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TITLE:

Visualizing Astrocyte Morphology Using Lucifer Yellow Iontophoresis

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Lucifer yellow, astrocyte, morphology, dye filling, end-feet, aquaporin-4, 3D reconstruction, hippocampus

SUMMARY:

Astrocytes are morphologically complex cells, exemplified by their multiple processes and bushy territories. To analyze their elaborate morphology, we present a step-by-step protocol to perform intracellular Lucifer yellow iontophoresis in lightly fixed tissue.

ABSTRACT:

Astrocytes are essential components of neural circuits. They tile the entire central nervous system (CNS) and are involved in a variety of functions, which include neurotransmitter clearance, ion regulation, synaptic modulation, metabolic support to neurons, and blood flow regulation. Astrocytes are complex cells that have a soma, several major branches, and numerous fine processes that contact diverse cellular elements within the neuropil. In order to assess the morphology of astrocytes, it is necessary to have a reliable and reproducible method to visualize their structure. We report a simple protocol to perform intracellular iontophoresis of astrocytes using fluorescent Lucifer yellow (LY) dye in lightly fixed brain tissue from adult mice. This method has several features that are useful to characterize astrocyte morphology. It allows for three-dimensional reconstruction of individual astrocytes, which is useful to perform morphological analyses on different aspects of their structure. Immunohistochemistry together with LY iontophoresis can also be utilized to understand the interaction of astrocytes with different components of nervous system and to evaluate the expression of proteins within the labelled astrocytes. This protocol can be implemented in a variety of mouse models of CNS disorders to rigorously examine astrocyte morphology with light microscopy. LY iontophoresis provides an

experimental approach to evaluate astrocyte structure, especially in the context of injury or disease where these cells are proposed to undergo significant morphological changes.

INTRODUCTION:

Astrocytes are the most abundant glial cells in the central nervous system (CNS). They play roles in ion homeostasis, blood flow regulation, synapse formation as well as elimination, and neurotransmitter uptake[1]. The wide range of astrocyte functions is reflected in their complex morphological structure[2,3]. Astrocytes contain several primary and secondary branches which divide into thousands of finer branchlets and leaflets that directly interact with synapses, dendrites, axons, blood vessels, and other glial cells. Astrocyte morphology varies across different brain regions, which may hint at their ability to perform their functions differentially in neuronal circuits[4]. Moreover, astrocytes are known to alter their morphology during development, during physiological conditions, and in multiple disease states[3,5,6].

A consistent, reproducible method is needed to accurately resolve the complexity of astrocyte morphology. Traditionally, immunohistochemistry has been used to visualize astrocytes with the use of astrocyte specific or astrocyte enriched protein markers. However, these methods reveal the pattern of protein expression rather than the structure of the astrocyte. The commonly used markers, such as glial fibrillary acidic protein (GFAP) and S100 calcium binding protein β (S100β), do not express in the entire cell volume and thus do not resolve complete morphology[7]. Genetic approaches to express fluorescent proteins ubiquitously in astrocytes (viral injections or transgenic mouse reporter lines) can identify the finer branches and overall territory. However, it is difficult to differentiate individual astrocytes, and analyses may be biased by the astrocyte population targeted by the specific promoter[8]. Serial section electron microscopy has been used to reveal a detailed picture of the interactions of astrocyte processes with synapses. Due to the thousands of astrocyte processes contacting synapses, it is currently not possible to reconstruct an entire cell with this technique[9], although this is expected to change with the use of machine learning for data analysis.

In this report, we focus on a procedure to characterize mouse astrocytes using intracellular iontophoresis with Lucifer yellow (LY) dye, using the CA1 stratum radiatum as an example. The method is based on pioneering past work by Eric Bushong and Mark Ellisman[10,11]. Astrocytes from lightly fixed brain slices are identified by their distinctive soma shape and filled with LY. The cells are then imaged with confocal microscopy. We demonstrate how LY iontophoresis can be used to reconstruct individual astrocytes and perform detailed morphological analyses of their processes and territory. Also, this method can be applied in conjunction with immunohistochemistry to identify spatial relationships and interactions between astrocytes and neurons, other glial cells, and brain vasculature. We consider LY iontophoresis to be a very suitable tool to analyze morphology in different brain regions and mouse models of healthy or disease conditions[7,12,13].

PROTOCOL:

The animal experiments in this study were performed in accordance with the National Institute

of Health Guide for the Care and Use of Laboratory Animals and were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles. Adult mice (6−8 weeks old) of mixed gender were used in all experiments.

1. Solution preparation

1.1. Artificial cerebrospinal fluid (ACSF) solution

1.1.1. Prepare fresh ACSF solution (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 14.7 mM $NaHCO_3$, 11 mM D-glucose, 1.25 mM Na_2HPO_4, and 2 mM $CaCl_2$) before each experiment. Add $MgCl_2$ and $CaCl_2$ in the final step. Dissolve the components in high quality deionized water.

1.1.2. Incubate the ACSF solution at 35 °C in a water bath and bubble with 95% O_2/ 5% CO_2 for at least 30 min before the experiment.

1.1.3. Add 2% lidocaine hydrochloride to reach a final concentration of 0.02% in ACSF (in 100 mL).

1.2. Fixative solution

1.2.1. Use 10% formalin buffered phosphate.

1.3. LY dye solution

1.3.1. Prepare 1.5% LY dye solution by dissolving LY CH dilithium salt or LY CH dipotassium salt in 5 mM KCl. Vortex thoroughly. A volume of 1 mL is sufficient for several experiments.

1.3.2 Centrifuge for 10 min at 16,800 x *g*. Then, filter the supernatant with a 0.2 µm syringe filter into a new tube. Aliquots can be kept at 4 °C for up to 3 months.

NOTE: It is critical to centrifuge and filter the dye solution to prevent aggregation of the dye particles, which may clog the electrode.

2. Mouse transcardial perfusion and brain dissection

2.1. Preparation of equipment and mouse for transcardial perfusion

NOTE: C57/BL6 mice of either sex can be used. It has been empirically observed that for the method to work reliably, it is important that the mice are not older than 3 months (6−8 weeks old is ideal). The perfusion protocol is described below. An additional reference is provided for more details[14].

2.1.1. Clear perfusion tubing with ACSF solution to remove any air bubbles in the line. Set up surgery tools in order of use (tweezers, curved, blunt scissors, iris scissors).

2.1.2. Deeply anesthetize mouse by putting it into an isoflurane induction chamber, after adding 2−3 mL of isoflurane to the chamber. Allow 1−2 min for anesthetic to take effect. Ensure that breathing does not stop.

2.1.3. Test for toe pinch reflex. Proceed when the mouse is unresponsive to pain stimulus and the reflex is absent. Secure the animal in the supine position with the head placed in the breathing cone with a supply of 5% isoflurane in oxygen and the limbs and tail taped down inside a chemical fume hood.

2.2. Transcardial perfusion

2.2.1. Using tweezers, lift up skin beneath rib cage. Make a 5−6 cm incision with the curved, blunt scissors through the skin to expose the abdominal cavity. Cut upwards through the abdominal wall until liver and diaphragm are visible.

2.2.2. Gently move the liver away from the diagram. With iris scissors, make a 3−4 cm lateral incision in the diaphragm.

2.2.3. Cut through the rib cage along the both sides of the body to expose the chest cavity. Be careful not to damage heart and avoid the lungs. Lift the sternum up to expose the heart.

2.2.4. Once the heart is visible, inject 0.05 mL of heparin sodium solution (1,000 USP per mL) into the left ventricle as an anticoagulant. Then, insert perfusion needle carefully into the left ventricle. Make sure that the needle stays in the ventricle and does not pierce through to other heart chambers. Make a small incision in the right atrium with iris scissors.

2.2.5. Perfuse with ACSF solution at a rate of approximately 10 mL/min until the fluid existing the body is cleared of blood (1−2 min).

2.2.6. Switch from ACSF solution to fixative solution without introducing any air bubbles. Perfuse with fixative solution for 10 min at 10−20 mL/min.

CAUTION: Take care that no fixative solution is draining from the nose. This indicates that the needle reached the right ventricle and the fixative is traveling to the lungs rather than through the systemic circuit to the rest of the body.

2.3. Dissection of the brain

2.3.1. Remove the head of the mouse with scissors and carefully dissect out the mouse brain from the skull. Place in fixative solution for 1.5 h at room temperature for a short post-fixation period.

NOTE: A good perfusion is necessary for successful LY iontophoresis. Brain should be white-colored and absent of blood in brain vasculature. Body and limbs should appear stiff.

3. Slice preparation

3.1. Preparation of hippocampal slices

3.1.1. Wash the brain with 0.1 M phosphate buffered saline (PBS) at room temperature for 5 min. Then, dry off the brain with filter paper and remove the olfactory bulb and cerebellum with a sharp razor blade.

3.1.2. Mount the brain on the vibratome tray using cyanoacrylate glue and fill the tray with PBS at room temperature. Cut hippocampal coronal sections of 110 µm thickness.

NOTE: Adjust the setting of the vibratome to make sure that slices have the same thickness and even surface. For this experiment, a speed setting of 4.5 and a frequency setting of 8 were used, which are arbitrary settings on the instrument (**Table of Materials**). Users may need to experiment with the settings on other devices.

3.1.3. Collect the sections from the tray and place in a dish of PBS on ice.

4. Electrode preparation

4.1. Prepare a sharp electrode with appropriate resistance.

4.1.1. Use a borosilicate glass single barrel electrode with filament (O.D. 1.0 mm, I.D. 0.58 mm). Pull an electrode on a micropipette puller (**Table of Materials**). Ideal electrodes filled with 1.5% LY in 5 mM KCl should have a resistance of 200 MΩ when placed into a bath of PBS.

NOTE: Puller setting varies depending on the apparatus (type of machine used and puller filament). A higher heat setting usually gives longer and finer tips. For the micropipette puller used in this experiment, the settings were: heat: 317, pull: 90, velocity: 70, and delay: 70. A trough type filament was used.

4.1.2. Store electrodes in a closed container to prevent dust from entering the tip. Keep electrodes elevated from the bottom of the box to prevent the tip from breaking.

4.2. Fill the electrode with LY dye solution.

4.2.1. Place an electrode in vertical position with the tip facing downward. Pipette 1−2 µL of LY solution into the back of the electrode and wait for 5−10 min for the solution to move to the tip via capillary action.

4.2.2. Gently secure the filled electrode in the electrode holder connected to manipulator.

NOTE: The silver wire of the electrode holder needs to be in contact with the LY inside the

electrode. Adjust volume of the LY solution if needed, depending on the wire length.

5. Filling astrocytes with iontophoresis

5.1. Test the electrode.

5.1.1. Place a brain slice gently into a glass bottom dish filled with 0.1 M PBS at room temperature. Hold the slice in place with a platinum harp with nylon strings.

5.1.2. Ensure that the electrode is connected to a voltage source and place the ground electrode into the bath containing the brain slice.

5.1.3. Move the objective to the brain region of interest.

5.1.4. Lower the electrode into the solution. Under the bright field, move it to the center of the field of view and examine it carefully with the 40x water immersion lens to ensure that it appears clear and without debris or bubbles. If there is anything clogging the electrode tip, replace it with a new one.

NOTE: Clogging is a concern for the 200 MΩ electrode. With centrifugation and filtration of the dye solution before every experiment, this should not be a frequent problem. However, because the tip of the electrode is small, tissue may sometimes become stuck in the opening. The authors have not found a way to prevent this, but it can be easily dealt with by simply using a new electrode.

5.1.5. Observe the tip of the electrode under the confocal laser-scanning microscope with the 488 nm laser. Then, test dye ejection by turning on the stimulator at 12 V. Note a large fluorescent dye cloud around the tip of the electrode while the stimulator is on. If no or little dye is ejected upon voltage stimulation, replace the electrode.

5.1.6. Under the bright field, slowly lower the electrode toward the slice stopping just above the surface.

5.2. Fill astrocyte with iontophoresis.

5.2.1. Identify astrocytes 40−50 µm below the slice surface with the infra-red differential interference contrast (IR-DIC). Look for cells with elongated, oval-shaped somata about 10 µm in diameter. Once an astrocyte is chosen, move it to the center of the field of view.

NOTE: A good cell for iontophoresis has clear, defined borders around it. Do not choose cells too close to the surface of the slice because they cannot be completely filled. It takes some practice over a few days to be able to routinely identify astrocytes for dye filling. Depending on the brain area used for the experiment, the number of astrocytes may vary. This may affect the time needed to identify an astrocyte before filling.

5.2.2. Slowly lower the electrode tip into the slice, navigating through the tissue, until it is on the same plane as the cell body.

NOTE: Move electrode slowly to avoid damaging the tissue.

5.2.3. Once the cell body of the astrocyte is clearly visible and outlined, slowly and gently advance the electrode forward. Move electrode until the tip impales the soma of the cell. Move the focus of objective slowly up and down to note if the electrode is inside the soma.

NOTE: The electrode tip must be inside the cell body, and a small indentation on the soma should be observed. Do not move the electrode any further to avoid the tip going through the cell.

5.2.4. Once the electrode tip is inside the cell, turn on the stimulator at ~0.5−1 V and continuously eject current into the cell. Using the confocal microscope, watch the cell fill. Increase the digital zoom to see the details of the cell and make sure that the tip of the electrode is visible inside the cell.

NOTE: Lower the voltage if it appears that dye is leaking out of the cell or filling other cells in the vicinity. If it appears that dye is still leaking out of the cell, pull electrode out slowly and find another cell. It is important that the dye does not leak to have a high signal/ background ratio in the final image.

5.2.5. Wait for about 15 min until the finer branches and processes appear defined, turn off the voltage and gently withdraw the electrode tip from the cell.

5.3. Image the filled cell.

NOTE: Imaging can be performed immediately after filling or following staining with immunohistochemistry. An objective with a higher numerical aperture (NA) results in better resolution.

5.3.1. Wait until the cell returns to original form before imaging (15−20 min) with 40x objective. To image the cell, adjust the setting on the confocal to make sure that the finer branches and processes appear defined.

5.3.2. Set up a z-stack with a step size of 0.3 µm. While imaging, move the objective until there is no signal from the cell and set that as the top. Then, move the objective down (focusing through the cell) until there is no signal, set that as the bottom.

5.3.3. After the imaging is complete, check the electrode for dye ejection. If a large dye cloud appears, it can be used for the next slice. Otherwise, replace it with a new electrode.

NOTE: Dye filling of a single astrocyte and imaging takes about 45 min to 1 h. For this specific

experiment, it was possible to obtain about 3−6 cells per mouse. If needed, multiple cells can be labeled on the same slice. However, to distinguish individual astrocytes, be sure to keep a distance of about 200 µm between cells.

6. Staining with immunohistochemistry (optional)

6.1. Once the imaging is done, immediately place the slice in 10% formalin on ice to preserve for immunohistochemistry. Keep brain slices in the dark and store overnight at 4 °C.

6.2. Wash brain sections 3x in 0.1 M PBS with 0.5% nonionic surfactant (i.e., Triton X 100) for 5 min each. Then incubate in a blocking solution of 0.1 M PBS with 0.5% nonionic surfactant and 10% normal goat serum (NGS) for 1 h at room temperature with gentle agitation.

6.3. Incubate the sections with agitation in primary antibodies diluted in 0.1 M PBS with 0.5% nonionic surfactant and 5% NGS for 2 days at 4 °C.

NOTE: Because of the thickness of the brain slices, the incubation period of the primary antibodies needs to be extended for better penetration and the antibody concentration may be increased. In this experiment, a 1:500 dilution was used for anti GFAP antibody and a 1:500 dilution was used for anti aquaporin-4 antibody.

6.4. Wash the sections 3x in 0.1 M PBS with 0.5% nonionic surfactant for 10 min each and then incubate with secondary antibodies diluted in 0.1 M PBS with 0.5% nonionic surfactant and 10% NGS for 6 h at room temperature.

NOTE: Do not choose a secondary antibody excited by 488 nm, which is the wavelength used to visualize the LY dye. In this experiment, a 1:1000 dilution was used for Alexa Fluor 546 goat anti-chicken IgG (H+L) and Alexa Fluor 647 goat anti-rabbit IgG (H+L).

6.5. Rinse the sections 3x in 0.1 M PBS for 10 min each. Then mount the sections on glass microscope slides in mounting media suited for fluorescence. Seal slides. Image cells at a step size of 0.3 µm.

REPRESENTATIVE RESULTS:

The data reported in this study are from 7−12 cells from 4 mice in each experiment. Average data are reported in the figure panels where appropriate.

To assess astrocyte morphology, we performed intracellular iontophoresis using LY dye to fill astrocytes in the CA1 stratum radiatum, which is summarized in **Figure 1**. **Figure 2** depicts a representative astrocyte and its elaborate morphological structure. The cell was imaged post-fixation with the 60x oil immersion lens on a confocal laser-scanning microscope using the 488 nm laser line (step size of 0.3 µm and 3.0−3.5x digital zoom). The photomultiplier tube (PMT), offset, and gain functions of the confocal microscope were adjusted to create a high signal/ background ratio in the final image. In **Figure 2A**, single optical plane images from different z-

steps (shown every 10 µm, labeled in order from 1−6) reveal the central soma and several major branches which divided into a dense network of processes. By generating a maximum intensity projection (stack size of 85 µm) we observed a detailed view of the structure of the astrocyte and its domain **(Figure 2B)**. The main components of the astrocyte's structure have also been noted. **Figure 2C** shows a zoom in (x4) maximum intensity projection of one major branch, several secondary branches, and the distribution of the surrounding branchlets and leaflets.

Morphological analyses and reconstructions of astrocytes were performed with image analysis software (**Table of Materials**) using the post-fixation images of LY-filled astrocytes (other software such as Image J could also be used). After the reconstruction of each astrocyte was completed, the volumes of the soma, major branches, processes, and territory were quantified. The soma was created first with a surface smoothing set to the x-y plane resolution limit (0.25 µm). The minimum object diameter was set at 3.0 µm to remove other objects not associated with the cell body. To create the major branches, the intensity of the soma was masked, due to its brightness relative to the rest of the cell. The surface smoothing and minimum object diameter of the major branches was set to 0.3 µm (z plane step size). To create the processes, the intensity of the major branches was also masked. Surface smoothing was set to 0.18 µm and minimum object diameter was set to 0.3 µm. The territory of the astrocyte was created using a lower intensity threshold and surface smoothing set to 0.75 µm. **Figure 3A** shows the original image of a CA1 astrocyte. The cell body, the major branches, the processes, and the territory volume enclosed by the astrocyte are reconstructed in **Figure 3B-E**. After the reconstructions of the cells were created, the volumes of the soma, entire cell, and territory were quantified and the number of major branches was counted (**Table 1**). CA1 astrocytes from the stratum radiatum had an average soma volume of 488.91 μm^3, average of ~7 primary branches, average cell volume of 5.58 x 10^3 μm^3, and average territory volume of 2.94 x 10^4 μm^3.

The well-characterized astrocyte marker, GFAP, is a cytoskeletal protein that labels the intermediate filaments of an astrocyte[8]. After astrocytes were dye filled, we performed immunostaining for GFAP to visualize expression in individual astrocytes (**Figure 4**). We found that GFAP was expressed in the cell soma, major branches, and some secondary branches of astrocytes, but not in the finer branches and processes (**Figure 4A**). No significant difference was found in the number of primary branches labeled by GFAP and those visualized by LY ($p = 0.1573$; **Figure 4B**). The cell area and volume of the astrocyte labeled by GFAP were significantly smaller than the area and volume visualized with LY ($p < 0.0001$; **Figure 4C,D**). This demonstrates that GFAP is a reliable marker for labeling major branches, but is not useful for determining the overall area or volume of the cell.

An important feature of astrocytes is their endfeet, which contact blood vessels and are proposed to help regulate blood flow in the CNS. To further understand the spatial relationship between astrocytes and brain vasculature, we stained with antibodies against aquaporin-4 following dye filling. Aquaporin-4 is a water channel protein found on astrocytes and ependymal cells, and is highly expressed in areas near ventricles and blood vessels[15]. We found that aquaporin-4 is expressed on astrocyte endfeet in close proximity to brain vasculature (**Figure 5A**). The image depicts three astrocyte endfeet contacting a blood vessel at different locations (indicated by the

white arrows). In the CA1 stratum radiatum, the average number of endfeet per astrocyte was ~2 (**Figure 5B**). Interestingly, the branches containing endfeet were significantly thicker than the other primary branches of the astrocyte, using the major branch reconstructions ($p = 0.0038$; **Figure 5C**). We also measured the length of the branches containing endfeet from the center of the soma to the blood vessel and compared that to the shortest, direct path to the blood vessel. The actual length of the branches to the blood vessel was significantly greater than the shortest path ($p = 0.0333$; **Figure 5D**), which suggests that these branches tend to take a longer, circuitous route to the blood vessel. **Movie 1** depicts a movie of a reconstruction of a LY-filled astrocyte and aquaporin-4 staining. The different structural components of the astrocyte (soma, major branches, processes, and territory) are represented in three dimensions. The LY-filled astrocyte together with the aquaporin-4 staining depict the astrocyte endfeet encircling the blood vessel. From the reconstruction of the major branches and the blood vessel, the branches that contain endfeet can be visualized extending from the soma to the vessel.

In the preceding sections, where p values are reported we used an unpaired Student's t test, with significance at $p < 0.05$.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of workflow in LY iontophoresis. Schematic representation of protocol highlighting the critical steps. After the mouse was perfused with fixative, the brain was dissected. Following a short post-fixation period, coronal sections were cut with a vibratome. Electrode was backfilled with 1.5% LY dye. Astrocytes were identified in the CA1 stratum radiatum using IR-DIC on a light microscope. The soma of the cell was impaled by the electrode, and dye was injected into the cell by applying 0.5−1 V until the finer processes were completely filled. The slice was imaged with confocal microscopy using a 40x water immersion lens and then processed for immunohistochemistry. Further imaging was completed with a 60x oil immersion lens to perform cell reconstruction and morphological analysis. Shown is an example reconstruction of the processes.

Figure 2: LY-filled astrocyte of CA1 stratum radiatum. (**A**) Single optical plane images from an astrocyte shown every 10 µm (labeled 1−6). (**B**) Maximum projection of the astrocyte, depicting the cell soma, several major branches, and numerous processes which make up its bushy territory. (**C**) Maximum projection (zoom x4) of one major branch (from section outlined in yellow), two secondary branches, several branchets, and the organization of the surrounding processes. Scale bar = 10 µm.

Figure 3: Reconstruction of a LY-filled astrocyte and its components. (**A**) CA1 astrocyte filled with LY. (**B-E**) Three-dimensional reconstruction of the soma (**B**), soma and major branches (**C**), processes (**D**), and territory (**E**) at 0° and 45° orientation. Scale bar = 10 µm.

Figure 4: GFAP immunostaining in LY-filled astrocytes. (**A**) Representative z-projection of LY (green) and GFAP (magenta) staining. GFAP is expressed primarily in the primary and some secondary branches of the astrocyte, but was not found within the entire astrocyte territory.

Scale bar = 10 µm. (**B**) Graph of number of primary branches labeled by LY and GFAP. (**C**) Cell area denoted by LY and GFAP staining. (**D**) Cell volume denoted by LY and GFAP staining. Open circles are raw data with closed squares indicating mean ± SEM. Data was collected from 12 cells from 4 mice.

Figure 5: Aquaporin-4 immunostaining in LY-filled astrocytes. (**A**) Representative z-projection of LY (green) and aquaporin-4 staining (magenta). Aquaporin-4 is expressed mainly in the endfeet of the astrocyte. White arrows denote the three endfeet as they contact a nearby blood vessel. Scale bar = 10 µm. (**B**) Number of branches with endfeet per astrocyte. (**C**) Thickness of branches with endfeet in comparison to the other primary branches of astrocytes. (**D**) Length of branches with endfeet in comparison to the shortest path to the blood vessel. Open circles are raw data with closed squares indicating mean ± SEM. Data was collected from 7 cells from 4 mice.

Movie 1: Reconstruction of a LY-filled astrocyte and aquaporin-4 staining. Representative movie of a CA1 astrocyte in proximity to a blood vessel. The soma, major branches, processes, and territory were reconstructed to analyze the morphology of the cell. The reconstruction of the major branches together with aquaporin-4 depict two astrocyte endfeet directly contacting the blood vessel.

Table 1: Morphological analysis of astrocyte structure. The astrocyte soma volume, number of primary branches per astrocyte, astrocyte cell volume, and volume enclosed by astrocyte territory are shown. Data was collected from 14 cells from 7 mice.

DISCUSSION:

The method outlined in this paper describes a way to visualize astrocyte morphology using intracellular iontophoresis of LY dye in lightly fixed brain slices. There are several critical factors highlighted in this protocol that influence the quality of the LY iontophoresis and morphological reconstruction of the cells. One factor is the quality and reproducibility of the images, which is determined largely by the age of the mouse and the outcome of the perfusion. In this study, we used C57/BL6N mice about 6−8 weeks old. A successful perfusion (highly dependent on the proper placement of the perfusing needle and noted by a white-colored brain and absence of blood in brain vasculature) is necessary for the most detailed and clearly filled cells. After impalement by an electrode, the cell membrane should maintain a tight seal around the tip of the electrode and prevent dye from leaking out. Despite best efforts, occasionally other structures not part of the cell will be filled as the pipette is advanced through brain tissue: we excluded these cells from the analysis. The resistance of the electrodes is an additional key factor. The high resistance only allows a steady ejection of dye from the electrode tip upon voltage stimulation. More technically, it is important to maintain gentle, slow movements when impaling the soma of the cell with the electrode. Electrode penetration through the cell can lead to dye leakage from the soma. A successful impalement followed by voltage application should result in almost immediate dye filling of the cell body and processes (i.e., within a few seconds). The time required to completely fill an astrocyte is related to the territory it encompasses; however, wait to ensure the finer processes are completely filled, at least 15 minutes.

We found this protocol to be a most faithful way to study astrocyte morphology in detail, nonetheless, the method has its limitations. Identifying a cell can be time-consuming and error prone. Under IR-DIC, one should identify distinctive features that mark the specific cell type (soma shape and size). Alternatively, the expression of red fluorescent reporters specifically in astrocytes, by viral injection or a transgenic mouse reporter line, allows for easier identification of these cells before filling. Also, LY is limited as a cytosolic dye because it cannot be used to label the astrocyte membrane, in comparison to labeling with a membrane-tethered GFP, such as Lck-GFP[16]. Lck-GFP would give a more accurate representation of the entire territory area, as LY iontophoresis reveals the internal volume of an astrocyte. However, depending on the experimental design, LY iontophoresis is better suited to resolve the entire astrocyte internal volume, develop three-dimensional reconstructions, and quantify the anatomical components that make up an astrocyte's structure[6]. Finally, as with all forms of confocal microscopy, spatial resolution is limited by diffraction, noted as the point spread function of the microscope optics[17]. Altering components of the imaging system, such as decreasing pinhole diameter, will help improve the images, but the true resolution is determined by the wavelength of light and the numerical aperture of the objective lens. In our case, we estimate the best resolution possible is probably around 300 nm, which is likely to be worse in the z-axis.

LY iontophoresis offers several advantages over other commonly used methods to label astrocytes. The protocol can be applied in any established mouse model, cell population, or brain region[18,19,20] as it is not limited by an astrocyte specific promoter or a transgenic mouse line. Genetic approaches to express fluorescent proteins ubiquitously in astrocytes by viral injections or transgenic mouse reporter lines (i.e., td-Tomato) require the use of an astrocyte promoter, which, in some regions, can be expressed in other cell types or not include all the astrocytes[21]. LY iontophoresis is also time-efficient, as viral injections of fluorescent proteins require surgery and time to express the specific virus, and transgenic mouse lines require breeding. Finally, LY iontophoresis is a useful method to distinguish individual cells, while other strategies would also need to be combined with a method for sparse labeling to visualize the territories of individual astrocytes[22,23,24]. However, no one method is a panacea and the choice of which is used needs to be tailored to the specific question being addressed.

Future studies can employ specific experimental manipulations and examine different components of astrocyte structure (soma, branches, processes, territory, etc.) to answer morphological questions. This could provide insight and direction into astrocyte morphology and its functional implications, which could be analyzed further by super resolution light microscopy or electron microscopy[4]. For example, LY iontophoresis provides a means to visualize fine astrocyte processes, which contact thousands of synapses and are involved in synaptic functions. Studying how the structure of these processes changes in different pathological conditions could help elucidate the roles of astrocytes in health and disease. LY iontophoresis is an important technique to visualize detailed cell morphology and characterize astrocyte properties to better understand their functions in the central nervous system.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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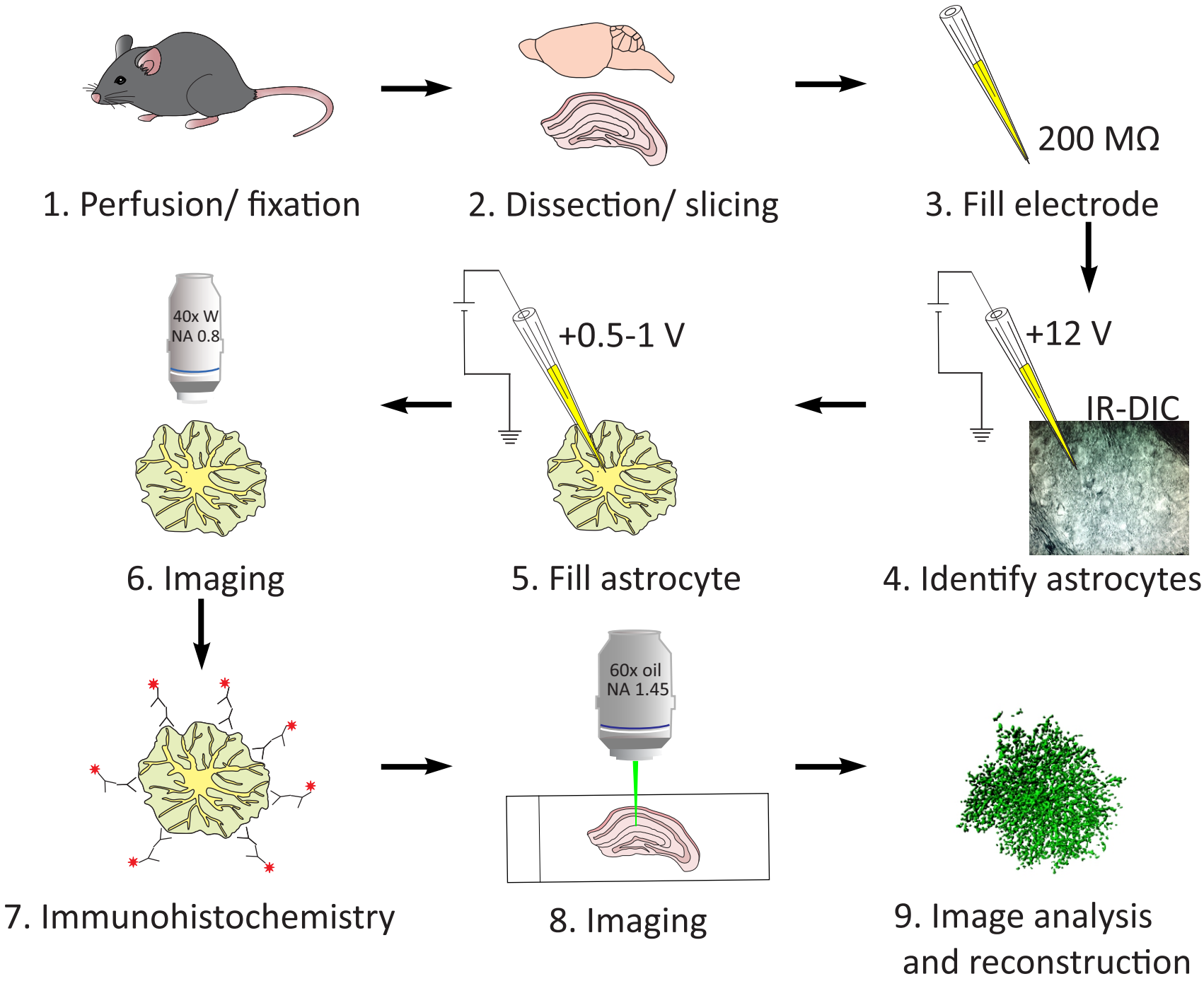

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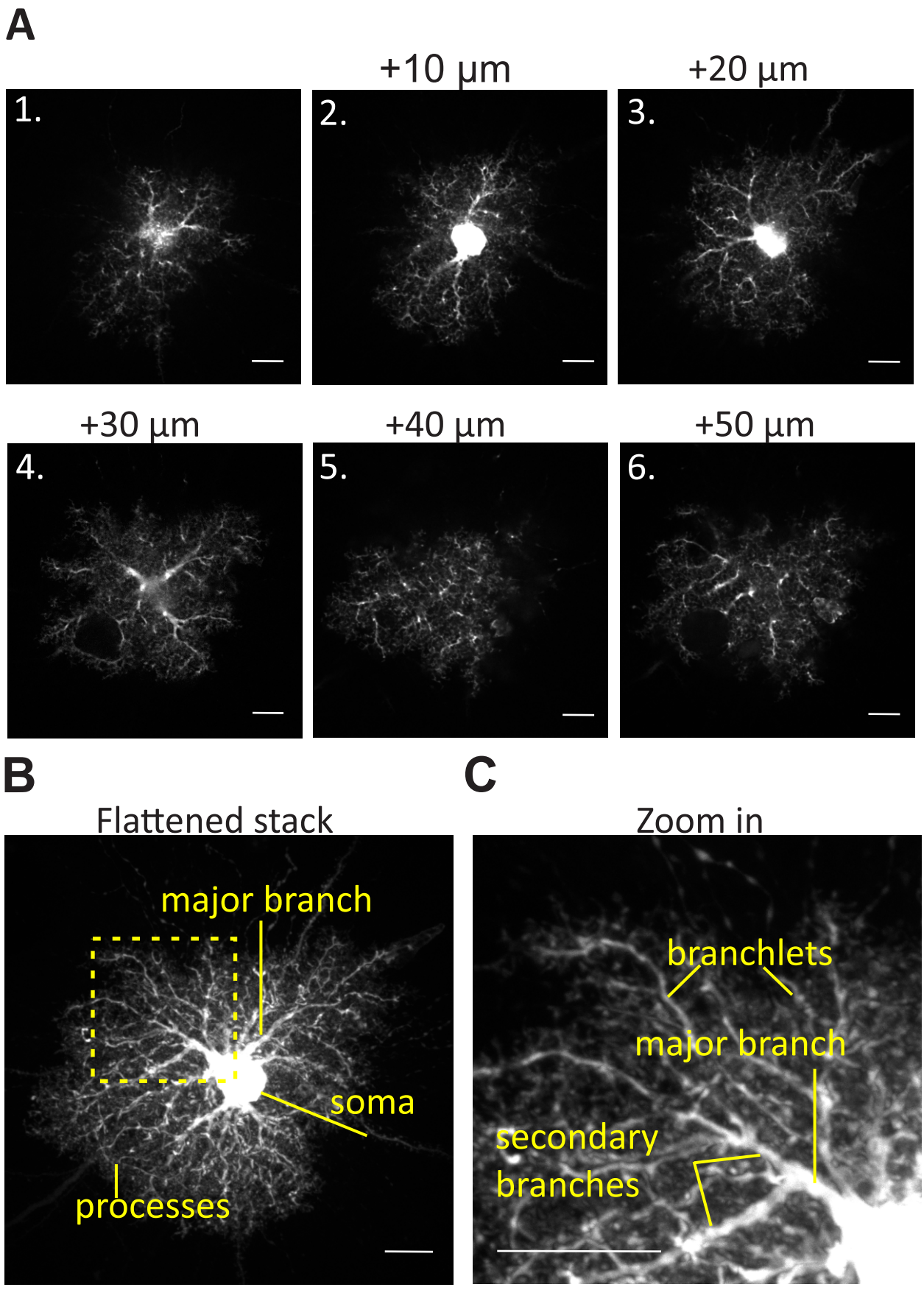

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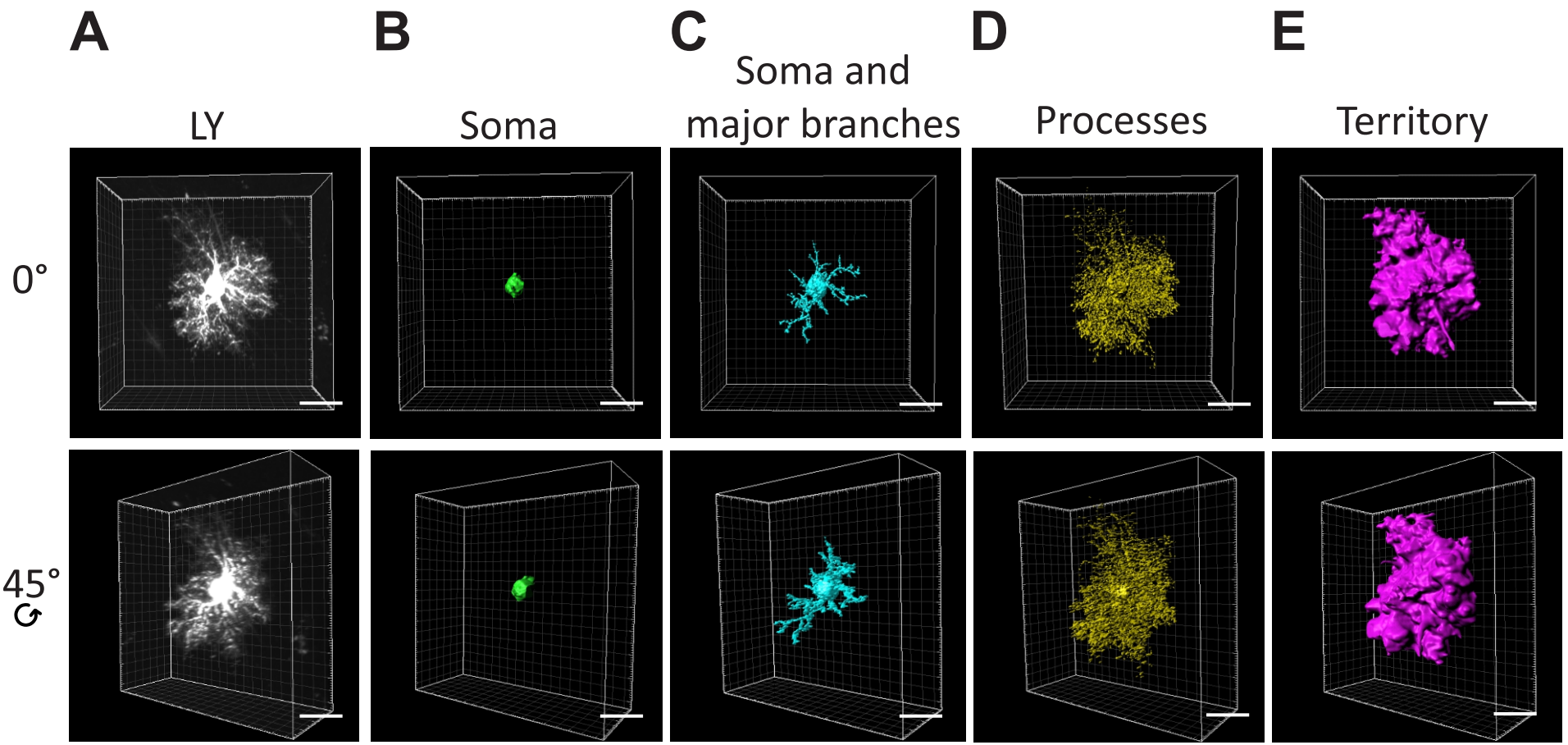

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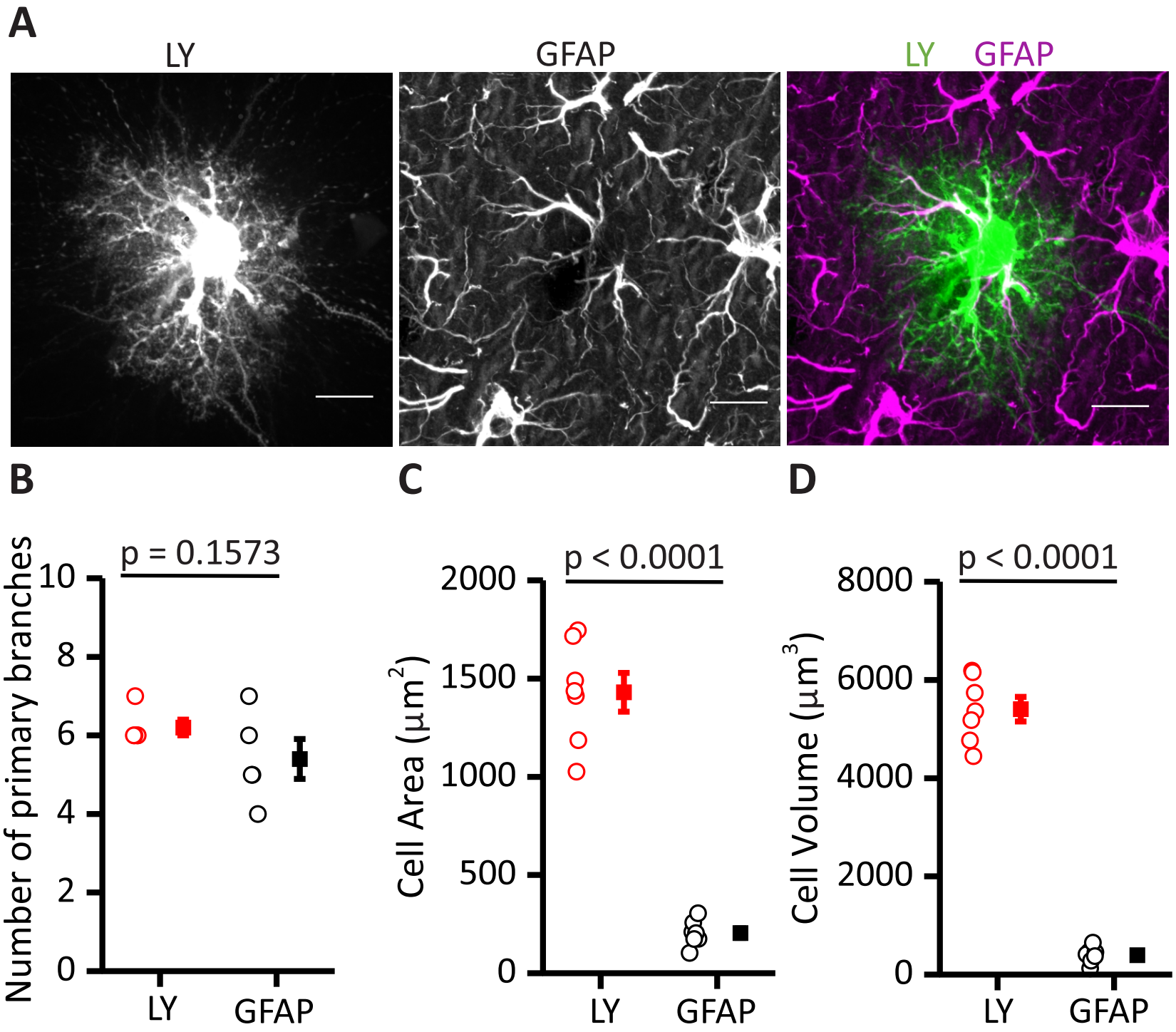

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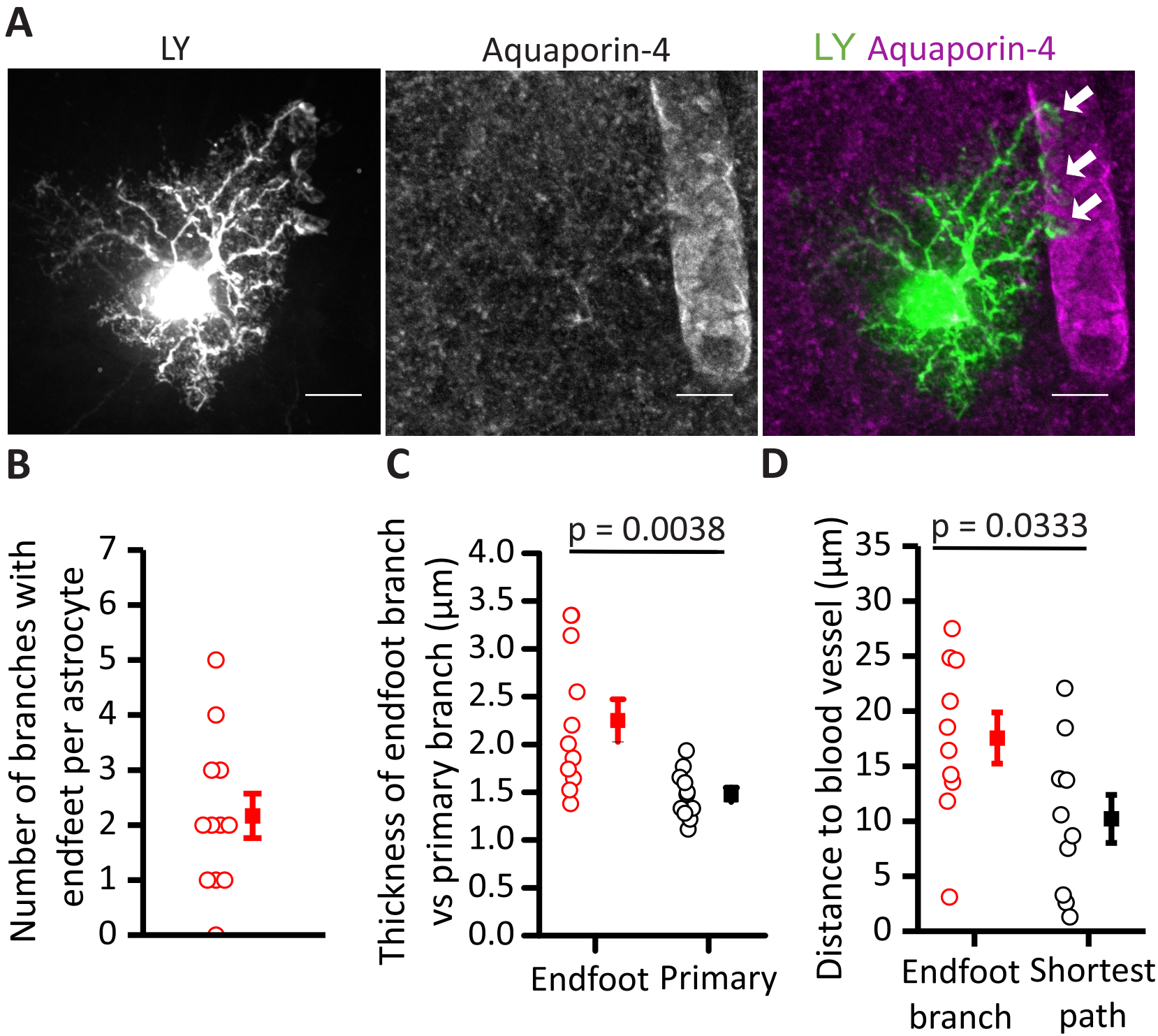

Movie 1

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Video or Animated Figure

Moye et al., LY-filled astrocyte with aquaporin 4.mp4

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Morphological characteristics	Mean ± SEM
Soma volume (μm^3)	489 ± 30
Number of primary branches	7.0 ± 0.5
Cell volume (μm^3)	5580 ± 425
Territory volume (μm^3)	29391 ± 8150
Number of cells	14

Click here to access/download;Table of Materials;Moye et al. 2019 JoVE_Materials (revised).xlsx

Name of Material/ Equipment	Company	Catalog Number
10% Buffered Formalin Phosphate	Fisher	SF 100-20
Acrodisc Syringe Filters with Supor Membrane	Pall	4692
Ag/AgCl ground pellet	WPI	EP2
Alexa Fluor 546 goat anti-chicken IgG (H+L)	Thermo Scientific	A-11040
Alexa Fluor 647 goat anti-rabbit IgG (H+L)	Thermo Scientific	A27040
Anti Aquaporin-4 antibody	Novus Biologicals	NBP1-87679
Anti GFAP antibody	Abcam	ab4674
Borosilicate glass pipettes with filament	World precision instruments	1B150F-4
C57BL/6NTac mice	Taconic Stock	B6
Calcium Chloride	Sigma	21108
Confocal laser-scanning microscope	Olympus	FV1000MPE
D-glucose	Sigma	G7528
Disodium Phosphate	Sigma	255793
Electrode puller- Model P-97	Sutter	P-97
Fluoromount-G	Southern Biotech	0100-01
Heparin sodium injection (1,000 USP per mL)	Sagent Pharmaceuticals	400-10
Imaris software (Version 7.6.5)	Bitplane Inc.	
Isofluorane	Henry Schein Animal Health	29404
Lidocaine Hydrochloride Injectable (2%)	Clipper	1050035
Lucifer Yellow CH dilithium salt	Sigma	L0259
Lucifer Yellow CH dipotassium salt	Sigma	L0144
Magnesium Chloride	Sigma	M8266
Microscope Cover Glass	Thermo Scientific	24X60-1
Microscope Slides	Fisher	12-544-2
Normal Goat Serum	Vector Laboratories	S-1000
Objective lens (40x)	Olympus	LUMPLFLN 40XW
Objective lens (60x)	Olympus	PlanAPO 60X
PBS tablets, 100 mL	VWR	VWRVE404
Pipette micromanipulator- Model ROE-200	Sutter	MP-285 / ROE-200 / MPC-200

Potassium Chloride	Sigma	P3911
Sodium Bicarbonate	Sigma	S5761
Sodium Chloride	Sigma	S5886
Stimulator- Model Omnical 2010	World precision instruments	Omnical 2010
Triton X 100	Sigma	T8787
Vibratome- Model #3000	Pelco	100-S

Comments/Description

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Author(s):	Stefanie L. Moye, Blanca Diaz-Castro, Mohitkumar R. Gangwani, and Baljit S. Khakh

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Rebuttal Letter
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General comments to the editor and the reviewers

Thank you for the careful review of our JoVE submission (#JoVE60225). In the sections that follow we have addressed all the points raised and have shown the changes in the revised files using blue text. The suggestions have improved our manuscript and we are grateful for the comments we received.

All the best,
Bal Khakh
(on behalf of all authors) 15th June 2019

Editorial comments

1. *"Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version."*

Thank you. We have carefully proofread the manuscript.

2. "*Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc."*

As suggested by the editors, we have changed the numbering of the protocol.

3. *"Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below."*

Thank you. We have added references and more detail to the protocol steps.

4. *"Line 103: Please list an approximate volume to prepare."*

We have modified the line 107 to add "A volume of 1 mL is sufficient for several experiments."

5. *"Line 106: What happens after centrifugation? Is the supernatant transferred to another container?"*

We have modified line 109, "Then, filter the supernatant with a 0.2 µm membrane filter into a new tube.

6. *"Line 117: Please specify the age, gender and type of mouse. What concentration of isoflurane is used?"*

We have modified line 116-118 to read,

"C57/BL6 mice of either sex can be used. We have empirically observed that for the method to work reliably, it is important that the mice are not older than 3 months (6-8 weeks old is ideal)."

In line 125, we state,

"Deeply anesthetize mouse by putting it into an isoflurane induction chamber, after adding 2-3 mL of isoflurane to the chamber."

7. *Please specify surgical tools used throughout the protocol.*

A reference to a perfusion fixation protocol for rodents was added in line 118. The surgical tools were also added to the protocol.

8. *"Lines 126, 129: How large is the incision?"*

In line 136 we state,

"Make a 5-6 cm incision with the curved, blunt scissors through the skin to expose the abdominal cavity."

In line 140 we state,

"With iris scissors, make a 3-4 cm lateral incision in the diaphragm."

9. "*Line 253: Please describe how."*

Line 280 states,

"Set up a z-stack with a step size of 0.3 µm. While imaging, move the objective until there is no signal from the cell and set that as the top. Then, move the objective down (focusing through the cell) until there is no signal, set that as the bottom."

10. *"Please discuss critical steps in the discussion section"*

We have included discussion on critical steps beginning at line 441,

"There are several critical factors highlighted in this protocol that influence the quality of the LY iontophoresis and morphological reconstruction of the cells. One factor is the quality and reproducibility of the images, which is determined largely by the age of the mouse and the outcome of the perfusion. In this study, we used C57/BL6N mice about 6-8 weeks old. A successful perfusion (highly dependent on the proper placement of the perfusing needle and noted by a white-colored brain and absence of blood in brain vasculature) is necessary for the most detailed and clearly filled cells. After impalement by an electrode, the cell membrane should maintain a tight

seal around the tip of the electrode and prevent dye from leaking out. Despite best efforts, occasionally other structures not part of the cell will be filled as the pipette is advanced through brain tissue: we excluded these cells from the analysis. The resistance of the electrodes is an additional key factor. The high resistance only allows a steady ejection of dye from the electrode tip upon voltage stimulation. More technically, it is important to maintain gentle, slow movements when impaling the soma of the cell with the electrode. Electrode penetration through the cell can lead to dye leakage from the soma. A successful impalement followed by voltage application should result in almost immediate dye filling of the cell body and processes (i.e. within a few seconds). The time required to completely fill an astrocyte is related to the territory it encompasses; however, wait to ensure the finer processes are completely filled, at least 15 minutes."

11. *"Please describe how image analysis and reconstruction is done. These steps do not have to be filmed."*

We have included a section on image analysis and reconstruction in the results section beginning at line 336,

"Morphological analyses and reconstructions of astrocytes were performed with Imaris software (Version 7.6.5) using the post-fixation images of LY-filled astrocytes (other software such as Image J could also be used). After the reconstruction of each astrocyte was completed, the volumes of the soma, major branches, processes, and territory were quantified. The soma was created first with a surface smoothing set to the x-y plane resolution limit (0.25 µm). The minimum object diameter was set at 3.0 µm to remove other objects not associated with the cell body. To create the major branches, the intensity of the soma was masked, due to its brightness relative to the rest of the cell. The surface smoothing and minimum object diameter of the major branches was set to 0.3 µm (z plane step size). To create the processes, the intensity of the major branches was also masked. Surface smoothing was set to 0.18 µm and minimum object diameter was set to 0.3 µm. The territory of the astrocyte was created using a lower intensity threshold and surface smoothing set to 0.75 µm. **Figure 3A** shows the original image of a CA1 astrocyte. The cell body, the major branches, the processes, and the territory volume enclosed by the astrocyte are reconstructed in **Figure 3B-E**. After the reconstructions of the cells were created, the volumes of the soma, entire cell, and territory were quantified and the number of major branches was counted **(Table 1)**. CA1 astrocytes from the stratum radiatum had an average soma volume of 488.91 μm^3, average of ~7 primary branches, average cell volume of 5.58 x 10^3 μm^3, and average territory volume of 2.94 x 10^4 μm^3."

12. *"Figure 3: Please describe different panels in the figure legend."*

We have included descriptions of individual panels in lines 409-411.

"**A.** CA1 astrocyte filled with LY. **B-E.** Three- dimensional reconstruction of the soma (B), soma and major branches (C), processes (D), and territory (E) at 0° and 45° orientation. (Scale bar = 10 µm)."

13. *"Please upload Table 1 to your Editorial Manager account as an .xlsx file."*

Table 1 is now updated as an .xlsx file.

14. *"Table of Materials: Please sort the materials alphabetically by material name."*

We have changed the list to alphabetical order.

15. *"References: Please do not abbreviate journal titles; use full journal name."*

We have updated the reference list to include the full journal names.

Reviewer #1

Major Concerns:

"When using a 60x oil objective, the image stack may not cover the whole astrocytes. A single astrocyte may have a size up to 100 um (diameter if it is considered as a sphere structure). The image quality up to 50 um deep may significantly decrease with confocal microscopy. This can be discussed."

The 60x oil objective is sufficient to image an entire mouse astrocyte which has a diameter of 40-60 µm on average (Khakh, B. S., & Sofroniew, M. V., 2015). We completely agree with the reviewer's comment about the z-resolution in confocal microscopy. We have included this point in the discussion of revised manuscript (lines 467-470).

"Finally, as with all forms of confocal microscopy, spatial resolution is limited by diffraction, noted as the point spread function of the microscope optics[17]. Altering components of the imaging system, such as decreasing pinhole diameter, will help improve the images, but the true resolution is determined by the wavelength of light and the numerical aperture of the objective lens. In our case, we estimate the best resolution possible is probably around 300 nm, which is likely to be worse in the z-axis."

Minor Concerns:

"The author may want discuss the advantages and disadvantages of using astrocyte specific FP labeling method as compared with dye filling of current protocol. Dye filling with iontophoresis of fresh slice needs a good skill, which is difficult for many people."

Thank you for this suggestion. We have included some discussion about astrocyte specific fluorescent protein labeling methods in the discussion section beginning at line 478.

"LY iontophoresis offers several advantages over other commonly used methods to label astrocytes. The protocol can be applied in any established mouse model, cell population, or brain region[18, 19, 20] as it is not limited by an astrocyte specific promoter or a transgenic mouse line. Genetic approaches to express fluorescent proteins ubiquitously in astrocytes by viral injections or transgenic mouse reporter lines (i.e., td-Tomato) require the use of an astrocyte promoter, which, in some regions, can be expressed in other cell types or not include all the astrocytes[21]. LY iontophoresis is also time-efficient, as viral injections of fluorescent proteins require surgery and time to express the specific virus, and transgenic mouse lines require breeding. Finally, LY

iontophoresis is a useful method to distinguish individual cells, while other strategies would also need to be combined with a method for sparse labeling to visualize the territories of individual astrocytes[22, 23, 24]. However, no one method is a panacea and the choice of which is used needs to be tailored to the specific question being addressed."

Reviewer #2

Major Concerns:

"As mentioned above, addressing other approaches for astrocyte imaging in the discussion is merited. The authors state in the discussion that LY iontophoresis offer several advantages over other commonly used methods, including the fact that it has a unique ability to resolve the entire astrocyte structure in 3 dimensions, and that it can be combined with immunohistochemistry, and applied to any established mouse model, cell population, or brain region. However, it is unclear to me how these advantages do not apply to other methods for 3D imaging of astrocytes, particularly tdTomato and LckGFP. If the authors wish to make this claim, it would be important to demonstrate this advantage by direct comparison with these methods. Certainly, individual labeling by iontophoresis provides an enhanced level of control over the number of cells labelled. Relatedly, it would be useful to the reader to provide citations (space limitations permitting) which put this astrocyte imaging approach in the context of others."

We agree with the reviewer's suggestion, and have added some discussion about astrocyte specific fluorescent protein labeling methods from lines 465-489.

"Also, LY is limited as a cytosolic dye because it cannot be used to label the astrocyte membrane, in comparison to labeling with a membrane-tethered GFP, such as Lck-GFP[16]. Lck-GFP would give a more accurate representation of the entire territory area, as LY iontophoresis reveals the internal volume of an astrocyte. However, depending on the experimental design, LY iontophoresis is better suited to resolve the entire astrocyte internal volume, develop three-dimensional reconstructions, and quantify the anatomical components that make up an astrocyte's structure[6]. Finally, as with all forms of confocal microscopy, spatial resolution is limited by diffraction, noted as the point spread function of the microscope optics. Altering components of the imaging system, such as decreasing pinhole diameter, will help improve the images, but the true resolution is determined by the wavelength of light and the numerical aperture of the objective lens. In our case, we estimate the best resolution possible is probably around 300 nm, which is likely be worse in the z-axis.

LY iontophoresis offers several advantages over other commonly used methods to label astrocytes. The protocol can be applied in any established mouse model, cell population, or brain region[18, 19, 20] as it is not limited by an astrocyte specific promoter or a transgenic mouse line. Genetic approaches to express fluorescent proteins ubiquitously in astrocytes by viral injections or transgenic mouse reporter lines (i.e., td-Tomato) require the use of an astrocyte promoter, which, in some regions, can be expressed in other cell types or not include all the astrocytes[21]. LY iontophoresis is also time-efficient, as viral injections of fluorescent proteins require surgery and time to express the specific virus, and transgenic mouse lines require breeding. Finally, LY iontophoresis is a useful method to distinguish individual cells, while other strategies would also need to be combined with a method for sparse labeling to visualize the territories of individual

astrocytes[22, 23, 24]. However, no one method is a panacea and the choice of which is used needs to be tailored to the specific question being addressed."

Minor Concerns:

1). *"The authors state that 7-12 cells from 4 mice in each experiment were used for the reported study. Approximately how long does it take to individually label this number of cells, either overall or for 1 slide or mouse? This will be useful for anyone who would like to adopt this approach."*

We have included this information in lines 244-246,

"Depending on the brain area used for the experiment, the number of astrocytes may vary. This may affect the time needed to identify an astrocyte before filling." Also, line 282 states, "Dye filling of a single astrocyte and imaging takes about 45 minutes to an hour. For this specific experiment, it was possible to get about 3-6 cells per mouse."

2). *"If multiple cells are labelled, is there any distance between cells which need to be allowed, in order to get good individual images of fine branches?"*

We included a note about this in lines 287-289,

"If needed, multiple cells can be labeled on the same slice. However, to distinguish individual astrocytes, be sure to keep a distance of about 200 µm between cells."

3). *"What dilution of Aquaporin-4 primary antibody was used for IHC? (I apologize if I missed it)."*

Line 300 states

"we used a 1:500 dilution for Anti GFAP antibody and a 1:500 dilution for Anti Aquaporin-4 antibody."

4). *"How much of a concern is clogging for a 200 MOhm electrode? Does this happen with any frequency?"*

We addressed this comment in the text lines 224-227,

"Clogging is a concern for the 200 MΩ electrode. With centrifugation and filtration of the dye solution before every experiment, this should not be a frequent problem. However, because the tip of the electrode is small, tissue may sometimes become stuck in the opening. We have not found a way to prevent this, but it can be easily dealt with by simply using a new electrode"

Overall, many thanks to the editor and reviewers for their comments – which have improved our manuscript.