Video Article

Assessment of Hippocampal Dendritic Complexity in Aged Mice Using the Golgi-Cox Method

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Abstract

Dendritic spines are the protuberances from the neuronal dendritic shafts that contain excitatory synapses. The morphological and branching variations of the neuronal dendrites within the hippocampus are implicated in cognition and memory formation. There are several approaches to Golgi staining, all of which have been useful for determining the morphological characteristics of dendritic arbors and produce a clear background. The present Golgi-Cox method, (a slight variation of the protocol that is provided with a commercial Golgi staining kit), was designed to assess how a relatively low dose of the chemotherapeutic drug 5-flurouracil (5-Fu) would affect dendritic morphology, the number of spines, and the complexity of arborization within the hippocampus. The 5-Fu significantly modulated the dendritic complexity and decreased the spine density throughout the hippocampus in a region-specific manner. The data presented show that the Golgi staining method effectively stained the mature neurons in the CA1, the CA3, and the dentate gyrus (DG) of the hippocampus. This protocol reports the details for each step so that other researchers can reliably stain tissue throughout the brain with high quality results and minimal troubleshooting.

Video Link

The video component of this article can be found at https://www.jove.com/video/55696/

Introduction

Dendrites are the largest portion of neurons that receive and process presynaptic input[1]. Their dendritic processes have a complex geometry, where the proximal branches have a larger diameter than the distal branches. As dendrites develop, they form several connections with other neurons in a process referred to as dendritic arborization. The extent and pattern of this branching determines the amount of synaptic inputs that a dendrite can adequately process[2].

Dendritic arborization is a necessary process for activity-dependent plasticity and proper development of neuronal circuits. Extension, retraction, branching, and synaptogenesis are intricate processes that include intrinsic genetic programs and influences from extrinsic factors. The morphological and branching variations of the neuronal dendrites within the hippocampus are implicated in cognition and memory formation[3,4]. The alterations in dendritic complexity are associated with pathophysiological and behavioral changes[5]. Abnormalities are related to several disease states, including Fragile X Syndrome and Down Syndrome[6].

Dendritic spines are the specialized subcellular compartments of the dendritic arbors that receive excitatory input within the central nervous system. There are three morphological classes of dendritic spines, with the name of each class based on their size and shape: 1) mushroom spines, which have complex postsynaptic densities with more glutamate receptors than other spines[7]; 2) stubby spines, which lack a stem; and 3) thin spines, which consist of a protracted, narrow stem and a globular head[8]. The dendritic spine volume is used in part to define them, with thin spines generally smaller ($0.01 \ \mu m^3$) compared to mushroom spines ($0.8 \ \mu m^3$)[9,10]. The spines stabilize with maturation. For example, the thin spines either retract after a few days or develop into mushroom spines. Alternatively, the mushroom spines are relatively stable and can survive for an extended period. The strength of the neuronal connections is thought to be based on the number of spines and/or their volume[11,12,13].

The classical Golgi staining method and its more modern variations have all been useful for examining the dendritic spine morphology and density. One unique aspect of the Golgi staining is that it randomly stains about 5% of the total neurons, which allows for the tracing of individual neurons[14,15]. Although the exact mechanism in which the Golgi method stains individual neurons is still unknown, the principle of the method is based on the crystallization of silver chromate (Ag_2CrO_4)[16,17]. There are three main types of the Golgi method: the rapid Golgi, the Golgi-Cox, and the Golgi-Kopsch[18,19]. All three methods start with an initial incubation phase in chromium salts for several days to months, but there are certain key differences between them. The rapid Golgi uses osmium tetroxide in the first step, whereas the Golgi-Kopsch includes paraformaldehyde. The staining in both the rapid-Golgi and the Golgi-Kopsch is followed by an incubation in a 1-2% silver nitrate solution for about 7 days. The Golgi-Cox method uses mercuric chloride and potassium dichromate instead of silver nitrate and has an impregnation time of 2-4 weeks. The tissues are then sectioned and quickly placed in a diluted ammonia solution, followed by a photographic fixer to remove salts.

Of the three types, the Golgi-Cox method is thought to be the best at staining the dendritic arbors without much background interference, in part, because the crystal artifacts do not occur on the surface of the tissue (unlike in the rapid Golgi method)[17,20,21].

The present method is a slight variation of the protocol provided with a commercial Golgi staining kit, and was designed to assess how a relatively low dose of the 5-Fu would affect the dendritic morphological characteristics and the spine density. Any data acquired could provide further insight into how chemotherapeutic treatment affects the neuronal circuitry.

Protocol

Experiments were conducted in accordance with the ethical standards approved by the Institutional Animal Care and Use Committee at UAMS.

1. Animals and 5-Fu Injection Paradigm

1. Purchase 6-month-old male C57Bl6/J wild-type mice and house them under a constant 12 h light/dark cycle until they reach 1 year of age.
2. Dilute the 5-Fu in 0.9% sterile saline. Use 60 mg/kg as the required dose per mouse.
3. **Give the intraperitoneal injections of the 5-Fu (once per week for three weeks).**
    1. Give the intraperitoneal injections around the same time on each day. For example, between 0900-1200 h.
4. Euthanize the animals and extract the brains 30 days after the final injection.

2. Euthanasia Procedure and Brain Extraction

1. Restrain the rodent and grab the base of the tail. With the other hand, place the thumb or first finger at the base of the rodent's neck. Quickly put pressure on the rodent's neck and push forward while the other hand holding the tail pulls backwards.
2. Hold the rodent with one hand and the large surgical scissors with the other. Place the rodent's neck between the two blades and quickly decapitate the head.
3. Take the severed mouse head, and use the fine scissors to trim the fur above the skull, up to the eyes. Next, place the tips of the scissors into each eye socket and close the scissors with slight force.
4. Use spring scissors to cut the skull to the left and right of the cerebellum.
5. Use forceps to lift the portion of the skull surrounding the cerebellum directly up and off.
6. Use the spring scissors to cut along the midsagittal line of the skull.
7. Use the forceps to lift the remaining portion of the skull off the brain.
8. Use a spatula and a probe to scoop out the brain into the desired container. Using a stainless steel blade, cut each brain along the midsagittal plane. Perform the following Golgi-Cox method on the right hemispheres.

3. Golgi Staining and Tissue Preparation

1. Immerse the freshly harvested half-brains into 5 mL mercuric chloride-based solution (solution A from the kit) in a 10 mL conical tube. The mercuric chloride solution is light-sensitive; cover the tube with foil. Store the sample in the dark at room temperature.
   NOTE: Solution A is provided in the kit that is listed in the table of materials. Caution! Solution A (also called mercuric chloride solution) contains toxic reagents such as potassium dichromate, mercuric chloride, and potassium chromate. Wear protective gloves and work in a fume hood.
2. After 1 day, slowly decant the solution into a temporary container (such as a large weigh boat), and dispose of it in a biohazard waste container. Immerse the brains (still in the original 10 mL conical tubes) with 5 mL of the mercuric chloride solution, and return the sample to the dark at room temperature for 13 more days.
3. **Add 30 g post-impregnation buffer to 90 mL distilled or deionized water (dH$_2$O). Fill each well of a 6-well plate with 6.5 mL of the post-impregnation buffer, one well per brain**
   NOTE: Post-impregnation buffer is provided in the kit that is listed in the table of materials.
    1. After 14 days (in total) in the mercuric chloride solution, rinse the tissue with dH$_2$O, then transfer them into 6-well plates with post-impregnation buffer. Cover the plates with foil and store them at room temperature in the dark.
4. Pipette out the post-impregnation buffer after 1 day of immersion and renew with fresh post-impregnation buffer; store for 1 day in the dark at room temperature. If required, store this at 4 °C for up to 1 month[22].

4. Sectioning

1. Label the 12-well plates on their lids with numbers in ascending order from left-to-right and up-to-down.
2. **Prepare two or three 12-well plates per brain if sectioning the entire brain.**
    1. Label the top of each 12-well plate lid with the identification number of the brain sectioned.
    2. Pipette 2 mL of 1x PBS into each well of each plate.
3. **Set up the stage and turn on the vibratome; ensure that there is enough 1x PBS in the container to cover the tissue.**
    1. Cut two pieces of plastic paraffin film and fold them over twice. Place them over the holes for the specimen holder knobs to prevent 1x PBS from leaking onto the counter.
    2. Put tape down on the tissue block of the specimen holder and then put cyanoacrylate adhesive glue (see table of materials) on it.

4. **Remove the brain from the 6-well plate and place it on a plastic or glass dish. Use the stainless steel double-edged blade to cut off about half of the cerebellum.**
   1. Cut the cerebellum caudally. Make sure that the portion of the cerebellum remaining is even.

5. **Immediately place the flat surface of the remaining cerebellum on the glue.**
   NOTE: The dorsal part of the tissue (the cerebral cortex) should be facing left or right relative to the blade. The rostral end that previously contained the olfactory bulb attached should face upwards.
   1. Wait at least a few minutes to make sure that the glue fully dries.

6. **While the glue that is holding the brain in place is drying, pour 1x PBS into the specimen bath. After the glue is dry, place the specimen holder with the tissue into the specimen bath.**
   1. If the tissue sample is not fully covered, pour more 1x PBS into the specimen bath.

7. Attach the blade to the blade holder of the vibratome and slowly lower the blade into the specimen bath until it is fully submerged in 1x PBS. Continue lowering the blade until it is slightly below the top of the tissue.

8. **Set the vibratome speed to 7, the amplitude to 6, and the cutting angle to 12° (see table of materials for vibratome model). Start the vibration and cut 200 μm sections[23].**
   1. Use a large paintbrush to move the tissue sections from the specimen bath into each designated well of the 12-well plates.

9. Continue to cut the tissue until the desired number of tissue sections is reached.

5. Post-staining

1. For each of the following staining steps and rinses, use 2 mL of the indicated solution per well. Use a motorized pipet filler (see table of materials) to transfer the solutions into the wells. Use a transfer pipette (see table of materials) to transfer solutions out of the wells and into the proper waste containers.
2. Rinse the sections in one 30 min wash of 0.01 M PBS-T (add 3.0 mL of detergent (see table of materials) to 1,000 mL 0.01 M PBS).
3. **Dilute ammonium hydroxide solution (Caution) 3:5 by volume with dH$_2$O immediately before use. Stain the sections in the diluted ammonium hydroxide solution for 19-21 min.**
   1. Protect the light-sensitive ammonium hydroxide solution with foil.
   NOTE: Ammonium hydroxide within solution has a concentration of ~10% and it is classified as "hazardous." Ammonium hydroxide solution may produce burns if it contacts the skin. It may cause respiratory tract irritation if inhaled. Wear protective gloves and work in a fume hood.
4. Stain the sections in the post-staining buffer (add 90 g of reagent D in 500 mL dH$_2$O) for 19-21 min. The post-staining buffer is light-sensitive; protect it with foil[22].
5. Rinse the sections in three, 5-10 min washes of 0.01 M PBS-T.

6. Mounting, Cleaning, and Covering

1. Mount the sections on 1% gelatin-coated slides with the large paintbrush. Allow them to dry for 20-30 min at room temperature, and then place them in a Coplin jar overnight.
2. Remove the slides with gloved hands from the Coplin jar and place them in a plastic rack (see table of materials). Place the plastic rack in the staining dish (see table of materials) containing 100% ethanol. Dehydrate them with three 5 min washes.
3. Wash the slides twice for 5 min each in 99% xylene. Place the slides in a glass rack and lower the rack into a glass staining dish containing xylene with a metal handle (see table of materials).
   NOTE: Caution, Xylene is harmful if inhaled and causes irritation if it touches the skin. It is highly flammable in liquid and vapor states. Wear protective gloves and work in a fume hood.
4. **Remove the slides from the xylene one at a time and quickly cover the tissue with ~0.25 mL of mounting media (see Material Table). Next, take the slide covers and lay them over the media.**
   1. Push any trapped air bubbles underneath the slide covers to the edge with a blunt object, such as the end of a paintbrush.
5. Place the slides in an area away from sunlight and allow them to dry for 2-3 days.
6. Proceed to acquiring images using a microscope and analyzing with the appropriate software.

7. Acquire Image Stack

1. Open the imaging program (see **Materials Table**). Place the slide onto the stage of the microscope. On the menu at the top of the program, click "Acquisition" and then click "Live Image."
2. Set the microscope to 10X. Identify the neuron of preference and then bring the cursor, which appears like a red X, onto the center of the soma. Left click to set the reference point.
3. Set the microscope to 40X. Click "Move" from the menu at the top of the program and then click "To Reference Point."
4. **Begin creating the image stack by manually scrolling with the fine objective above the tissue until it is slightly out of focus.**
   1. Click "Set Top" in the bottom right hand corner of the program under the heading "Image Acquisition."
   2. Scroll slightly under the tissue until it is slightly out of focus and then click "Set Bottom" under "Image Acquisition." Next, click "Acquire Image Stack."
5. **After the image stack is acquired, type the desired name for image file in the window that appears. Save as "MBF Ascii file."**

1. When prompted, select the file type as "MBF JPEG2000 stack file" and then click "Save."

8. Neuronal Tracing

1. In the toolbar underneath the menu, click the box titled "Contour Name." Select "Soma" from the drop down menu.
2. Manually trace the soma. Then right click and select "Finish Cell Body" when tracing is complete.
3. Select "AutoNeuron" to bring up another tab titled "AutoNeuron Workflow."
4. **Under "Configuration Type," select "New." Set the parameters and then click "Next Step" to bring up "Soma Detection" subheading.**
   1. To set the parameters, select "Brightfield". Next, under "Max Process Diameter", click "Start Measuring". Using the cursor, go to the base of the dendrite and manually set the diameter.
5. Click "Yes" in the window that requests the user to trace the entire image. Manually trace the soma. Unclick and then click "Next step" to bring up "Seed Placement" subheading.
6. Click "Validate Seeds" and then click "Next Step" to bring up "Neuron Reconstruction" subheading.
7. Under "Neuron", click "Interactive." Perform interactive tracing by following the direction from the program of the dendrites and right-clicking to complete the highlighted area traced by the program. After tracing all the dendrites, click "Next Step."
8. After a new window appears, save the new configuration with a desired title. Click "Save."

9. Analysis

1. Open the analysis program (see the table of materials).
2. Click "File" from the top menu and then click "Open Data File". Select the file of interest.
3. Under the analysis subheading, select "Sholl Analysis."
4. In the "Sholl Analysis" window, set the starting radius to 10 µm. Under "Analysis," click the boxes "Dendrites" and "Branch Orders."
5. Right click the newly opened windows and click "Excel Export." Click "Save."
6. Under the analysis, click "Branched Structure Analyses." Select "All Possible Analyses."
7. Right click the newly opened window and click "Excel Export"[24].

Representative Results

The effects of the 5-Fu treatment on the dendritic arborization and complexity in the hippocampus of Golgi-stained brain sections were quantified and traced using a commercially available imaging software. After tracing, the dendritic arborization, the spine density, and the spine morphology were analyzed using Sholl analysis and the dendritic complexity index (DCI). Sholl analysis is a quantitative analytical method that can be used to determine the dendritic arbor morphology[25]. Starting from the soma, circles 10 µm apart from each other overlay the dendritic tracings. The length of the dendrites is determined by the number of circles that the dendrites cross over. The branch point analysis, a method to ascertain the complexity of a dendritic arbor, is based on the total number and order of branch points.

A branch point is defined as when a branch splits into two sub-branches. The branch order is a measure of the complexity based on how many times the branches divide. For example, a first-order branch point would be the original dendrite extending from the soma, while a second-order branch point would be the point at which the branch divides into two sub-branches. By examining both the branch points and the branch order, the complexity of the dendritic arbor (based on the number of branch points) and at what points the branching occurs (a smaller branch order is closer to the soma, while a larger branch order is further from the soma) can be determined. These parameters were applied to the DCI. The CA1 and CA3 were divided into apical and basal portions and analyzed independently[26,27].

The dendrites of the CA1 and CA3 pyramidal neurons and granule neurons in the DG were analyzed. All neurons selected for each experimental group fulfilled the following criteria: 1) the dendrites had dark and consistent Golgi staining across their entire length, 2) the dendrites were visibly in-tact, and 3) each neuron had enough space between them to prevent interference during the analysis[28]. **Figure 1** demonstrates a neuron that fulfills the above criteria. Data were expressed as mean ± SEM. To determine the statistical differences between the sham and the 5-Fu groups, a paired two-tailed t-test was used. All the statistical analyses were conducted using analytical software (see table of materials), and $p <0.5$ was considered significant. A mixed-factors analysis of variance (ANOVA) was used to evaluate the effects of the 5-Fu treatment and the Sholl radius. Fisher LSD post-hoc tests were used following ANOVA when appropriate[29].

In both CA1 ($t = 7.68$, $p <0.01$; **Figure 2A**) and CA3 ($t = 7.54$, $p <0.01$; **Figure 3A**) apical pyramidal dendrites, there was a significant overall reduction in spines following the 5-Fu treatment. While the 5-Fu treatment did not significantly affect the basal spine density of CA1 ($t = 1.79$, $p = 0.15$; **Figure 2B**), it did significantly decrease CA3 basal pyramidal dendritic spines ($t = 5.57$, $p < 0.05$; **Figure 3B**). Furthermore, the Fisher LSD post-hoc tests revealed a decrease in the dendritic arborization of the CA1 apical pyramidal dendrites at 40-100 µm (Fisher's LSD, $p < 0.001$; **Figure 2C**) and 130-160 µm (Fisher's LSD, $p <0.05$; **Figure 2C**) away from the soma compared to the saline treated controls. A similar phenomenon was seen in the CA1 basal apical dendrites at 30-60 µm (Fisher's LSD, $p < 0.001$; **Figure 2D**) and 70-110 µm (Fisher's LSD, $p <0.05$; **Figure 2D**) away from the soma[29].

Regarding the CA3 apical pyramidal dendrites, there was not a significant difference in the segmental dendritic length following the 5-Fu treatment compared to the saline treated controls ($F (28,87) = 0.91$; $p = 0.59$; **Figure 3C**). However, for the CA3 basal pyramidal dendrites, there was a significant difference between the 5-Fu treatment and the saline treated controls in the segmental dendritic length ($F (25, 78) = 1.85$; $p <0.05$; **Figure 3D**). The Fisher's LSD revealed that the 5-Fu treatment decreased the dendritic arborization at 90-100 µm ($p <0.01$; **Figure 3D**), 70-80 µm and 110-120 µm (Fisher's LSD, $p <0.05$; **Figure 3D**) away from the soma[29].

To determine the dendritic complexity, a branch order analysis was used to compare the 5-Fu treated and the saline treated control groups. Within the DG, the branch order analysis showed a significant difference between each treatment and branch order ($F_{(8, 36)} = 25.61$, $p < 0.001$; **Figure 4**). This was further analyzed using the Fisher's LSD, which indicated that there was decreased length at the 4th and 6th order following the 5-Fu treatment ($p < 0.001$; **Figure 4**)[29].


Figure 1: Golgi stained neuron that represents the criteria for imaging and further analysis. All three spine types can easily be seen following the Golgi staining. Staining is consistent across the entire length of the dendrite and the dendrite is isolated from other neurons. Scale bar, 5 μm. Please click here to view a larger version of this figure.
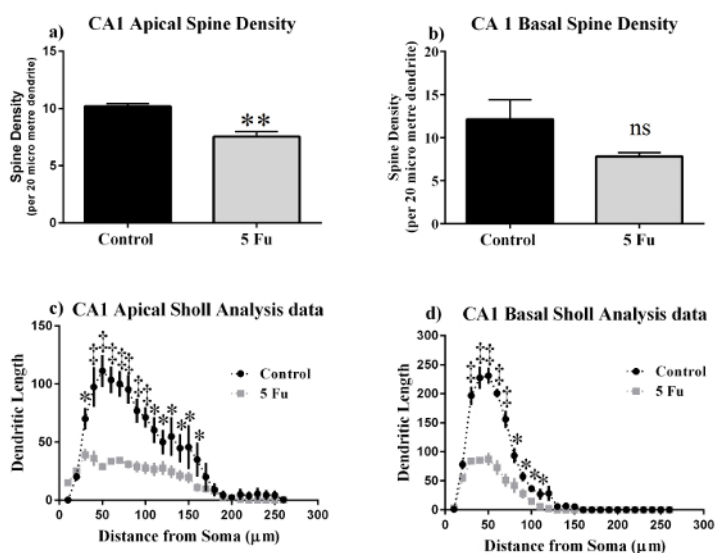
Figure 2: Golgi stained neurons demonstrated significant differences in spine density between the 5-Fu treated and the saline treated groups in the apical CA1, but not the basal CA1. There was a significant difference in the spine length between 5-Fu treated and saline treated groups in both apical and basal CA1. (**a**) In the CA1 apical pyramidal dendrites, 5-Fu significantly decreased spine density. (**b**) In the basal dendrites, there were no significant changes in the overall density of spines. (**c**) Sholl analysis revealed that the 5-Fu treatment significantly decreased dendritic arborization at 40-100 μm and 130-160 μm away from the soma in the CA1 apical pyramidal dendrites. (**d**) In the basal dendrites, the 5-Fu treatment decreased the dendritic arborization at 30-60 μm and 70-110 μm away from the soma. Average ± SEM (n = 6). *p <0.05; **p <0.01, ANOVA[29]. Please click here to view a larger version of this figure.
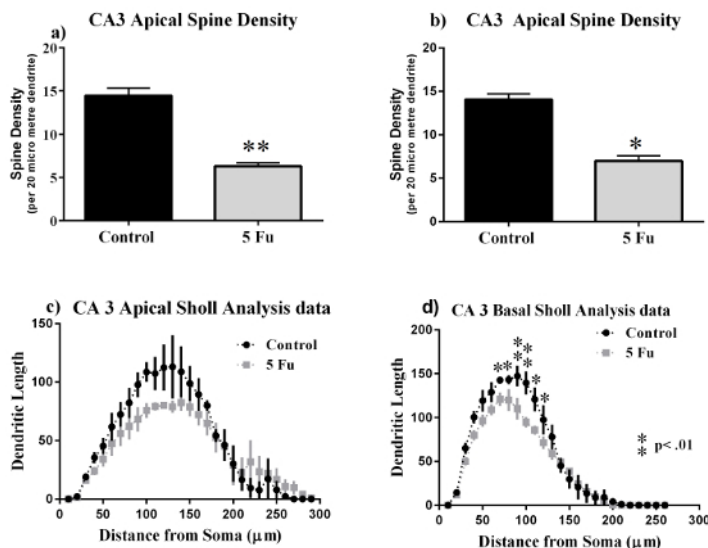
Figure 3: Golgi stained neurons demonstrated significant differences in spine density between 5-Fu treated and saline treated groups in both apical and basal CA3. There was a significant difference in spine length between 5-Fu treated and saline treated groups in apical CA3, but not basal CA3. (**a**) In the CA3 apical pyramidal dendrites, 5-Fu significantly decreased spine density. (**b**) In the basal dendrites, 5-Fu significantly decreased the overall density of spines. (**c**) There was no significant effect of 5-Fu treatment in the CA 3 apical area. (**d**) In the basal dendrites, 5-Fu treatment decreased dendritic arborization 70-120 µm away from the soma. Average ± SEM (n = 6). *, $p <0.05$; **, $p <0.01$, ANOVA[29]. Please click here to view a larger version of this figure.
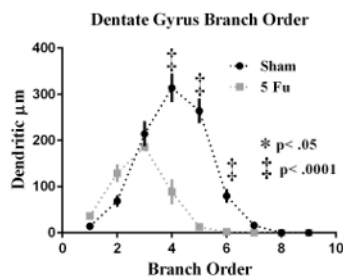
Figure 4: Golgi stained neurons within the DG showed decreased length at the 4th and 6th order following 5-Fu treatment compared to the saline treated group. Length per branch order in pyramidal neurons of the CA1 hippocampal region. Average ± SEM (n = 6). *$p <0.05$; **$p <0.01$, ANOVA[29]. Please click here to view a larger version of this figure.

Discussion

Compared to more modern techniques, the Golgi-Cox method has several advantages that make it the preferred method for examining spine morphology: 1) The staining can be used for essentially any tissue, 2) A basic light microscope setup is all that is needed to acquire Golgi-based images, 3) The Golgi-Cox imaging is faster than confocal imaging, and 4) Golgi stained sections are viable for several months to years longer than samples that are fluorescently-labeled. Even with these advantages, the Golgi-Cox method still has certain limitations. First, the entire process is very time consuming, requiring several weeks of staining followed by analyzing the images. As seen with this protocol, it takes 14 days in solution A and 1 day in solution B before the sectioning can begin. Additionally, performing sectioning, post-staining, and mounting will take 2-3 hours per brain. Each slide must dry for 1 day before cleaning and covering them. After which, the amount of time it will take to image and analyze the sections will depend on the individual. Second, there may be issues of consistency between the data from analysts due to differences in categorizing spines[30]. For this reason, it is recommended that one person performs the imaging and analysis portion of each experiment, which further extends the amount of time for completion of the project.

There are several critical steps of the Golgi-Cox method that require exact timing and attention. When staining the sections with the ammonium hydroxide solution, it is imperative that the sections remain in the solution for at least 19 min, but not over 21 min. If the sections are not in the ammonium hydroxide solution for enough time, they will not be properly stained, meaning that the neurons will look less defined when imaging, thus making it harder to analyze them. If the sections are in the ammonium hydroxide solution for over 21 min, they may become over-stained, thus making it difficult to identify the individual neurons. Therefore, when switching out the solutions, the researcher must move quickly. Another important step is the transfer of the sections onto slides. Before moving the sections with the large paintbrush, it is best to first add a small amount of the PBS-T onto the slide. This allows the sections to easily be placed onto the slide and maneuvered without potentially breaking apart. One more critical step is the drying step after the sections are placed on a slide. If the slides do not dry for at least 20 min at

room temperature, then certain sections will most likely fall off during the dehydration steps. If the slides dry for longer than 30 min, they will most likely become brittle and break apart during the dehydration steps.

When performing this procedure, there are certain steps that may be troublesome. For example, if the entire cerebellum is cut off before placing the brain on the specimen holder and sectioning, the brain may crack apart before the DG is fully sectioned, rendering any further sections unusable. Therefore, it is important to only cut caudally through about half of the cerebellum. Another step that may negatively affect the sections is the vibratome speed and the amplitude. While the recommended settings are for 7 and 6 respectively, upon cutting, certain sections may disintegrate in the PBS or appear uneven. To correct this, finely adjust either the vibratome speed or the amplitude, one at a time until the sections are in-tact and even, between settings of 6-8.

Even though commercial kits for Golgi staining are available, they often lack exact details for each individual step. With this protocol, we report in detail all the steps that were used to allow other labs to reliably stain tissue at a high quality with minimal troubleshooting. The materials used are available to most neuroscience labs. Aberrations in dendrite morphology and alterations in the number of dendritic spines are related to a multitude of neurological disorders[31]. Furthermore, these perturbations of the dendritic arborization might represent a significant morphological hallmark of the chemotherapy induced injury in the brain[32]. In the future, we will examine how the structural alterations induced by 5-Fu may relate to the behavioral, cellular, and molecular changes.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

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