouse back to the induction chamber and repeat above step.
- 8 Switch on the isoflurane for the stereotaxic and put mouse on the heating pad of the stereotaxic frame, gently pull the tongue to the side to avoid choking, put upper teeth into the hole of tooth bar and cover nose/mouth with the nose cone.

- 9 The heating pad settings should be adjusted so that the temperature probe placed under the mouse should read a body temperature between 33-37 °C.
- 10 Apply ophthalmic ointment over eyes.
- 11 Inject appropriate volume (based on mouse weight and desired dosage) of analgesic according to institutional protocols; an appropriate amount of saline can also be injected to prevent dehydration during the procedure.
- 12 Once the mouse is deeply anesthetized (~1 breath/second, no pain reflex with toe pinch), carefully insert and secure the ear-bars and tighten the screw for the nose cone. The position of the mouse head will be verified and adjusted once the skull is exposed, but it is recommended to make sure that the head is not visibly tilted and easily moved. Recommend to keep the numbers on two ear bars equal. Tooth bar can be adjusted closer or further from mouth if ear bars not entering the ears and not hitting the hard structure.

Note the anesthesia status of the mouse needs to be checked periodically during the whole surgery time and adjust the isoflurane level as needed.
- 13 Clean the area of the incision with the povidone-iodine swab followed by the ethanol swab, repeat 2 more times.
- 14 It is preferred to apply line-block anesthetic (0.15% bupivacaine) under the skull skin before starting the procedure rather than applying EMLA cream on the sutured skin at the end of the surgery.

Intracranial Viral Injection - the Procedure

- 15 With the fine scissor, expose the skull by making an anterior-posterior incision.
- 16 Clean the skull surface with cotton swabs and visually identify bregma and lambda.
- 17 Fill a glass pipette with a small volume of non-toxic food dye, mount it on the stereotaxic arm holder and lower it onto the skull.

- 18 Mark bregma by gently touching the intersection of the coronal/sagittal sutures with the pipette tip, and zero the coordinates on the reader.
- 19 Lift up the pipette and move it to lambda (intersection of lambdoid and sagittal sutures) and measure its position relative to bregma.
- 20 Minimize the deviation of dorso/ventral (D/V) and medio/lateral (M/L) distance between lambda and bregma by adjusting the position of the head. Depending on the type of stereotaxic frame used, this can be done through adjusting the ear bars and tooth bar height, or rotating the frame (e.g., when using KOPF Model 1900).
- 21 Re-zero the coordinates at bregma and repeat bregma/lambda measurements until satisfactory. Recommend to keep the differences within 0.05 mm.
- 22 It is recommended to use the measured anterior/posterior (A/P) distance between bregma and lambda to calculate an adjustment factor for the final coordinates: the measured B-L distance will be divided by the reference distance of 4.2. For an adult mouse, the obtained value ("adjustment ratio") should be close to 1, and in this case no coordinates adjustment is required (but still optional). For smaller mice, the reference coordinates should be multiplied by the calculated adjustment ratio to obtain the final coordinates for the specific mouse.
- 23 Move the pipette to the spot indicated by the adjusted A/P and M/L coordinates and mark it.
- 24 Whether performing uni-lateral or bi-lateral injections, it is recommended to mark the spots on both sides of the skull, and to measure their relative dorso-ventral position. Their relative D/V deviation should be minimized by adjusting the position of the head.
- 25 Once the desired spot has been marked, the marker pipette can be removed, and a hole (~1mm diameter) is drilled in the skull at the indicated position.
- 26 Blood and debris are cleaned with sterile saline and sterile cotton swabs. Use a bent syringe needle to carefully remove dura.
- 27 Insert micropipette with volumetric references and connect it to a syringe (recommended: 3 ml syringe) to apply positive/negative pressure.
- 28 Place a piece of parafilm over the skull and put a couple of microliter viral solution on it.

- 29 Lower pipette into the viral solution and draw up desired volume of viral solution in the syringe by applying negative pressure, then release the pressure and discard the parafilm. Make sure the tip is inside the viral solution for the whole time of loading to avoid drawing up bubbles.
- 30 Lower pipette loaded with the viral solution onto the bregma first and zero the coordinates.
- 31 Lift the pipette and move it to the injection site. The pipette should be able to go through the tissue under the hole without bending. If it bends, move the pipette away and clean the hole.
- 32 Gradually lower the pipette tip into the brain until the desired dorso-ventral coordinate is reached.
- 33 Replace the 3 ml syringe to a 1 ml syringe. Slowly inject the desired volume of viral solution (recommended: ~150nl/min) by gently and gradually applying positive pressure to the syringe.
- 34 Release pressure and leave the pipette in position for ~5-10 min so that the viral solution can spread and be absorbed by the tissue.
- 35 Slowly retract viral injection pipette and discard it in an appropriate waste collection bin.
- 36 Inject some sterile saline to the skull surface and clean it with a cotton swab.
- 37 Suture Skin.
- 38 Use the povidone-iodine swab to disinfect the suture.

Intracranial Viral Injection - After the Procedure

- 39 Remove animal from stereotaxic frame and place it in the clean, empty cage on heating pad until deambulatory in about 5-10 minutes.
- 40 Once awake and deambulatory, mouse can be moved to the clean cage with food and water, also on heating pad.

- 41 Clean up the working area and sterilize/autoclave surgery tools.
- 42 24 hours after the surgery, a second dose of Metacam is administered and antibiotic ointment is applied on the sutured skin.
- 43 The health status of the mouse is monitored over the following days. If needed, additional doses of Metacam or saline can be administered.
- 44 Mouse is normally kept in a cage on heating pad for at least 4 days and is then moved to standard housing.
- 45 Mice are sacrificed for experiments at least 4 weeks after surgeries.

Perfusion and Fixation - Before the Procedure

- 46 Set up equipment and Solutions:
 - PBS can be prepared from 10X concentrated solution
 - 4% PFA solution is prepared by diluting the concentrated PFA stock with PBS. For better results, it is recommended to prepare a fresh 4% PFA solution in PBS right before the procedure.
- 47 Under the hood, pour PBS and PFA solutions in 50 ml tubes inserted in a ice bucket.
- 48 Attach the perfusion needle to the connector at the end of the tubing. Start running PBS through the tubing.
- 48.1 If possible, it is recommended to have a connector system with a switch that allows to pre-load the PFA solution and the PBS solution in the respective collection tubing and quickly switch from one to the other avoiding the need to move a single collection tube from one solution to the other, interrupting the procedure.

Perfusion and Fixation - the Procedure

- 49 Terminally anesthetize the mouse according to institutional protocols.

- 50 Bring the anesthetized mouse to the dissection tray and verify that the mouse is fully anesthetized. This can be performed by pinching one of the posterior paws and observing the presence (or lack of) pain reflex. The mouse must be fully anesthetized before starting the trans-cardiac perfusion.
- 50.1 Once full anesthesia is achieved, the mouse can be positioned on the dissection area and needles can be inserted in its paws to avoid movement.
- 50.2 The mouse should be positioned in a supine position, with the head oriented away from the operator. If the operation area is slightly inclined, the mouse should be oriented so that the head is facing downward.
- 51 Holding the skin just below the sternum with a tweezer, cut the skin just below, exposing the peritoneal cavity and the rib cage. The diaphragm should remain intact.
- 52 Expand the cut with the scissors to cut the fascia connecting the skin to the rib cage. Once the rib cage is clearly visible, carefully cut the diaphragm without damaging the beating heart. Cut the chest cage and lift it toward the head. A needle can be used to hold it in position while operating. The liver should be visible in the abdominal cavity.
- 53 Carefully insert the needle connected to the peristaltic pump (where PBS is circulating) in the left ventricle of the heart, and rapidly pinch the right atrium with the spring scissor. Dark-red blood should start flowing out of it immediately. Hold the needle in position, while the solution washes out the blood from the mouse.
The heart should still be beating. A wash water bottle can be used to remove excess blood and see more clearly.
- 54 After perfusing 20 ml of PBS and the liver turns whiter, shift the perfusing solution to PFA.
- 54.1 Maintain the needle in position and keep perfusing.

Perfuse mouse with 30 ml of PFA solution.

As PFA reaches the tissues, some appendages of the mouse might appear to be moving or contracting. This normally indicates that the fixation is working. If the perfusion is done correctly, this should start shortly after changing the perfusion solution to PFA.
- 55 Once 30 ml PFA has run through, stop the perfusion, remove the needle and release the mouse. The carcass should be very stiff.



- 56 Decapitate the mouse with the scissor.
- 57 With the fine scissors, cut the skin and expose the median line of the skull. Cut off the posterior part of the skull. Then, carefully cut along the median line, towards the rostral part of the head. Past bregma, apply two diagonal cuts toward the eyes, and two other later cuts along the lambdoidal sutures.
- 58 Carefully open the skull with the help of the tweezers and expose the brain.
- 59 With the tweezers, gently remove the brain and slide it into the 15ml falcon tube filled with 10 ml PFA. Close the falcon tube and gently shake it.

Perfusion and Fixation - After the Procedure

- 60 Disposal & Clean up:
 - Properly dispose of the mouse carcass in the carcass bag.
 - Dispose of all the PFA waste (liquid and solid) and sharps according to institutional guidelines.
 - Clean/wash all the tools/equipment.
- 61 Keep the brain in PFA for post-fixation for 1.5-2 hours at 4°C. Shaking is recommended.
- 62 Once the post-fixation time has passed, transfer the brain with a spatula to a new 15 ml falcon tube filled with 10 ml PBS containing 0.1% sodium azide.
- 63 Store brain for a variable of days at 4°C until sectioning. To minimizing fading of fluorescence in brain cells, we recommended storing the brain under dark and sectioning/imaging the brain in a few days to a month.

Brain Sectioning

- 64 Set up the vibratome with blade. This vibratome should be specific for PFA use.
- 65 Mount brain onto specimen plate using glue.



- 66 Place specimen plate into the slicing chamber and adjust specimen orientation as desired.
- 67 Mount the slicing chamber on the vibratome and fill the slicing chamber with PBS.
- 68 Cut the sections (50-60 μm thick) and collect the sections in a 24-well plate filled with PBS containing 0.1% sodium azide. It is important that the sections are collected consecutively in the correct sorting. Recommend to make room light dimmer when possible to avoid bleaching off the fluorescence.
- 69 After the brain region of interest has been sectioned, seal the 24-well plate with parafilm, wrap it with foil to avoid light, then store it upright at 4°C.
- 70 Remove the specimen plate from the slicing chamber and remove the remaining brain and glue.
- 71 Clean the vibratome according to the manufacturer's manual. Clean the tools.

Brain Sections Mounting

- 72 Fill a reusable glass petri dish with PBS.
- 73 Transfer a few brain sections to be mounted on one glass slide to the dish using a glass pipette with smooth end (burned on fire), or a brush.
- 74 Hold the end of a glass slide with white coating and immerse the remaining part into the PBS, gently bring one section onto the desired position of the glass slide using the glass pipette, then lift the slide with glass pipette holding the section in place. Can adjust the position by immersing the slide and section again.
- 75 Repeat the above step until all sections transferred to the glass slide.
- 76 Wipe the liquid off around the sections as much as possible, then leave the slide on the table with foil covered or place the slide into a drawer for a few minutes until no major liquid can be seen on or around the sections.
- 77 Squeeze mounting medium into a 0.5 ml tube, then slowing pipette up ~75 μl medium and drop that on all sections and the spaces in between sections, forming a line. Make

sure not to drop bubbles onto the sections.

- 78 Hold one end of the coverslip, allowing the other end touching the furthest section first, then slowly lower down the coverslip until touching all the sections and medium evenly spread across all sections.
- 79 Let slides dry in a drawer/under dark for 24 hours then transfer the slides into a slide container and store it at 4°C.

Confocal Imaging

- 80 Turn on the equipments and open the software.
- 81 Mount the slide with sections onto the stage, up side down for inverted scope.
- 82 Use low magnification objective (recommended 20x) and eyepiece to find the region of interest.
- 83 Take the slide off the stage, switch the objective to 60x (recommended 1.42-1.49NA lens), put a drop of oil onto the objective, then mount the slide back onto the stage.
- 84 Find a isolated dendrite that can be traced back to the soma using eyepiece and software.
- 85 Measure with the software to find a segment of ~30 μm long and ~30 μm away from the soma for the near end.
- 86 Optimize settings. Use 256×512 to just include the dendrite of interest. Generally use 1-10% for power and 20-35% for gain but it depends on the brightness of sections and confocal used. Use 1.0 airy unit and Nyquist if there are such options. Use ~6.4ms dwell time and average 8 times. Check saturation to make sure no signals saturated.
- 87 Set the top and bottom levels to capture z-stack images. Use 0.125 μm as step size.
- 88 Capture the images.
- 89 After finishing imaging, remove the slide, wipe the oil off from both objective and slide with a lens paper and further wipe them with lens cleanser-dipped lens paper.



- 90 Close the software and turn off the equipments.

Spine Reconstruction

- 91 Open NIS Elements software and load Nikon z-stack images.
- 92 Denoise and deconvolve the Nikon images: under NIS ai choose 'denoise.ai' to denoise, then deconvolve with 'automatic' deconvolution.
- 93 Open Imaris 10.0.0 software and load denoised and deconvolved images.
- 94 Add Surface. Uncheck 'classify surfaces', 'object-object statistics' and 'start creation with slicer view', or can just load favorite creation parameters at this step (caution sometimes it doesn't work well when applying parameters to different images). Click blue next.
- 95 Smooth→surfaces detail, use 1x pixel size would give a tighter (better) surface. So use 0.09 µm for images captured with Nyquist (0.094).

Check 'Labkit for pixel classification' then click blue next. A new window will appear. The idea is to go through different z planes to train what are negative and positive signals. Click the pencil tool on top, click background (blue) on left, draw some blue scratches on the background area (scroll to a different plane will make the scratches disappear). Keep the brush size at 1 (thicker line will penetrate to other z levels). Then click foreground (red) on left, zoom in by pressing Ctrl and Shift, scroll center mouse scroll wheel, draw on spine necks as well as spines and dendrite, pan around with right click under pan (top left arrows) mode, go back to check background and draw lines right outside the dendrite. (can undo drawn lines by selecting the 'blob' sign, then left click on the drawn lines, also can 'erase'/brush size can be increased) Then click 'labkit pixel classification' on middle left, and click the setting sign next to it. In the new window, check after 'use GPU acceleration', then click ok. Then click the black play button next to the setting. A small window 'training in progress' will show. After that is done, blue (noise) and red (signal) will be shown (if red covers a bigger area than actual signals, just means a bigger surface later and not a big deal, but it is suggested to keep the surface tighter). If the spine neck is still not shown in red (click 'segmentation' eye sign on and off to check if the spine neck is included), draw more lines and train again. Do 2-3 rounds (checking different z levels to make sure; more time needed for better result). When satisfied, click 'compute result and send it to Imaris' on bottom left. (For more info on using labkit, refer

to <https://www.dropbox.com/s/g6bxn8c6hu26bva/2022-03-16%20-%20Labkit%20Demo%20tutorial%20video.mp4?dl=0>

If you don't have Labkit, Imaris support recommends using this version of Fiji for Labkit pixel classification that contains the necessary update sites:

<https://www.dropbox.com/s/ilesetv2hywx6yc/Fiji.app.zip?dl=0>. Place the unzipped Fiji folder on the C drive in a location that is not in the Downloads folder and link the Fiji.exe location within Imaris under File > Preferences > Custom Tools. Do not update this version of Fiji. Then do the above step in the last paragraph.

- 96 Back to Imaris, switch between slice view and volume view to check if spines are connected now. If importing the saved parameters for surface, then it will skip the Fiji step. If still not connected, you can draw the spine later. Not check 'enable', click blue next.
- 97 In the bottom left, filter type, can use any but can just use default 'Number of voxels lmg=1' (play with the threshold to leave out other junks). Click green next.
- 98 If just need to analyze part of the dendrite, go to edit tab, under pointer selection mode on top right, navigate to the part to cut, hold shift and left click to draw a blue line, then select 'cut surface' on left, repeat for the other location, but make sure the blue line not falling on other parts of dendrite, rotate the image to avoid the cuts on other sites.

On top right, click the circle selection tool, Ctrl+scroll to enlarge the circle, then select those that are not considered part of the dendrite/spines interested, don't select those disconnected spines. Go to the bottom left edit tab, click 'delete', do multiple rounds to delete all. Can use Ctrl+hold left and move to select many. Note those deleted do not look like 3D shape as those kept. If accidentally deleted the spines to be kept, Ctrl+Z to undo. If not deleting all other stuff, that's fine and the image just does not look so clean and also needs more work for later training.

Then at bottom left, still under the edit tab, select the part to analyze, hold shift and left click to select those disconnected spines also, then click 'mask selection' on bottom left. Use the default setting in the new window, then click ok. Click on the channel (top right) to rename or recolor. To add or delete a channel, under 'edit' menu, click 'add channels' or 'delete channels'.

- 99 Check the first mask channel as well as unchecking surfaces 1 on top left, then the selected dendrite/spines will be shown.
- 100 Add filament. Select the last detection type (without blue starting point and with green spines). Uncheck 'object-object statistics'. Click blue next.

- 101 Uncheck surfaces. Back to filament, in 'seed points for segments' (for dendrite) appeared, select the correct mask channel to analyze. Check 'multiscale points', put values for thinnest diameter and largest diameter after measuring them, follow pro tip or type value. Click blue next.
- 102 Adjust seed points threshold to make sure seed points are distributed throughout the dendrite and also sizes/positions are correct (can be edited later, so not necessary to adjust here). It is ok if seed points on spines and that will be classified later. Seed points need to be at the ends of the dendrite segment to be analyzed. If not, in the slice rendering mode, follow the pro tip to manually add seed points there, adjust the box/seed point size by Ctrl and center scroll mouse wheel. To delete one circle, the box size needs to be bigger than the circle. Leave 'classify seed points' checked.
- For 'segment diameter smooth strength', if you want to make sure the segment diameter is dynamic across filaments to reflect that, use lower smooth strength. Click blue next. The default can be fine.
- 103 Classify seed points. The manually added seed points may not be shown but they are there. Select those on spines to discard, and select those on the dendrite to keep (follow pro tip to swipe selection). Train and predict. Do a final check in the end and manually edit if needed. When satisfied, click blue next.
- 104 A 3D dendrite structure will be shown. If there are some extra parts than the main dendrite, just select and discard. Click blue next and the structure will be smoothed. Click blue next.
- 105 Spine detection. Select the correct mask channel. Measure the size of the thinnest spine head (e.g., 0.188) at the brightest section by Ctrl and center scroll mouse wheel to autofill the thinnest diameter. For maximum length, measure the length of the longest spine at slice view then fill the value (e.g., 5). If there are no branched spines, then don't check 'allow branch spines'. Recommend to keep the parameters consistent across dendrites. Click blue next.
- 106 Adjust seed points threshold to make sure points on all spine heads (can add new dots with shift+left click). Should try to have seed points on the end of the spine heads, otherwise 3D cones may not cover the whole spine. Click blue next.
- 107 Classify spine points. Follow the pro tip to select those on spine heads to keep and just keep one point per spine head. Select those on dendrite or elsewhere to discard. Train and predict. Do a final check in the end and manually edit if needed. When satisfied, click green next.
- 108 If some spines are still not detected, manually add/draw them. Click the draw tab (third tab) on bottom left, select the correct source channel, check 'spine', 'automatic diameter' and 'automatic center'. When pointing to 'autopath', you can see multiple options and



follow the second option. Switch to slice rendering mode, navigate to the center location where the spine should be added, start at the base and shift/ctrl/right click to restart and shift/left click to end. It is ok if the start point is inside the segment since the only part counted as spine is what's outside the segment. The newly added spine will be shown in the stats→detailed→Filament No. Spine Segments. If a spine is not drawn/connected correctly, under edit tab and correct channel, can select it then 'delete', then go back to the draw tab to redraw it.

109 The 3D structure is usually not good enough to capture the actual size and so to classify spines later, specifically outlines are bigger than actual signals when viewing under slice rendering mode, and that should affect the statistics. So you should edit the filament (segment+spines). Version 9.9 Imaris does a better job than version 10 so can use the old version but keep consistent for all dendrites. Select the 'filaments 1' on top left, click the edit tab to edit segments or spines. Select the correct source channel. At bottom left near 'filaments' (without any one spine highlighted in yellow), click 'center' ~3 times (going through centering algorithm), then click 'diameter' ~3 times to correct the positioning and sizes of the cones for the whole 3D structure. If just want to edit one or some spines, click to select one spine or Ctrl+left click to select a few (will be highlighted in yellow), select the correct source channel, near 'selection', click 'center' ~3 times, then click 'diameter' ~3 times. Note 'diameter' value above is the smallest diameter for the spine that should be used.

110 When satisfied, go to tools tab (last tab), select 'classify spines'. Can change the rules for each spine type. Click 'classify spines' and different spine types will be colored differently. On top left, can check/uncheck each spine type to show/unshow those spines.

111 To look at statistics, with 'filaments 1' or 'filaments 1 classified spines' highlighted and checked, on bottom left, select the Statistics tab. Click Detailed tab for detailed measurements.

To export all the data, click 'export all statistics to file'. That will generate a folder containing a number of excel files for each type of variables.

Go to help menu→statistics reference for more info. For example, click on filament (module of interest) to look for the definitions. Go to file→ preferences→statistics→spine to add more spine measure when needed.

112 To calculate spine density, use the total number of 'Spine Part Mean Diameter Head' values (number of spines) from the excel file ending with Mean_Diameter_Head to divide the 'Segment Length' value (dendritic segment length) from the excel file ending with Segment_Length.

113 If you want to use the same parameters for other images or batch processing, go to the creation tab, click 'store parameters for batch' for later importing and analysis. Can leave



both Arena (for batch processing) and favorite creation parameters (for one at a time) checked. Can save in the end or another time as long as this whole file is saved. Same for surface including Labkit.

Protocol references

<https://www.protocols.io/view/mouse-stereotaxic-surgeries-for-intracranial-viral-81wgby191vpk/v1>