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Corresponding Author:	Brandon Pekarek UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	brandon.pekarek@bcm.edu
Order of Authors:	Brandon Pekarek
	Patrick Hunt
	Gary Liu
	Benjamin Arenkiel
	Benjamin Belfort
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TITLE:

Imaging and Quantification of Intact Neuronal Dendrites via CLARITY Tissue Clearing

AUTHORS AND AFFILIATIONS:

Brandon T. Pekarek[1], Patrick J. Hunt[1,2], Benjamin D. W. Belfort[1,2], Gary Liu[2], Benjamin R. Arenkiel[1,3]

[1]Department of Genetics and Genomics, Baylor College of Medicine, Houston, TX, US.
[2]Medical Scientist Training Program, Baylor College of Medicine, Houston, TX, US.
[3]Department of Neuroscience, Baylor College of Medicine, Houston, TX, US.

Email addresses of co-authors:

Brandon T. Pekarek	(Brandon.Pekarek@bcm.edu)
Patrick J. Hunt	(PJHunt@bcm.edu)
Benjamin D. W. Belfort	(Benjamin.Belfort@bcm.edu)
Gary Liu	(Gary.Liu@bcm.edu)
Benjamin R. Arenkiel	(Arenkiel@bcm.edu)

Corresponding authors:

Brandon T. Pekarek	(Brandon.Pekarek@bcm.edu)

SUMMARY:

Neuronal dendritic morphology often underlies function. Indeed, many disease processes that affect the development of neurons manifest with a morphological phenotype. This protocol describes a simple and powerful method for analyzing intact dendritic arbors and their associated spines.

ABSTRACT:

Brain activity, the electrochemical signals passed between neurons, is determined by the connectivity patterns of neuronal networks, and from the morphology of processes and substructures within these neurons. As such, much of what is known about brain function has arisen alongside developments in imaging technologies that allow further insight into how neurons are organized and connected in the brain. Improvements in tissue clearing have allowed for high-resolution imaging of thick brain slices, facilitating morphological reconstruction and analyses of neuronal substructures, such as dendritic arbors and spines. In tandem, advances in image processing software provide methods of quickly analyzing large imaging datasets. This work presents a relatively rapid method of processing, visualizing, and analyzing thick slices of labeled neural tissue at high-resolution using CLARITY tissue clearing, confocal microscopy, and image analysis. This protocol will facilitate efforts toward understanding the connectivity patterns and neuronal morphologies that characterize healthy brains, and the changes in these characteristics that arise in diseased brain states.

INTRODUCTION:

Understanding the spatial organization, patterns of connectivity, and morphology of complex

three-dimensional biological structures is essential for delineating the functions of specific cells and tissues. This is especially true in neuroscience, in which tremendous effort has been dedicated to building high-resolution neuroanatomical maps of the central nervous system[1,2]. Close examination of the neurons that comprise these maps yields varied morphologies, with connections and locations that reflect the function of these diverse sets of neurons[3,4]. Moreover, investigation of subcellular structures, especially dendritic spines, can inform the maturity of synapses, thereby reflecting developmental processes and neurological disease states[5–7]. Thus, approaches that improve imaging resolution and throughput are essential toward better understanding brain function at all scales.

Recent advances have expanded the molecular and genetic toolkit for marking and manipulating populations of neurons. The development of new fluorescent markers, combined with new methods of introducing these markers into neurons, allows for differential labeling of populations of interacting neurons within the same animal or brain sample[8–11]. Because light is scattered by opaque lipids, and given the high lipid content of brain tissue, imaging neuronal populations has primarily been limited to thin sections or has relied on advanced microscropy techniques (e.g., confocal, multi-photon, and light-sheet microscopy) to image deep structures. However, these efforts have been greatly bolstered by advances in tissue clearing techniques. Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/immunostaining/*in situ* hybridization-compatible Tissue-hYdrogel (CLARITY) is one such technique, in which tissues of interest are infused with hydrogel monomers (acrylamide and bis-acrylamide) and then washed with detergents[12]. The hydrogel monomers hybridize to create a stable 3D hydrogel scaffold that is optically transparent and permeable to macromolecule labels. Nucleic acids and proteins are maintained within the hybridized matrix, whereas lipids are removed by the detergent washes (**Figure 1**). This results in a stable tissue that is rigid enough to maintain the original shape and orientation of cells and non-lipid molecules, while optically transparent enough to easily image deep structures at high resolution. This maintenance of tissue structure and orientation allows for the imaging of thick slices, thereby preserving cell-to-cell connections and spatial relationships. Moreover, because the location and availability of proteins and nucleic acids is maintained during the clearing process, cleared tissues are able to hold expression-based markers, as well as exogenous labels. Thus, CLARITY lends itself as a potent method for imaging large amounts of deep brain structures and the connections between these structures at high resolution.

The use of CLARITY greatly improves approaches to imaging neuronal populations. This technique is especially adept at generating large amounts of imaging data. CLARITY works well with multiple forms of protein-based fluorescence. This protocol utilizes a lentiviral-based approach to sparsely label cells with EGFP and tdTomato; however, transgenic reporter alleles expressing tdTomato or EGFP to label cells for reconstruction have been routinely used. It is important to choose a fluorophore that is both photo-stable and bright (e.g., EGFP or tdTomato). Additionally, using a strong promoter to express the fluorophore yields superior contrast and image quality. The drawbacks of this technique arise as properly analyzing this large amount of data can be both labor- and time-intensive. Specialized microscopes can help improve throughput and decrease workload. However, building, owning, and/or operating advanced microscopes are often cost-

prohibitive for many laboratories. This work presents a high throughput, relatively rapid, and simple method of visualizing large amounts of neural tissue at high-resolution using CLARITY tissue clearing of large sections, combined with standard confocal microscopy. This protocol describes this approach through the following steps: 1) dissecting and preparing the neural tissue, 2) clearing the tissue, 3) mounting the tissue, 4) imaging the prepared slices, and 5) processing full slice images using microscopy visualization software reconstruction and analysis (**Figure 2**). These efforts result in high-resolution images that can be used to analyze populations of neurons, neuronal connection patterns, 3D dendritic morphology, dendritic spine abundance and morphology, and molecular expression patterns within intact brain tissue.

PROTOCOL:

The following protocol follows all animal care guidelines for Baylor College of Medicine.

1. Dissection and tissue preparation

1.1. Euthanize the mouse with an overdose of isoflurane by placing the mouse in a closed container with a towel soaked in isoflurane (or by other IUCAC approved means).

1.2. Perfuse the animal transcardially using a 25 G needle with 10 mL of ice cold PBS, followed by 10 mL of 4% PFA.

1.3. Dissect the brain region (or tissue) of interest.

1.4. Place the dissected tissue into 4% PFA overnight at 4 °C. Proper fixation is key for this protocol. Do not skip or shorten this step.

1.5. After fixation in 4% PFA for at least 12 h, transfer the tissue to a 4% acrylamide hydrogel mixture for 24 h at 4 °C.

NOTE: When thawing the hydrogel, ensure that it has not polymerized, this can happen if the hydrogel becomes too warm. Thaw on ice to prevent premature polymerization.

2. Tissue clearing

2.1. Place the brain tissue (still submerged in hydrogel) in a vacuum incubator for 3 h at 37 °C with a -90 kPa vacuum. Leave the tube top unscrewed to allow the vacuum to form properly.

2.2. Wash the tissue with PBS for 10 min at room temperature (25 °C) with gentle shaking.

2.3. Place the polymerized tissue sample in the electrophoresis chamber, keeping note of the orientation of the tissue within the chamber.

2.4. Fill the chamber and reservoir with the supplied electrophoresis SDS buffer.

2.5. Run the sample at 70 V, 1 A, and 35 C, with constant current for about 2 h/mm of tissue.

2.5.1. Check the sample periodically; it may require more time in the chamber to properly clear. A good starting point for clearing is 1–2 h per mm of brain tissue. A whole mouse brain requires 8–10 h for sufficient clearing. Remember the orientation of the sample before removing it from the chamber and be sure to replace it back into the chamber in the same orientation.

NOTE: **Figure 3A** shows what a fully cleared brain will look like. The brain will look opaque and not clear at this stage due to the presence of dissolved SDS, but there should be little to no yellow tissue colored hue after lipid extraction.

3. Preparing and mounting the cleared tissue

3.1. After the sample has finished clearing and looks sufficiently clear, wash in PBS overnight at room temperature. Replace the PBS with fresh PBS as often as possible. This step is critical to remove residual SDS that can form precipitates in later steps.

3.2. Following the final wash in PBS, wash the tissue for 5 min in deionized water at room temperature three times. The tissue will become opaque at this step and may expand.

3.3. Incubate the tissue in the refractive index matching solution (see **Table 1**) for at least 4 h at room temperature. **Figure 3B** shows a piece of cleared tissue after incubation in refractive index matching solution.

3.4. During the incubation of tissue in refractive index matching solution, construct a suitable housing chamber to image the sample if necessary.

3.5. Constructing an imaging chamber for small tissue samples/slices

3.5.1. Using a glass slide as a base for mounting, lay down either rubber or plastic spacers and secure with super glue. If premade spacers are not available, use plastic rings made from conical tube cross sections.

3.5.2. Ensure to secure these pieces to the glass slide without any holes (**Figure 3C**).

3.5.3. Place the cleared tissue into the mounting chamber prefilled with refractive index matching solution.

3.5.4. Securely mount the tissue by placing a glass coverslip on top and sealing it with nail polish.

3.5.5. Image this tissue by adding a drop of refractive index matching mounting solution directly on top of the glass.

3.6. Large tissue imaging chamber

3.6.1. Construct this chamber if the tissue is larger than 5 mm thick (suitable for whole brains or hemispheres).

3.6.2. Using a 10 cm glass dish with a high wall, place a 50 mL conical tube in the center, making sure that the diameter of conical is large enough to accept the barrel of the objective lens used.

3.6.3. Make 3% agarose in water and pour it in the space between the glass dish and conical tube, allow to cool for 1 h (**Figure 3D**). This will form a ring of solid agarose (**Figure 3E**).

3.6.4. Securely adhere the tissue to the bottom of the chamber using super glue and fill the chamber with refractive index matching solution. Apply glue to adhere the tissue on a region that will not be imaged to allow reclamation of the tissue from the dish without damaging the regions of interest.

NOTE: This preparation is time sensitive, as the refractive index media may start to polymerize unless preserved from air and stored at 4 °C.

4. Imaging cleared tissue samples

4.1. Acquire the image using a confocal microscope fit with a 25x/0.95 NA objective with a 4 mm working distance.

4.2. Turn on all the relevant imaging equipment. Place the sample on the stage and place a drop of refractive index matching solution onto the top of the mounting chamber.

4.3. Carefully approach the immersion media with the objective and form a continuous column of media.

4.4. Using epifluorescence, find an appropriate imaging field.

4.5. Begin the image acquisition procedure by testing the appropriate settings.

4.5.1. Start by setting the resolution and scan speed settings using **Figure 4A** as a guide. If imaging using a confocal microscope, fully close the pinhole to obtain the smallest optical section and thus best z-resolution.

4.5.2. Gradually increase the laser power/sensor gain until a suitable image is obtained with a high signal-to-noise ratio.

4.5.3. If utilizing standard EGFP/tdTomato two-color imaging, set the light collection settings using **Figure 4B** as a guide.

4.5.4. Set the z-stack parameters based on the observed start and end points of the tissue. Set the step size based on the desired z-resolution using **Figure 4C** as a guide.

NOTE: Smaller step sizes will yield a greater z-resolution but will also introduce more laser dwell time, potentially leading to sample bleaching.

4.5.5. When satisfied with image acquisition settings, acquire the image.

4.5.6. Ensure that the image has a high signal-to-noise ratio and shows distinct boundaries of structures (**Figure 4D**).

5. Image processing and 3D quantification using microscopy analysis software

NOTE: Microscopic image analysis software packages are powerful tools for three-dimensional image visualization and processing. Many of these programs are perfectly suited for the handling of large datasets that are generated from imaging cleared tissue samples. The following steps and associated figures correspond to the Imaris software workflow.

5.1. Open the image stack and import it into the selected analysis software.

5.2. View the image in three-dimensional space and make any desired changes to the lookup tables using the display adjuster to better visualize the image.

NOTE: **Figure 5A** demonstrates the possible extreme imaging depth accessible through 2-photon microscopy paired with CLARITY tissue clearing. **Figure 5B** shows distinct dendrite processes as well as clearly visible spine morphologies from the z-stack presented in **Figure 5A**.

5.3. To filter out any consistent background, click on the **Image Processing** button and select the background subtraction filter.

NOTE: **Figure 5C** shows the image with a consistent hazy background signal before processing. **Figure 5D** shows the image after the background subtraction filter has been applied.

5.4. Observe the 3D image and become familiar with it by looking at it from multiple angles and zoom levels.

5.5. Start the dendrite tracing by first selecting the **Filament** tracer tool.

5.6. Click on **Edit the Filament Manually, Skip Automatic Creation**.

5.7. Set the mode to auto path and check **Auto-center** and **Auto-diameter** corrections.

5.8. Shift + right click on the cell body to set a starting point.

5.9. Trace the neuron along the entire length of the dendrite; left click on the end of the dendrite to set the termination point to allow the software to automatically calculate the path in between the start and end points.

5.10. Repeat this step for all the dendrites, and fully trace out the cell structure.

5.11. Visualize the traced cell and confirm its accuracy. Make manual adjustments as needed.

5.12. Select the **Creation** tab.

5.13. Select the **Recompute Dendrite Diameter** option.

5.14. Follow the wizard to completion for a more accurately traced dendrite.

5.15. Select the **Draw** tab.

5.16. Click on the **Spine** radio button to start drawing spines.

5.17. Set the approximate spine diameter as needed, and use the measurement tool to get an accurate representation of spine diameters.

5.18. Click on the center of the spine heads to add a new spine.

5.19. Repeat this for all spines on the dendrite.

NOTE: It is important to observe the dendrite from all possible angles when manually adding spines.

5.20. Check the newly added spines for accuracy and make changes as needed.

5.21. Select the **Creation** tab.

5.22. Select the **Recompute Spine Diameter** option to allow the software to determine the proper head and neck diameters, which are crucial for downstream data analysis.

5.23. Follow the spine diameter creation wizard to completion.

5.24. Observe the result of the computation and make any manual adjustments as needed. **Figure 5E** shows a fully traced neuron complete with spines.

5.25. To create a classified list of spines, select the **Tools** tab.

NOTE: The MATLAB extension must be installed for this to work.

5.25.1. To install the MATLAB extension, open the preferences window.

5.25.2. Select the **Custom Tools** option.

5.25.3. Add the appropriate MATLAB runtime MCR.

5.26. Click on **Classify Spines**.

5.27. Edit the desired parameters for spine classification.

5.28. Click on **Classify Spines** on the MATLAB extension. **Figure 5F** shows the dendrite overlaid with color-coded classified spines.

5.29. Select the **Statistics** tab.

5.30. Configure the desired statistics to quantify the data by clicking on the **Configure** button in the bottom-left corner of this tab.

5.31. Once the method of statistics representation has been chosen, export the data using the **Export Statistics on Tab Display to File** button located on the bottom right of this window.

5.32. Graph the statistics using a preferred graphing method.

REPRESENTATIVE RESULTS:

After image acquisition, the representative cell morphology was analyzed using embedded statistics and classifying scripts within the analysis software. The collected data (**Figure 6A**) reflects that neuron 2 has a larger dendritic structure with a higher density of spines. As a whole, the data suggests that neuron 2 has a more complex dendritic structure compared to neuron 1. To substantiate this result, standard Sholl analysis was performed, which affirms that neuron 2 is more dendritically complex than neuron 1 as denoted by the increased number of Sholl intersections at 50–100 μm from the soma (**Figure 6B**). Finally, dendritic spines of the two imaged neurons were classified into four main categories based on their overall shapes and sizes. Spines that exhibit more filopodia-like shapes are likely more immature spine subtypes. Spines with defined heads, called mushroom spines, likely contain more developed and mature synapses[7]. The analysis presented here shows that neuron 1 contains a larger proportion of filopodia-like spines as compared to neuron 2 (**Figure 6C**). Thus, based on morphology, neuron 2 is more developmentally mature as it is larger, more highly branched, and contains a higher density of mature spines.

FIGURE AND TABLE LEGENDS:

Figure 1: CLARITY protocol renders tissues transparent while maintaining inherent structure and molecule-molecule spatial relationships. (**A**) Original orientation of neuronal tissue and intercellular components prior to clearing. (**B**) Hydrogel monomers (purple lines) are infused into

the tissue and polymerized into a hydrogel mesh. The tissue and hydrogel mesh are crosslinked via formaldehyde fixation. (**C**) The tissue is then washed with ionic detergent solutions while exposed to electric fields. During this process, the detergent micelles remove lipid molecules from the tissue, leaving behind a crosslinked network of transparent hydrogel and biomolecules.

Figure 2: Flow chart of the protocol, diagramming the tissue preparation, clearing, mounting, imaging, and image processing.

Figure 3: Constructing mounting chambers for cleared tissue samples. (**A**) Cleared whole brain in PBS prior to immersion in refractive index matching solution. (**B**) Brain slice in refractive index matching solution. (**C**) Imaging chamber for large and small cleared tissue samples. The chambers can be made using a variety of materials including but not limited to 3D printed plastics and cut conical tubes. (**D**) Imaging chamber for whole brain or hemisphere imaging, using 50 mL conical tube as a relief before pouring agarose. (**E**) Whole brain or hemisphere imaging chamber with agarose fully set; this setup is optimal for large barrel immersion objectives.

Figure 4: Acquire high quality large datasets from cleared tissue samples. (**A**) Image acquisition settings used for whole cell high-resolution imaging. The pinhole was fully closed to enable fine optical sections. Scan speed was determined empirically based on optimal pixel dwell times. (**B**) Light path settings: these will depend on the fluorophore and equipment used. (**C**) Z-stack settings; a fine z-step was used to capture as much information in the z-direction as possible. (**D**) Representative max projection; scale bar represents 25 µm.

Figure 5: 3D analysis of dendrites in the analysis software. (**A**) Side-view of a two-photon acquired 600 µm z-stack acquired from a 1 mm thick tissue expressing tdTomato; scale bar represents 100 µm. (**B**) Top-view of the same z-stack acquired in panel A; scale bar represents 100 µm. (**C**) Confocal acquired image of tissue expressing EGFP. Image files can be directly imported into the analysis software and pre-processed for greater image quality; scale bar represents 25 µm. (**D**) Threshold subtraction is used to remove the consistent background signal present in C. (**E**) Filament tracing and spine identification: this process is best when performed semi-automatic with the auto-depth feature checked. Spines were then hand labeled after full dendrite reconstruction; scale bar represents 25 µm. (**F**) Spine classification using a built-in MATLAB extension. Spines have been color coded based on their morphology; stubby spines are red; mushroom spines are green; long thin spines are blue; filopodia are purple; scale bar represents 25 µm.

Figure 6: Representative results. (**A**) Table of common morphological measurements automatically generated by the selected analysis program. (**B**) Number of Sholl intersections generated by program statistics. (**C**) Pie chart representing the distribution of dendritic spine morphologies.

Table 1: Recipes for refractive image matching solution and hydrogel solution. The composition of the refractive index matching solution and the hydrogel are listed.

DISCUSSION:

Before the advent of contemporary tissue clearing techniques, studying neuronal morphology consisted of time-intensive sectioning, imaging, and reconstruction of adjacent very thin sections. Using electrophoretic tissue clearing in combination with confocal imaging provides an unobstructed view of complete neuronal morphology. From intact dendritic trees, down to the smallest synaptic bouton, imaging and quantifying neuronal morphology has never been more feasible.

The preparation of cleared brain tissue is straightforward and requires only one piece of specialized equipment. Tissue cleared and imaged using this protocol subverts the need for tedious thin sectioning, handling, and mounting, drastically decreasing the time from experimentation to image acquisition. Additionally, tissue imaged without sectioning remains more faithful to the original structures, as there are no sources of damage or necessary post-hoc image reconstruction. Finally, this protocol saves time by allowing simultaneous imaging of large-scale features such as dendritic trees alongside small-scale submicron features such as spines.

One important collection of steps in this protocol are the wash steps that follow the clearing process. These steps are critical for removing all traces of the SDS electrophoretic clearing buffer. If the cleared tissue is not sufficiently washed, precipitates will form during the mounting step. These precipitates can sometimes be redissolved by incubating the tissue at 37–55 °C for a short time. However, if the precipitates persist, they will scatter light, yielding poor imaging depth and quality.

Mounting large pieces of tissue presents a challenge compared to traditional thin slice imaging. Here we present multiple processes to mount large tissue, which depend on the imaging technique, objective lens properties, and the size of tissue sample. First, it is important to use an objective lens that is suitable for immersion in the specific refractive index matching media used, and which has sufficient working distance for imaging large tissue samples. This protocol is largely limited by the optical properties of the imaging platform used, specifically the working distance of objectives. Imaging at depth is readily achieved using this protocol. However, if an objective with sufficient working distance is not available, the tissue itself will present a physical barrier to acquiring large images. The next important decision is the imaging technique. Two-photon microscopy is typically used for its superb image quality, depth of imaging, and speed of acquisition. Two-photon microscopy enables imaging of up to 1 mm into CLARITY cleared tissue without loss of image quality as demonstrated in **Figure 5A**,**B**. However, very similar results can be achieved when using traditional confocal microscopy albeit with a sacrifice to imaging depth compared to two-photon microscopy (**Figure 5C**,**D**).

In summary, this method provides a robust and convenient platform for analyzing neuronal morphology at both large and small scales. Additionally, this method largely minimizes handling and processing time, while also providing more accurate and complete three-dimensional images. Cellular morphology is a commonly used proxy for assessing circuit function and health underlying many diseases and pathologies[13–15]. Imaging neuron morphology is powerful, straightforward, and well suited to assay in a multitude of disease models.

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DISCLOSURES:

The authors have nothing to disclose at this time.

REFERENCES:

1. Abbott, L. F. et al. The Mind of a mouse. *Cell*. **182** (6), 1372–1376 (2020).
2. White, J. G., Southgate, E., Thomson, J. N., Brenner, S. The structure of the nervous system of the nematode Caenorhabditis elegans. *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences*. **314** (1165), 1–340 (1986).
3. Jiang, X. et al. Principles of connectivity among morphologically defined cell types in adult neocortex. *Science*. **350** (6264) (2015).
4. Winnubst, J. et al. Reconstruction of 1,000 projection neurons reveals new cell types and organization of long-range connectivity in the mouse brain. *Cell*. **179** (1), 268–281.e13 (2019).
5. Araya, R., Vogels, T. P., Yuste, R. Activity-dependent dendritic spine neck changes are correlated with synaptic strength. *Proceedings of the National Academy of Sciences of the United States of America*. **111** (28) (2014).
6. Bosch, M., Hayashi, Y. Structural plasticity of dendritic spines. *Current Opinion in Neurobiology*. **22** (3), 383–388 (2012).
7. Martínez-Cerdeño, V. Dendrite and spine modifications in autism and related neurodevelopmental disorders in patients and animal models. *Developmental Neurobiology*. **77** (4), 393–404 (2017).
8. Arenkiel, B. R., Ehlers, M. D. Molecular genetics and imaging technologies for circuit-based neuroanatomy. *Nature*. **461** (7266), 900–907 (2009).
9. Kim, E. H., Chin, G., Rong, G., Poskanzer, K. E., Clark, H. A. Optical probes for neurobiological sensing and imaging. *Accounts of Chemical Research*. **51** (5), 1023–1032 (2018).
10. Weissman, T. A., Pan, Y. A. Brainbow: New resources and emerging biological applications for multicolor genetic labeling and analysis. *Genetics*. **199** (2), 293–306 (2014).
11. Haggerty, D. L., Grecco, G. G., Reeves, K. C., Atwood, B. Adeno-associated viral vectors in neuroscience research. *Molecular Therapy - Methods and Clinical Development*. **17** (June), 69–82 (2020).
12. Chung, K. et al. Structural and molecular interrogation of intact biological systems. *Nature*. **497** (7449), 332–337 (2013).
13. Kanning, K. C., Kaplan, A., Henderson, C. E. Motor neuron diversity in development and disease. *Annual Review of Neuroscience*. **33**, 409–440 (2010).
14. Ledda, F., Paratcha, G. Mechanisms regulating dendritic arbor patterning. *Cellular and*

Molecular Life Sciences. **74** (24), 4511–4537 (2017).
15. Falougy, H. E, Filova, B., Ostatnikova, D., Bacova, Z., Bakos, J. Neuronal morphology alterations in autism and possible role of oxytocin. *Endocrine Regulations*. **53** (1), 46–54 (2019).

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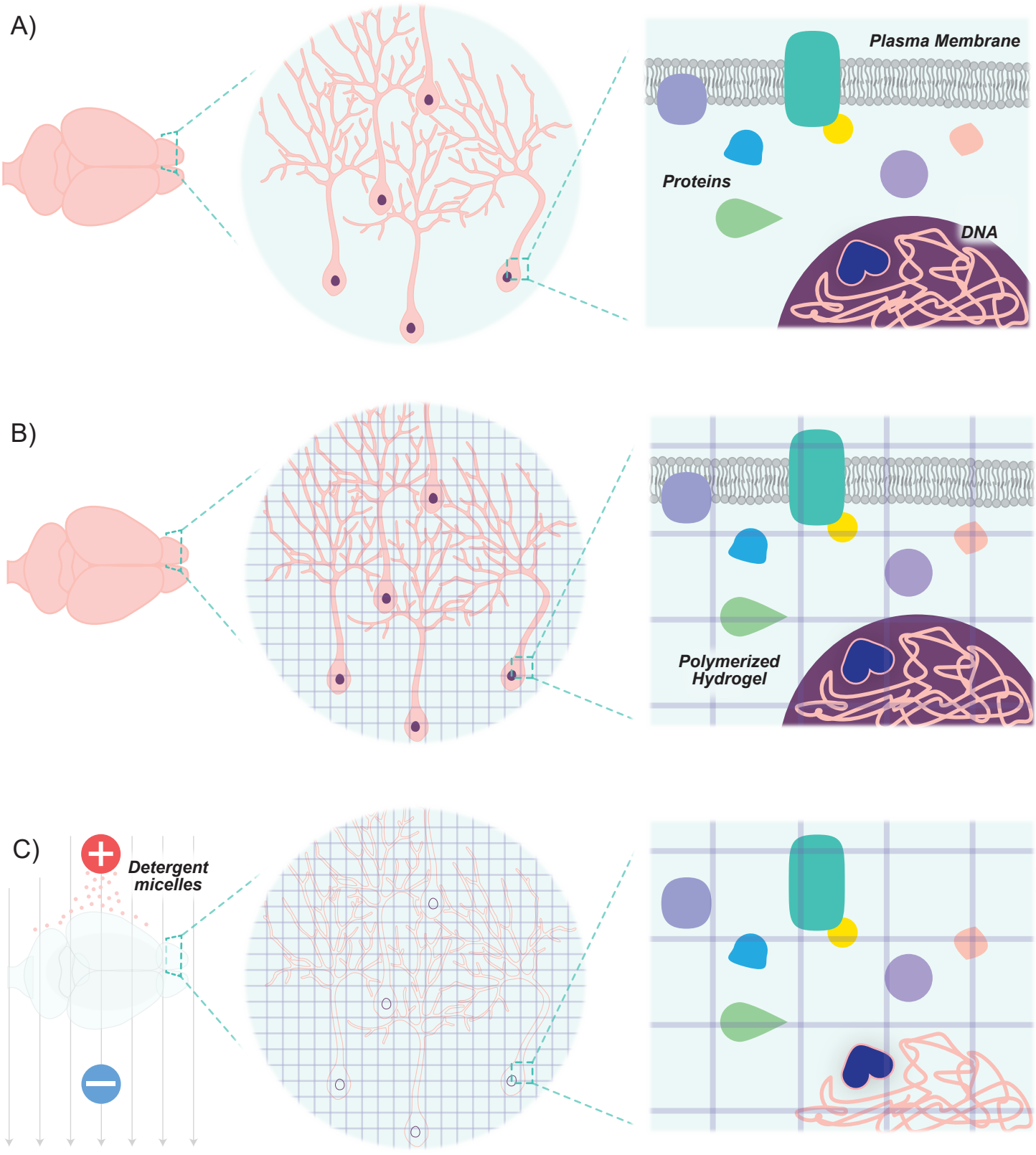

Figure

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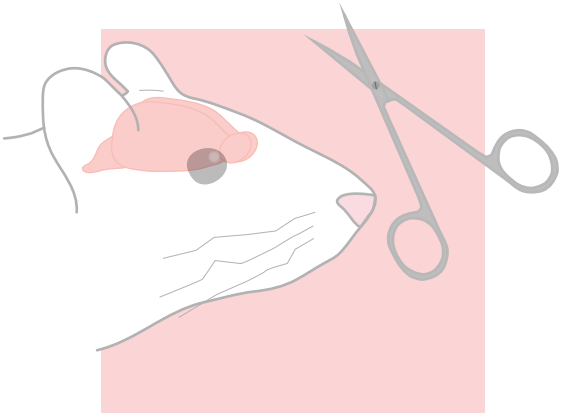

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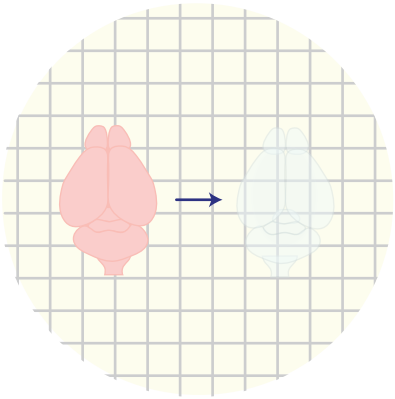

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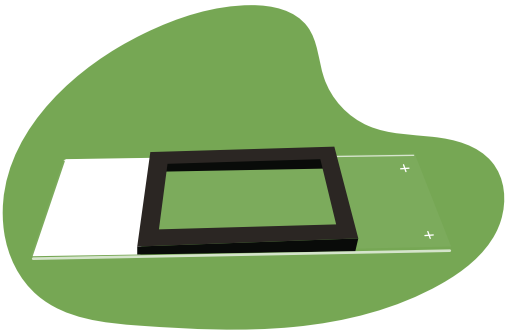

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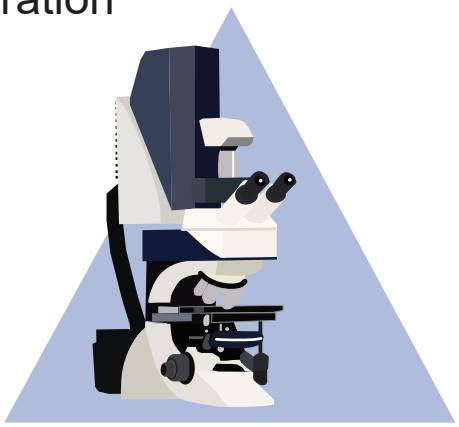

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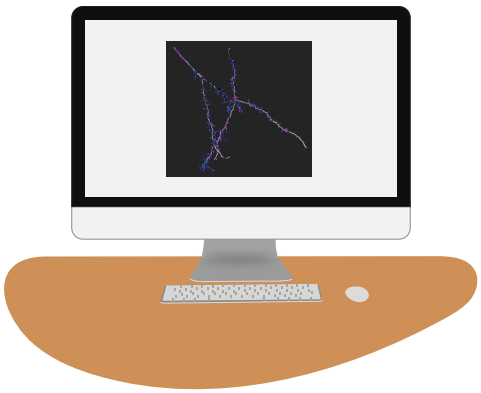

5. Image processing

Figure

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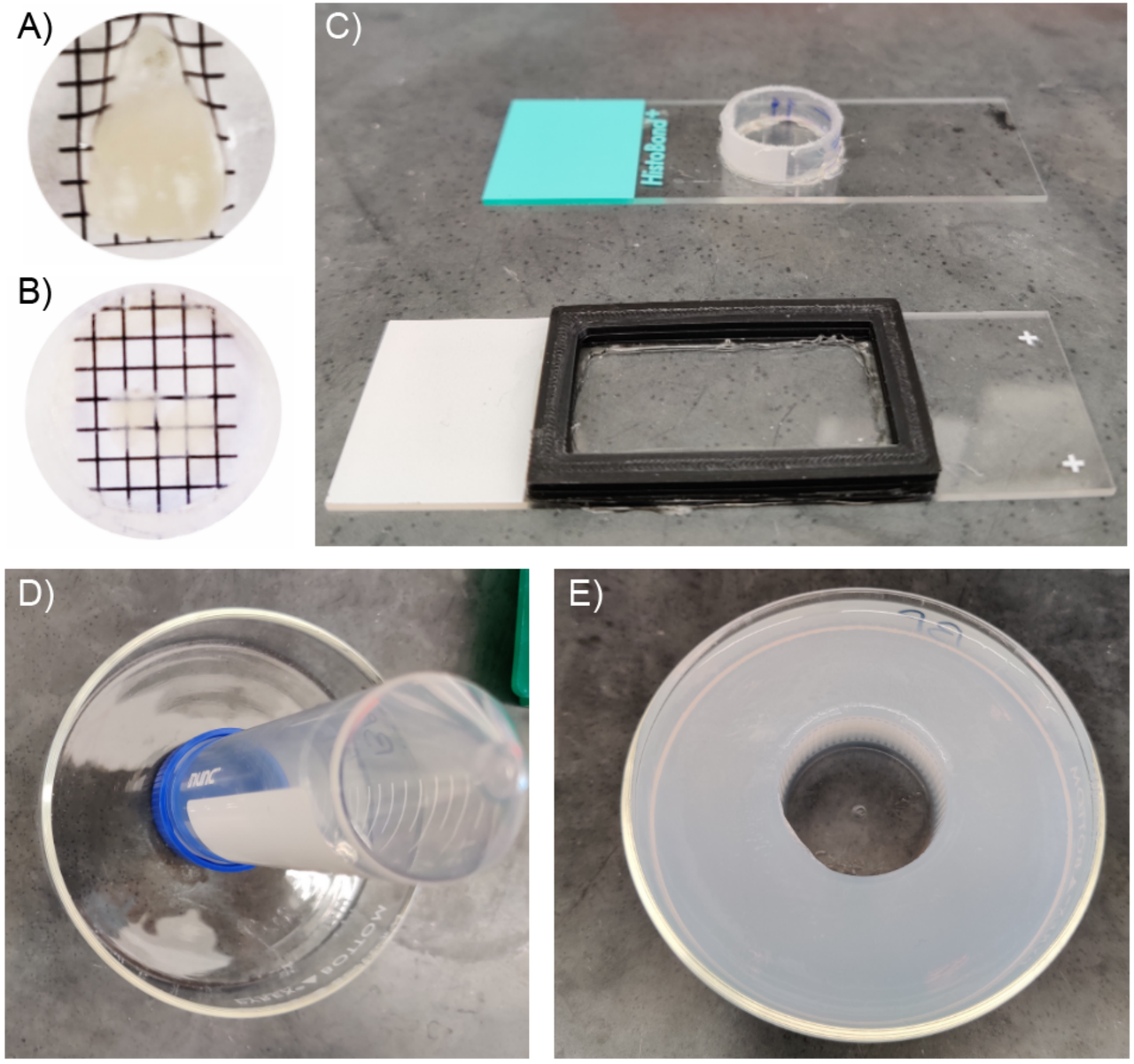

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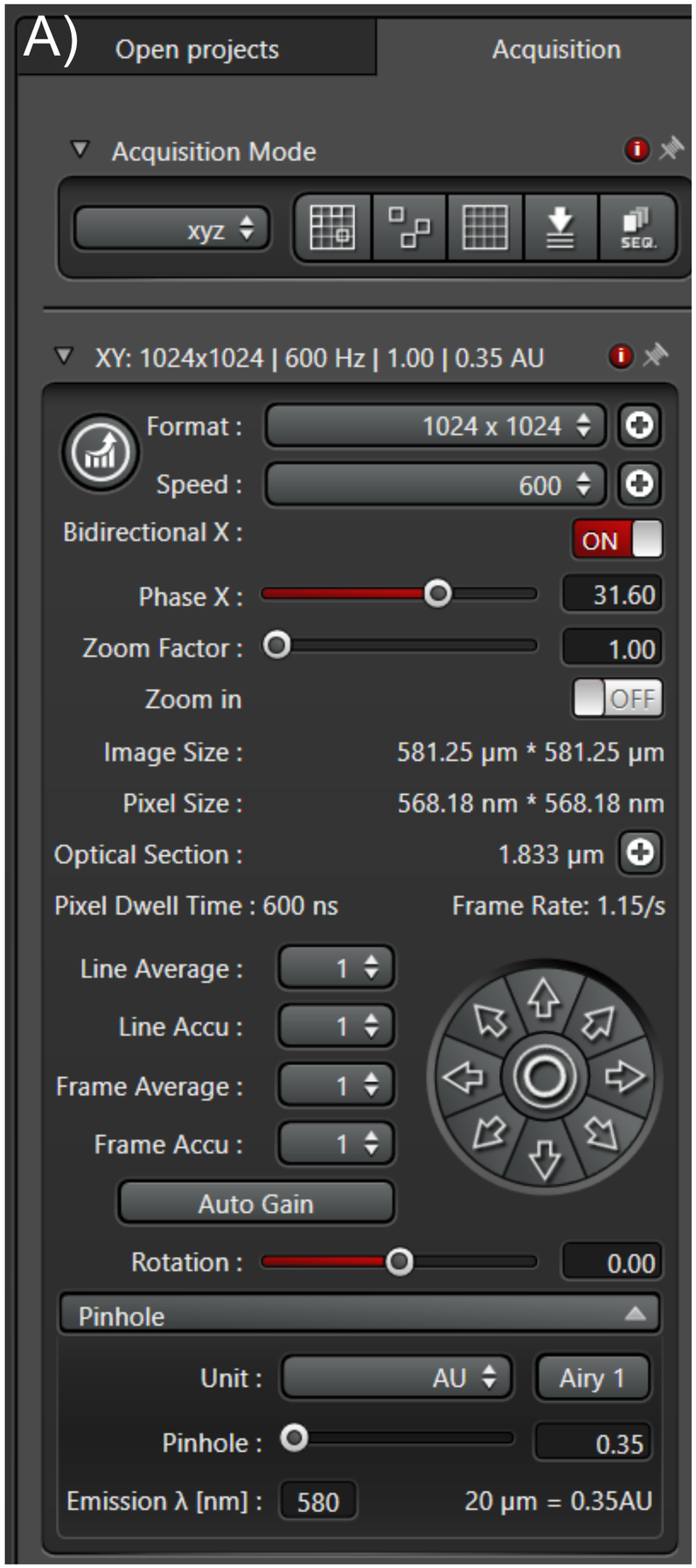
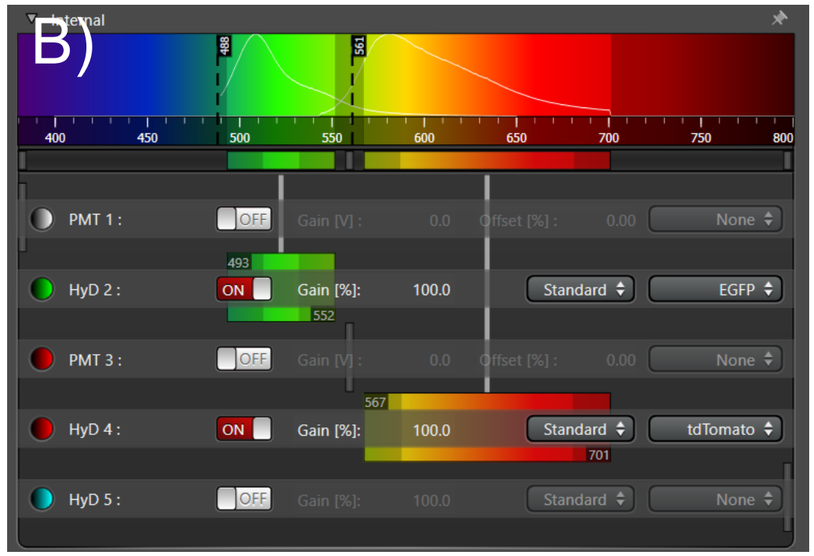
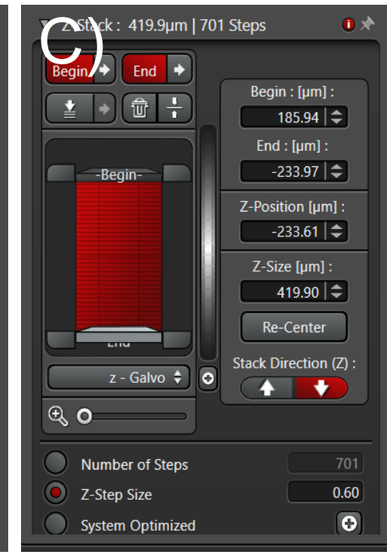
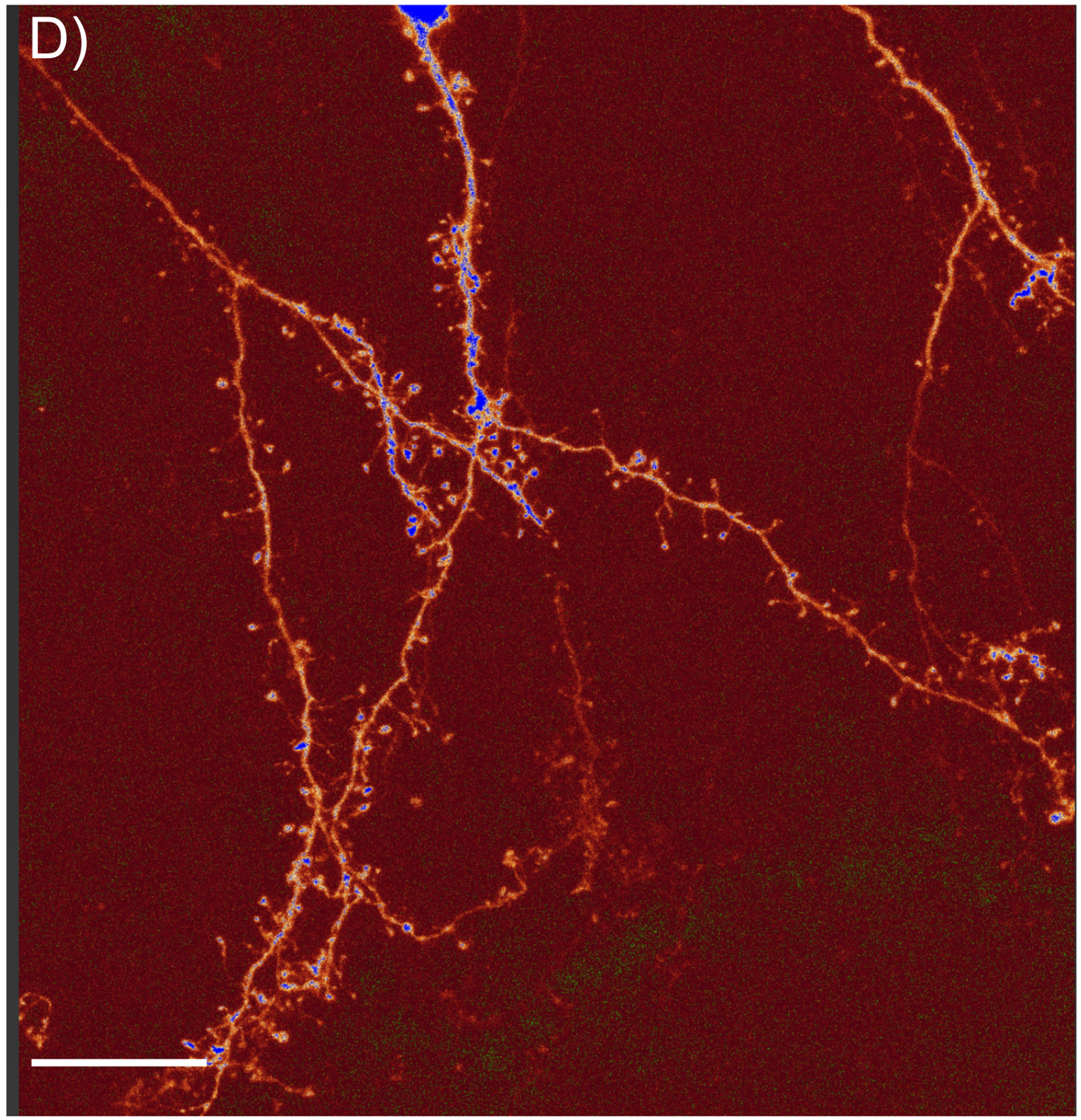


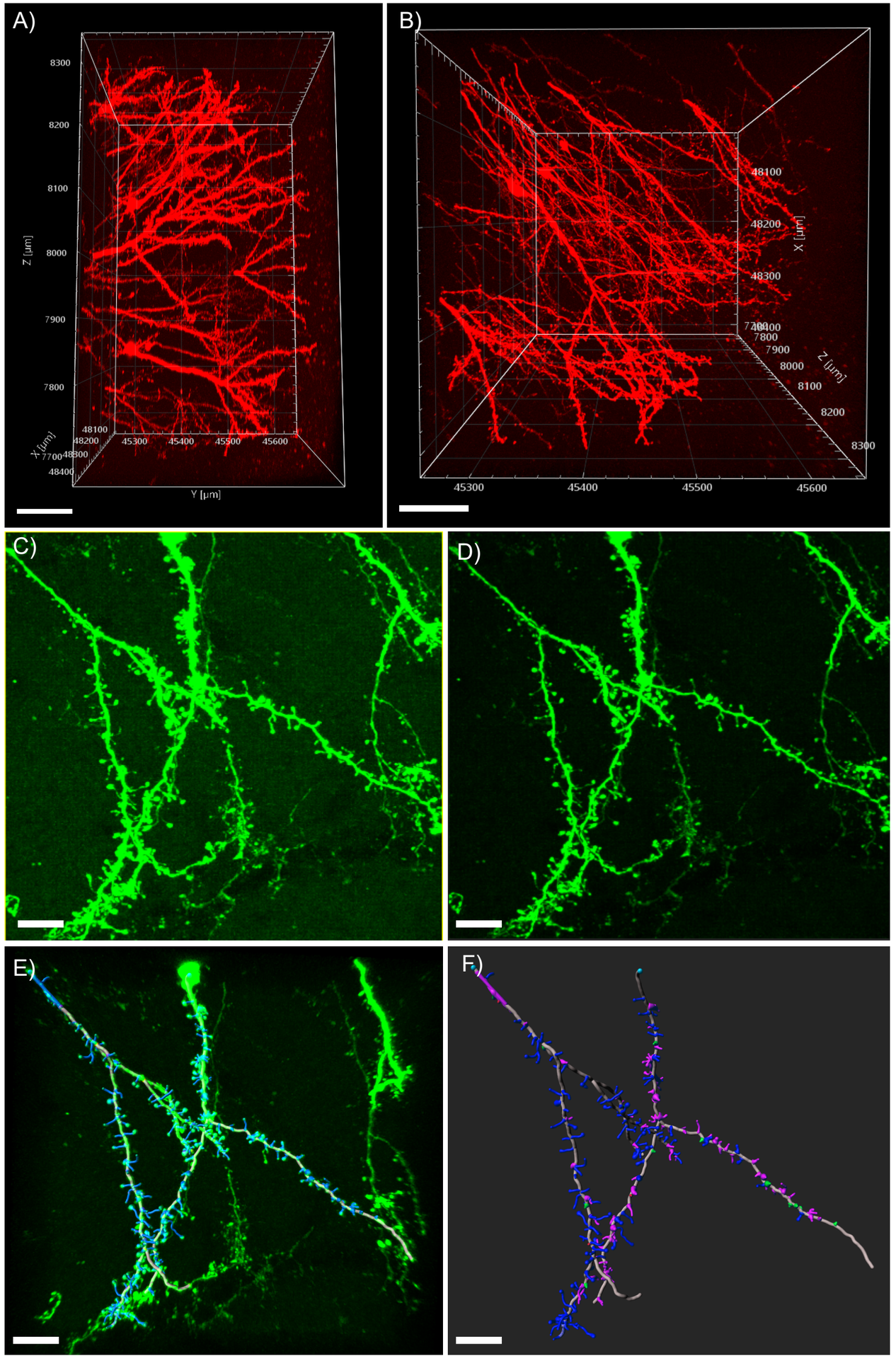






Figure

Click here to access/download;Figure;Figure 5.pdf



Click here to access/download;Figure;Figure 6 -revised.pdf

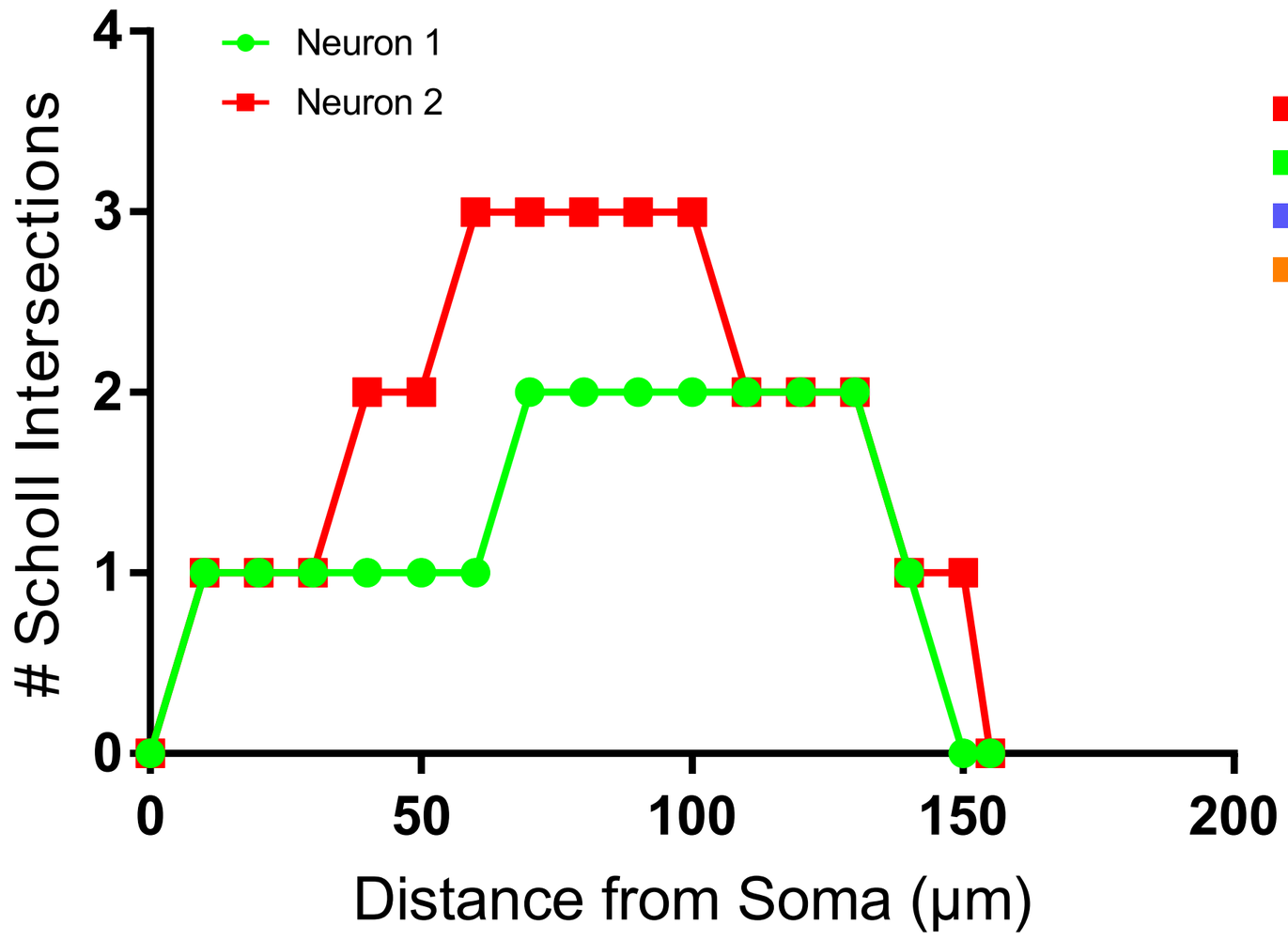
A)

Common morphology measurements

Measurement	Neuron 1	Neuron 2	Unit
Dendrite area	3075	4179	µm^2
Dendrite length	284	359	µm
Dendrite volume	502	642	µm^3
Number of branches	2	4	N/A
Number of branching spines	18	26	N/A
Number of spines	123	162	N/A
Primary dendrite spine density	2.99	2.77	1/10µm
Total spine density	4.33	4.51	1/10µm
Total spine volume	598	752	µm^3

B)

Number of Scholl Intersections



C)

Distribution of Spine Morphologies

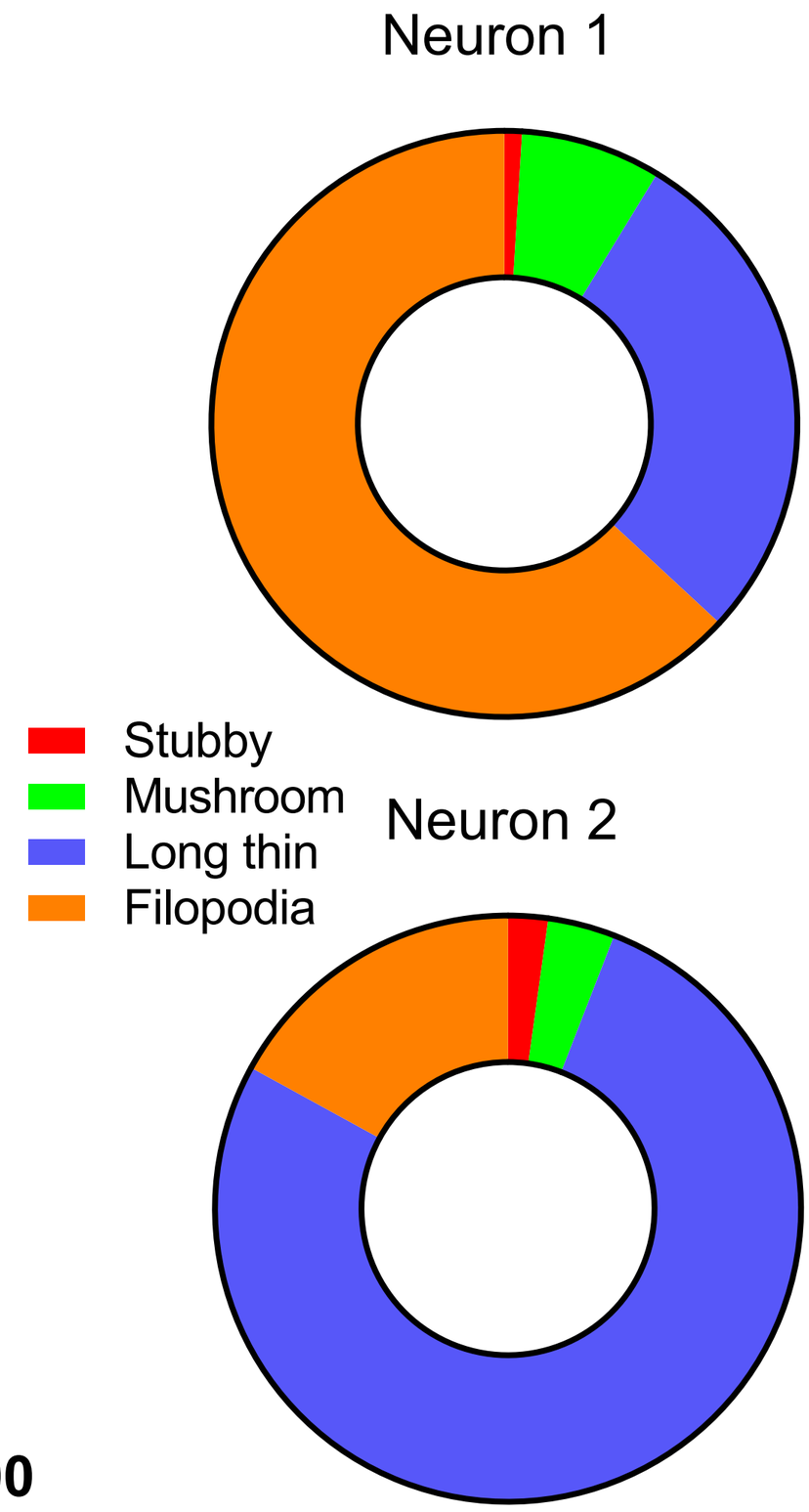


Table 1

Click here to access/download;Table;Solutions.xlsx

Solution	**Composition**
Refractive index matching solution	80 g of histodenz 60 mL of 0.02 M phosphate buffer 0.01% of sodium azide
Hydrogel	13.33 mL of 30% Acrylamide (no-bis) 10 mL of 10x PBS 250 mg of VA-044 76.66 mL of ddH_2O

Notes
pH to 7.5 with NaOH
Store at 4 °C
Adapted from Marx, V. Nature Methods volume 11, pages 1209–1214 (2014)

Mix together on ice, otherwise the solution may begin to polymerize
Aliquot and store at -20 °C

Table of Materials

Click here to access/download;Table of Materials;Table of Materials- 62532R1.xlsx

Name of Material/ Equipment	Company	Catalog Number
15 mL Conical Tube	Thermo Scientific	339650
25 G x 1" Needle	BD	305127
30% Acrylamide (No-Bis)	National Diagnostics	EC-810
50 mL Conical Tube	Thermo Scientific	339653
Electrophoretic Tissue Clearing Solution	Logos	C13001
Histodenz	Sigma	D2158-100G
Hydrogel Solution Kit	Logos	C1310X
Imaris	Oxford Instruments	N/A
Paraformaldehyde 16%	EMS	15710
PBS, 1x, 500 mL, 6 bottles/case	fisher	MT21040CV
VA-044	Wako	925-41020
X-CLARITY Polymerization System	Logos	C20001
X-CLARITY Tissue Clearing System II	Logos	C30001

Comments/Description

Rebuttal Letter
Click here to access/download;Rebuttal Letter;21-03-19 Response to Reviewers.docx

Response to Reviewers

Manuscript JoVE62532: **"Imaging and quantification of intact neuronal dendrites via clarity tissue clearing"**

Corresponding Author: Benjamin R. Arenkiel

We thank the reviewers for their helpful comments and suggestions to improve our manuscript entitled: **Imaging and quantification of intact neuronal dendrites via clarity tissue clearing.** In this manuscript we describe a rapid and high-throughput protocol for processing, visualizing, and analyzing thick slices of labeled neural tissue at high-resolution using tissue clearing, confocal microscopy, and image analysis.

We have read the editor's and reviewers' comments, and provide a response below outlining the changes that have been made in a revised version of our manuscript. These changes and our response to the reviewers are itemized there, and described in further detail in a point-by-point response below.

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have thoroughly proofread the manuscript and have fixed all spelling and grammar issues that we found.

2. Please provide an institutional email address for each author.

We have added the institutional email addresses for each author.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the text and removed all uses of personal pronouns.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Logos X-Clarity, etc.

We have revised the text and removed all commercial language. We have included this information in the Table of Materials.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We have added the following ethics statement “The following protocol follows all animal care guidelines for Baylor College of Medicine” in line 110 of the manuscript.

6. Line 85: Please add more details to your protocol steps. How is the anesthesia induced? What concentration/ percentage of anesthesia is used? Is the anesthetic used for euthanizing the animal?

We have included additional details to our protocol to better describe how the anesthesia was used to euthanize the mouse. The following text was added in line 115 of the manuscript: “Euthanize the mouse with an overdose of isoflurane by placing the mouse in a closed container with a towel soaked in isoflurane (or by other IUCAC approved means).”

7. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

We have revised the manuscript to abbreviate all forms of time less than 1 day.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have revised the manuscript and ensured that the protocol is written only in the imperative tense. We have removed all text that included “could,” “should,” or “would.”

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Please move the discussion about the protocol to the Discussion. (Lines 99-102, 112-114, 122-130, 133-169, etc.)

We have simplified the protocol to include only 2-3 actions per step with a maximum of 4 sentences per step. All additional text has either been removed or moved to the Discussion.

10. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be

visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have added the requested one-line space between each protocol step. Additionally, we have highlighted in yellow the essential steps of the protocol for the video.

11. Figure 5: Please define the scalebar and the magnification used in the Figure legends.

We have added and defined the scale bars and magnification settings used in Figure 5.

12. Please sort the Table of Materials in alphabetical order.

We have alphabetized the Table of Materials.

Reviewer #1:
Manuscript Summary:
Pekarek and colleagues have provided an rapid protocol of visualizing neural dendrites utilizing CLARITY tissue clearing pipeline, where clear description of tissue preparation, tissue clearing, tissue mounting, image acquiring and processing steps are provided in detail. Tissue clearing techniques have facilitated histological studies of neural morphology in an effort-saving, high-throughput manner and have the potential to produce profound insights into the biology of the brain. The notes and problems addressed in prepping and mounting the cleared tissue are useful experience dealing with different situations. The extensive elaboration of three-dimensional visualization and quantification of neurons after imaging acquiring will be of rewarding guidance for beginners processing the data.
However, there are several concerns which should be addressed before publishing.

Major Concerns:
1. While the technical protocol of tissue clearing followed by imaging acquiring and processing has been provided herein, the introduction of fluorescent labeling has been skipped, although its importance is mentioned in the relevant chapters. Unless fluorescent reporter mice are used, the labeling of non-fluorescent mice will be an imperative and troublesome part of the whole technical route.

We thank you very much for taking the time to review our manuscript. We greatly appreciate your feedback on our work. We have added the following text to the Introduction of our manuscript: “CLARITY works well with multiple forms of protein-based fluorescence. This protocol utilizes a lentiviral-based approach to sparsely label cells with EGFP and tdTomato, however we routinely use transgenic reporter alleles

expressing tdTomato or EGFP to label cells for reconstruction. It is important to choose a fluorophore which is both photo-stable and bright (ex. EGFP or tdTomato). Additionally, using a strong promoter to express the fluorophore yields superior contrast and image quality." This is intended clarify how the neurons were labeled prior to tissue clearing. We have intentionally left the section broad to accommodate the use of diverse fluorophores as well as the different methods of introducing these fluorophores to neurons.

Minor Concerns:

1. The authors have mounted tissues in specially-made chambers with sealed glass coverslip/ super glue and then imaged under ortho confocal microscope. However, this seems cumbersome and may result in one-time consumption of the samples. And immersing the objective lens directly into the medium may limit the options of lens (such as oil lens) and higher magnification. Did the authors consider the alteration of using inverted confocal microscopes and confocal dishes?

We acknowledge the concerns expressed here by the reviewers, however, the tissue can be reclaimed after imaging by removing the coverslip and accessing the tissue sample from the imaging chamber. If the tissue is glued to the chamber, the surface on which it was glued must be sacrificed. However, other areas of the tissue sample, including the area that you wish to image can be retained intact and undamaged. We have added the following sentence to clarify this point: "Make sure to adhere the tissue by gluing it on a region that will not be imaged. This will allow reclamation of the tissue from the dish without damaging regions of interest."

Immersion of the objective in refractive-index mounting media is imperative for matching the refractive indices of the lens, mounting solution, and tissue sample. Using an oil immersion lens will introduce a refractive index mismatch with the tissue sample, scattering light and ultimately compromising tissue clarity and imaging depth.

2. The accumulation of temperature-sensitive precipitates will affect the imaging quality and limit imaging duration, considering that it usually takes longer to collect images from cleared tissues. The recipe of refractive index matching solution may need further exploration and improvement. One suggestion for reference is to use a thermostatic microscope stage, however this may be limited by the microscope setup.

We thank you very much for offering these suggestions. We will consider using thermostatic microscope stages in our future work.

3. The time for tissues to achieve the endpoint of complete transparency is different. We suggest that this protocol lists the best time for tissues of different sizes to be completely transparent.

We have added the following text to our protocol: “A good starting point for clearing is 1-2h per mm of brain tissue. A whole mouse brain requires 8-10 hrs for sufficient clearing.” This discussion of the time required for clearing brain tissue of different thicknesses will assist readers in deciding the best time length to use in clearing their own tissue samples.

4. The authors may miss the latest published papers related to the topic of tissue clearing in Neuroscience, e.g. ACS Chemical Neuroscience 2021 12 (1), 5-29.

We thank the reviewer for bringing this manuscript to our attention. We are familiar with this study and will incorporate it into our own future work.

Reviewer #2:
The manuscript JoVE62532, "imaging and quantification of intact neuronal dendrites via clarity tissue clearing," provides a step by step protocol for detecting fluorescent signal in thick brain slices. The CLARITY technique has transitioned from difficult to routine of the years yet can still be finicky as it is cleared largely by "eye." Overall, the authors have produced well written paper that provides a straightforward protocol.

However, minor concerns are listed below:
Introduction. Line 69: "analyzing this large amount of data can be…" should also state a numerical value of storage based on tissue thickness for confocal stacks acquired. The latter is the rate limiting step in processing large volumes.

We thank you very much for taking the time to review our manuscript. We greatly appreciate your feedback on our work. We recognize that handling large sets of data can be a rate-limiting step. Our largest data set was imaged at 1024 x 1024 resolution, with a 600 μm z-stack at 0.9 μm z-steps and 16-bit color depth in the Leica imaging acquisition software. This process yielded a data set that is 2.8 GB in size. However, the size of data sets that result from thick tissue imaging varies widely based on the resolution, bit-depth, z-stack and z-step size, number of channels acquired, etc. chosen by the researcher. Thus, due to this large amount of variance, we have decided not to include a value for data set size based on tissue thickness.

Protocol. Line 108: the use of the Logos X-CLARITY clearing apparatus may, arguably, be the highlight of this paper as it hasn't been used often in literature. The authors suggest checking the sample periodically, but don't say how often.

We have added text clarifying the time needed for clearing based on tissue thickness: “A good starting point for clearing is 1-2 hrs per mm of brain tissue. A whole mouse brain requires 8-10 hrs for sufficient clearing.” This text will be useful in helping readers decide how often to check their samples during the clearing process.

This should be explained. Line 122: the authors describe incubating in the required refracting matching solution, but do not state what it is. I do no find the "(see recipes/alternatives below)" and it isn't listed in the Materials/Equipment section, along with the other CLARITY components. Line 125: It is unlikely that the refractive index matching solution precipitates, if they are made correctly- it's likely that the SDS still hasn't been washed out (I've never seen a CLARITY paper that needed to rinse with water?

We have added the recipes for making homemade RIMS (refractive index matching solution) and homemade hydrogel in the newly added Table 1. The precipitates we are referring to are from SDS. We have altered the text to clarify further. Rinsing with deionized water is done to remove any additional dissolved solids, including SDS, PBS, etc.

General Methodology. The authors do not mention the brain thickness of the slice imaged. What was the z-depth related to the shown dendrite figures? Considering the whole point of CLARITY is to image within large samples, this is important. As is, the shown images could be done in normal sections. The authors should, at least provide a z-stack (at higher magnification) demonstrating depth. Proving the images were taken well into the tissue would be more convincing.

The image used for analysis is a 70 μm z-stack taken from an original image that is 400 μm deep. To demonstrate imaging depth, we have added an additional z-stack view of another large stack (~600 μm) to Figure 5. We have added the following callouts to these new images in the protocol section: “Figure 5A demonstrates the possible extreme imaging depth accessible through 2-photon microscopy paired with CLARITY tissue clearing. Figure 5B shows distinct dendrite processes as well as clearly visible spine morphologies from the z-stack presented in Figure 5A.”