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TITLE:

Biocytin Recovery and 3D Reconstructions of Filled Hippocampal CA2 Interneurons

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SHORT ABSTRACT:

The protocol outlined here describes the immunofluorescence analysis, biocytin recovery and high-quality reconstructions of hippocampal CA2 interneurons following the intracellular electrophysiological recordings *in vitro*, allowing neuronal characterization and ultimately fine neuronal anatomy to be studied.

LONG ABSTRACT:

How cortical network activity processes information is of importance to a large number of basic and clinical scientific questions. The protocol described here identifies the basic building blocks of this circuitry. The in-depth studies of cortical regions will ultimately provide other scientists with the circuit components needed for an understanding of how the brain acquires, processes and stores information and what goes wrong in disease, while the electrophysiological and morphological data are widely used by computational neuroscientists in the construction of model networks that explore information processing. The protocol outlined here describes how biocytin-filled cells recorded in the CA2 region of the hippocampus are recovered and then reconstructed in both 2D and 3D. Additionally, the protocol describes the demonstration of calcium binding protein or peptide content in recorded interneurons.

INTRODUCTION:

The cortex and hippocampus are structures of such complexity that the classification of neuronal subtypes¹⁻⁴, maps of the connections between them⁵⁻¹¹ and how this circuitry supports cognitive functions¹²⁻¹⁵ are still under intense study and the subject of continuing debate. For example, to understand the details and complexities of the circuitry and to coordinate data obtained from many different studies, it is extremely helpful to be able to define and describe the components, but it remains a matter for debate how many different classes of neurons exist, or even whether it is possible to define all neurons as belonging to a specific class. Computational tools that can build and test circuits with varying degrees of

complexity are being developed¹⁶⁻¹⁸, but central to these endeavors is the need for detailed studies of the cell types and of the properties of the connections between them. A large amount of information about the local circuitry of the neocortex of adult rats has already been collected using the protocol described here^{10,19-22}. Although the “wiring diagram” is far from complete, some clear patterns or rules have emerged. Moreover, although some details vary, these rules are common to two mammalian species (rat and cat) and across several neocortical regions, allowing the development of basic building blocks that are likely to be equally applicable to human neocortex. The technique described here is used to extend our understanding of the functional map of cortical circuitry by identifying the presynaptic and postsynaptic neurons involved in connections in the regions that have not been studied in detail before, using a protocol that allows excellent tissue preservation and remarkable staining recovery in adult brain tissue. Data on the local circuitry in CA2 and neuronal properties in this subfield have been collected with this method by combining intracellular electrophysiological recordings (paired recordings with sharp microelectrodes) with biocytin filling, immunofluorescence, histological procedures and highly-detailed neuronal reconstructions, allowing direct comparison with the neighboring CA regions²³⁻²⁵.

The technique described in this article has been developed over the years to obtain detailed neuronal anatomy allowing the correct classification of the cells and high quality and accurate reconstructions of both their dendritic and axonal arbors, data that can be correlated with electrophysiological data collected from paired recordings using sharp electrodes. The histological protocol has been optimized to preserve the ultrastructure of the neurons and obtain excellent recovery of both dendritic (including spines) and axonal arbors. For example, the principle of the double fixation technique by firstly immersing in a fixative solution and secondly post-fixing in osmium tetroxide gives a good contrast for light microscopy²⁶. A small amount of glutaraldehyde and picric acid solution are added to the fixative solution to enhance antibody penetration and to preserve the cells’ ultrastructure as suggested in a previous study²⁷. The permeabilization of the brain slices using the freeze-thaw method combined with cryo-protection with sucrose, rather than a traditional detergent, also provides optimal preservation of the tissues for detailed morphological analysis of the recorded cells. In addition, the visualization particularly of very fine structures is improved by reducing background staining, with the incubations with hydrogen peroxide (H₂O₂) and sodium borohydride (NaBH₄). Adding nickel chloride (NiCl₂) to the horseradish peroxidase (HRP) reaction to obtain a black pigment reaction product also increases the contrast.

The following protocol describes the procedures used to ascertain excellent tissue preservation and highly detailed neuronal 2D and 3D reconstructions following intracellular recordings *in vitro*. The description of the slice preparation, intracellular paired recordings using sharp electrodes and subsequent histological procedures used in our laboratory have been previously reported²⁸. Although the protocol applies to the cells filled intracellularly with biocytin in 450-500 µm thick slices, the same protocol may be used following whole-cell recordings. However, the use of thinner slices will result in less complete reconstructions of the cells.

PROTOCOL:

All procedures used throughout this study were carried out according to the British Home Office regulations with regard to the Animal Scientific Procedures Act 1986.

1. Determination of Calcium Binding Protein or Protein Content of Interneurons and Biocytin Visualization Following Electrophysiological Recordings and Biocytin Filling

Note: At the end of the electrophysiological recordings, slices that contain biocytin-filled cells are fixed overnight prior to histological procedures. The fixative solution will be replaced with a single change of 0.1 M phosphate buffer the next morning, if the rest of the procedure is carried out on another day, in order to prevent tissue damage. The fixation solution (4% paraformaldehyde, 0.2% saturated picric acid solution, 0.025% glutaraldehyde solution in 0.1 M phosphate buffer (PB)) must be made fresh on the day of the recordings for best results.

1.1. At the end of the electrophysiological recording, carefully pick up the slice containing the recorded cell(s) with a paintbrush and place it in a pot containing artificial cerebral spinal fluid (ACSF).

Note: Details of the protocol used for slice preparation and intracellular recordings using sharp electrodes have been described previously²⁸.

1.2. In a fume cupboard, carefully pick up the brain slice with a paintbrush and roll it onto a small piece of fine quality filter paper. Place another piece of moistened filter paper onto the slice and place the two pieces of wet filter paper in a small plastic pot containing 5-10 mL of fixative solution and store in the fridge overnight at 4 °C.

1.3. Prepare a solution of gelatin in distilled water. Place 20 mL of distilled water in a beaker on a hot plate and heat it to 60 °C. Add progressively 2.4 g of gelatin to the water, wait until dissolved and allow it to cool to 35-40 °C before use to prevent tissue damage.

1.4. Replace the fixative solution with 2 mL of 0.1 M PB. Place the tissue in a Petri dish (diameter of 9 cm, height of 1.4 cm), ensuring that it lies flat with no folds or creases, and remove the excess buffer using a dry paintbrush.

1.5. Cover the tissue with the warm gelatin solution and place the Petri dish onto a frozen block to quickly cool the solution.

1.6. Using an angle-poise lamp to provide contrast, look through the side of the dish and keep the slice flat using light pressure from a fine paintbrush until the gelatin begins to set.

Note: The gelatin can be re-melted if the tissue does not lie flat. Any imperfections on the surface of the gelatin caused by the removal of the paintbrush as it solidifies may be removed by melting the surface layer gently by moving the bulb of the lamp close to the surface for a few seconds.

1.7. Move the dish of setting gelatin to the fridge and leave at 4 °C for 30-60 min.

1.8. In a fume cupboard, cut out a small block (~ 1 x 1 cm) containing the gelatin-embedded tissue of the dish using a scalpel blade, lift the block using a small spatula and carefully place it in the same but fresh fixative solution used to fix the slices for at least 30 min at 4 °C.

1.9. Wash the gelatin block in 5 mL of 0.1 M PB three times, dry it using a piece of paper tissue and stick the block side up (*i.e.*, with the tissue at the top) onto a vibratome chuck using superglue.

1.10. Remove excess glue with a piece of filter paper and use a scalpel blade to cut the corners of the block off, leaving a diamond shape.

1.11. Section the slice at 50 µm thickness using a vibratome and place each section carefully in a glass vial containing 10% sucrose.

1.12. Carefully pick up a section from the vial, place it flat into a Petri dish lid. Using a dissecting microscope and a fresh scalpel blade, remove the gelatin from around the section and return the section to a vial containing 2 mL of fresh 10% sucrose

Note: It is crucial to remove as much gelatin as possible at this stage to reduce tissue shrinkage during the dehydration step (Step 1.37).

1.13. Cryo-protect the sections in 0.1 M PB-based sucrose-glycerol solution at room temperature by incubating them for 10 min in 10% sucrose solution, 20 min in 20% sucrose-6% glycerol solution twice and finally 30 min in 30% sucrose-12% glycerol solution twice under constant agitation.

1.14. Place the sections flat onto a small rectangle of tin foil using a paintbrush. Remove any excess liquid from the sections and carefully fold the tin foil into a parcel.

1.15. Hold the tin foil close to the surface of liquid nitrogen without touching the surface for 30 s and then allow the sections to thaw completely for approximately 30 s. Repeat the freeze-thaw another two times.

1.16. Remove all sections with a paintbrush and place them in a glass vial containing 2 mL of 0.1 M PB under constant agitation to wash off excess sucrose.

1.17. Remove the PB with a Pasteur pipette and incubate the sections in 2 mL of 1% aqueous H₂O₂ for 30 min. Wash the sections in 2 mL of 0.1 M PB 3x 5 min.

1.18. Remove the 0.1 M PB with a Pasteur pipette and add 1 % sodium borohydride (NaBH₄) in 0.1 M PB.

Note: Do not cap the vial, as NaBH₄ solution gives off hydrogen gas.

1.19. Remove the sodium borohydride with a Pasteur pipette and wash the sections thoroughly in 2 mL of 0.1 M PB 5x 5 min.

1.20. Replace the 0.1 M PB with 10% normal goat serum (NGS) in 0.1 M PB for 30 min.

1.21. Remove the goat serum and incubate the sections overnight at 4 °C in a mixture of mouse monoclonal and rabbit polyclonal antibodies made up in ABC solution.

Note: A list of primary antibodies used in previous studies is displayed in **Table 1** in Botcher *et al.* (2014)²⁹.

1.22. Incubate the sections for 2 h in the dark in a mixture of fluorescently-labelled secondary antibodies (**Table of Materials**).

1.23. Mount the sections onto the slides in mounting medium and cover with a coverslip.

1.24. Take images of fluorescence labelling at 40X magnification (**Figure 1Bb**).

1.25. After the fluorescence imaging, place the slide into a glass Petri dish containing PBS and carefully remove the coverslip. Then wash the sections off the slide using gentle pulses of PBS from a Pasteur pipette. Place the sections into a clean glass vial containing PBS.

1.26. Perform the avidin-HRP reaction by firstly incubating the sections in ABC for at least 2 h to amplify the HRP reaction product.

1.27. Prepare the 3,5 diaminobenzidine (DAB) solution by adding one tablet to 5 mL of distilled water.

1.28. Wash the sections with PBS three times for 10 min and then with Tris buffer twice for 10 min. Remove the Tris buffer after the last wash.

1.29. Quickly add one drop of 8 % NiCl₂ solution to the DAB solution, pipette the solution in and out to mix and quickly add 1 mL of this solution over the sections. Incubate the sections in the DAB/NiCl₂ solution for 15 min.

1.30. Add 10 µL of 1% H₂O₂ to the DAB solution. Allow the reaction to proceed in the dark under constant agitation for about 1 to 2 min and monitor the labelling of the filled cells with a dissecting microscope.

1.31. Stop the reaction by removing the DAB/NiCl₂/H₂O₂ solution and wash the sections with Tris buffer twice for 5 min.

1.32. In a fume cupboard, place a small circle of filter paper into a Petri-dish and dampen it with 0.1 M PB. Lift the sections one at a time from the glass vial using a paintbrush, and place them carefully flat upon the paper.

1.33. Cover the sections with another moistened circle of filter paper and remove excess buffer by gently touching tissue paper to the surface.

1.34. Apply 8-9 drops of 1% osmium tetroxide in 0.1 M PB to the top paper, cover the dish and retain in the fume hood for at least 30 min, but no more than 1 h.

1.35. Open the Petri dish and lift the top filter paper. Lift the sections carefully one at a time with a paintbrush, place them in a glass vial and rinse them in distilled water twice.

1.36. Dispose of waste osmium tetroxide by submerging all contaminated equipment in a diluted solution of domestic bleach (to deactivate the osmium) then flush it down the sink in the fume hood with copious quantities of water. Rinse all disposable equipment and place them in appropriate bins.

1.37. Place each section flat onto a glass slide and coverslip the sections. Transfer the slide into a Petri dish, place an empty glass vial over the coverslip to retain it in place and cover with 50% alcohol. After 15 min, remove the slide from the solution and remove the sections from the slide. Place the sections back on the slide and then place the slide in 70% alcohol for 15 min. Repeat the same process with 95% and finally 100% alcohol solution.

1.38. Following the dehydration step, transfer the sections to a glass vial containing 100% alcohol on a shaker in a fume cupboard. Replace the alcohol solution with propylene oxide (C_3H_6O) and wash three times for 5 min. Following the last wash, keep ~2 mL of propylene oxide in the vial and add resin (1:1 ratio). Ensure that the resin is dissolved and keep the sections under constant agitation for 30 min.

1.39. Place each section in an aluminum planchette containing epoxy resin using a wooden stick and incubate overnight.

Note: Do not leave the sections in the resin longer than 24 h to avoid the risk of damaging the sections.

1.40. Place the planchette over a hot plate for approximately 10 min. Pick up each section with a wooden stick and place them on a clean slide. Keep the orientation of each section consistent using a dissecting microscope. Place a coverslip over the sections. Place the slide in the oven for 48 h at 56 °C for curing.

2. 3D Neuronal Reconstructions

Note: Neurolucida software is used. Instructions provided below only apply to a specific neurone reconstruction system (**Table of Materials**). The 6 or 7 sections obtained from the cutting are matched prior to the reconstructions using a dissecting microscope.

2.1. Place a slide on the stage and secure with stage clip and open the neuron reconstruction software. Click on **Acquire** tab and select **Live image**.

2.2. Measure the thickness of each section using 100X oil objective lens. Make a note of the value on the z-meter at the top and bottom of each section and calculate the section thickness as the difference of the two values.

2.3. Use a low-magnification objective to focus on the **Home** section containing the cell body. When the cell body is in focus, click in the center to mark the reference point.

2.4. From the **Trace** tab, select **Serial Section Manager**. Then select **Create New Section** (+ icon) in the **Serial Section Manager** window. Enter the number of sections. Name each section in the correct Z order and enter the cut thickness measured in Step 2.2.

2.5. To trace the soma in 3D using 100X objective, select **Contour** from the **Trace** tab and select the **Cell Body** contour. Use the joystick to move the focus to the very top of the cell body. Place the points by clicking around the perimeter of the part that is currently in focus. Right-click and select **Close Contour** to finish this first outline. Repeat this process at different z positions until the bottom of the cell body is reached (**Figure 2**).

Note: Select **3D visualize** in **Trace** tab to visualize the cell body in 3D.

2.6. To trace the cell body in 2D using 100X objective, select **Cell Body** from the **Neuron** menu in the **Trace** tab. Focus on the middle of the cell body. Place the points by clicking around the perimeter of the cell body. To complete the cell body, right-click and select **Finish Cell Body**.

2.7. To trace the dendritic arbor, select **Dendrite** or **Apical Dendrite** in the **Neuron** menu. First trace a short, initial segment for each dendrite. Trace along each dendrite using the joystick to move across the section and the mouse scroll wheel to adjust the diameter of the cursor to match the diameter of the dendrite.

2.8. Check the alignment of the tracing with the live microscope image and adjust if necessary, especially after moving using the joystick. In the **Trace** tab, select **Align Tracing**, click on the tracing and then click on the location where it is to be moved to.

2.9. When a node in the tree is reached, right-click and select **Bifurcating Node** or **Trifurcating Node** from the drop-down menu.

2.10. When the end of a branch has been reached, select an ending from the drop-down menu in the **Neuron** menu. Select the correct ending type, *i.e.*, **High Ending** or **Low Ending** to facilitate matching across sections.

2.11. Reduce the magnification on the microscope once all dendrites in the current section have been traced, select the **Joy Free** tab and move to a section that matches immediately above or below the completed section.

2.12. To identify matching points between the dendrites in a section that matches the completed section, click on the **Move** tab and select **Match points**. Select the number of points needed to be matched (three or more points is preferred) and then press **OK**. Click on the ending of a completed branch and then click on the branch. Repeat this for each match point. Repeat this process at 100X magnification to ensure accurate matching.

2.13. Add matching branches to the previous section directly by right-clicking on the ending. Select **Add to Ending** when the branch lines up with the completed traced branch and trace as previously described.

2.14. Once all the dendrites of each section have been traced, trace the axon using the same process by selecting **Axon** from the **Neuron** menu.

2.15. Go to the **Home** section, re-align the reconstruction with the live microscope image and select **Contours** from the **Trace** tab. Select a pre-defined contour/layer border/region border and click to trace along a contour. Select **End Open Contour**. Trace all desired layers and region outlines.

2.16. Use **3D visualize** in **Trace** tab to visualize the reconstructions in 3D.

2.17. To record the videos of 3D reconstructions (**Video 1**), open the 3D reconstruction and open **Create movies**. Set a file destination and desired rotation speed. It is recommended to set the rotation to 270°. Select **Start recording**, record for a few seconds, then click **Auto-rotate**. Select **Stop recording** when required. Edit the video with a video editing software (**Table of Materials**).

2.18. To export the files into Tiff or JPEG files, select **File | Export | Export tracing as image**. Choose a $\mu\text{m}/\text{pixel}$ ratio and select **Fit**. Select a background color and select **File**. Name the file, select Tiff or JPEG and press **Save**. To export the files into vector files, select **File | Export | Export tracing as vector files**.

2.19. Use a neuron reconstruction analysis software to perform morphometric analyses.

2.19.1. To analyze dendritic trees and obtain a dendrogram, open the file in Neurolucida Explorer. Select **Analyze | Structure** and select **Dendrogram** from the drop-down menu (**Figure 3**).

2.19.2. To perform a comprehensive morphometric analysis of the 3D reconstructions (branch complexity, volumes of branches and somas, surface area), open the file in the software. In the **View** tab, click **Select all**. Select the **Analyze** tab. Then select **Structure** and **Branch Structure Analysis**.

3. Troubleshooting

3.1. Change camera settings. For best results, set the exposure time to less than 100 ms and gain and offset as close to 0 as possible.

3.2. If the neuron reconstruction software does not automatically display the traced contours as a 3D cell body in **3D visualize** in **Trace** tab, select **Select objects** in **Trace** tab, hold down the **Ctrl** key on the keyboard and click on all the drawn contours, right click and then select **Set cell body** on the drop-down menu.

3.3. To adjust the z-parameters of an individual branch, select the tree (**Select objects** in **Trace** tab) and set the z-adjustment of all points via the right-click menu drop down. Select **Modify z position**, then select **Shift z value** and enter the required value.

3.4. Realign the reconstructions using **'Go To'** in the **'Move'** tab when it is necessary to move a large distance over the tracing.

3.5. Add additional nodes at any point during the reconstruction process. Select the branch where the node is to be placed into (**Select Object** in **Trace** tab and click on the branch), right-click, and select **Insert Node into Selected Tree**. Then click on the right location on the branch.

3.6. When reconstructing a complex neurone, trace matching branches individually and later splice them for easier identification of branches. Trace the branches in the new section individually and then attach these branches to the matching branches on the previous section by hovering over the ending of the branch to be spliced, right-click and select **Splice**, then hovering over the ending that the branch is to be spliced to and click.

3.7. If a branch is traced under the wrong tree type, select the tree, right-click and select **Change Tree Type**, and select the correct tree type.

3.8. If a point is incorrectly placed, perform **Ctrl + Z**, or click the **Undo** button in the **Trace** menu to remove the last point. However, this procedure will not work if the joystick is used to move across the section before attempting to remove a point. In this case, the point may be removed when the branch is ended. Select the branch (Press **Select Object** and click on the branch), hover over the point to be removed, right-click and select remove point.

3.9. If unsure whether two branches are mapped, either 1) use **3D Visualize** in **Trace** tab to check whether the branches match in the z-plane, or 2) use **Show Flanking** feature in the Serial Section Manager to display sections immediately above and below the current section in grey.

3.10. If a section is flipped, go to **Tools** in the **Trace** tab. Select **Adjust XY Scaling** and change X-axis to -1. Then select **Correct Z-shrinkage** and change z-axis to -1. Then trace branches as usual. Remember to reverse these changes when moving to a section which had the original orientation. Changing the x- and z-axis will not change branch ending labels, so take care when splicing that the correct endings are being used.

3.11. Correct Z-shrinkage can be corrected, if there is a significant difference between the section cut thickness and the mounted thickness measured. In the **Trace** tab, select **Tools**, then **Correct Z-shrinkage**, and enter the shrinkage factor for the z-axis as a decimal representation of the cut thickness divided by the mounted thickness.

REPRESENTATIVE RESULTS:

Neurons in hippocampal CA2 were filled with biocytin following electrophysiological recordings (**Figures 1Ac, Ad and 1Bc, Bd**). The slices were fixed overnight following the recordings and the neurochemistry and morphological characterization of neurons were revealed following the protocol described here.

The calcium binding protein or protein content of filled interneurons was determined by incubating the slices first with primary antibodies and then with fluorescently labelled secondary antibodies. The firing characteristics of the interneurons during the recordings will dictate the choice of primary antibodies used. Avidin-AMCA was used to visualize the biocytin-filled interneuron and anti-mouse fluorescein isothiocyanate (FITC) and goat anti-rabbit Texas Red (TR) were used to characterize the interneuronal neurochemistry (**Figure 1Bb**).

Following the fluorescence visualization, an HRP protocol was used to reveal the biocytin (**Figure 1Ab**). The fine detailed anatomy of CA2 interneurons was then drawn in 3D using a neuron reconstruction software (**Figure 2 and Figure 3a**). A video of a 3D reconstruction of a basket cell recorded and filled in CA2 is displayed in **Video**. Neuronal reconstructions were considered as complete if both the dendritic and axonal arbors were confined within the depth of the 450-500 μm slice. Poor axonal arbor staining was assessed by the presence of truncated branches with open endings at the top or the bottom of the slice or by a staining that was limited to the axon initial segment and very proximal branches. **Figure 4** represents the examples of a good and a poor HRP staining following biocytin visualization.

Morphometric analysis of 3D reconstructions (**Figure 3**) can be carried out to demonstrate branch complexity, soma surface area and the surface area and volume of the dendritic and axonal arbors.

FIGURE AND TABLE LEGENDS:

Figure 1: Neuronal reconstructions of two types of basket cells recorded and filled in the

hippocampal CA2 region and correlated electrophysiological data obtained following intracellular recordings *in vitro*. This figure has been modified from previous studies²³⁻²⁴. SO Stratum Oriens, SP Stratum Pyramidale, SR Stratum Radiatum, SLM Stratum Lacunosum Moleculare. (A) Aa: Reconstruction of a CA2 basket cell with restricted dendritic and axonal arbor using a drawing tube (1000X). The dendrites are in black, and the axon is in red. Ab: Image of the biocytin-filled basket cell following the avidin-HRP protocol described here. Ac: Representative trace of voltage responses to hyperpolarizing and depolarizing current injection of a CA2 basket cell with restricted dendritic and axonal arbor. Ad: Example of a CA2 pyramid to narrow arbor basket cell connections recorded using sharp electrodes. Composite excitatory post-synaptic potential (EPSP) averages show brief train depression apparent during responses to trains of three spikes. (B) Ba: 2D reconstruction of a CA2 basket cell with wide dendritic and axonal arbors using a drawing tube (1000X). The dendritic tree of this basket cell (in black) extended radially through all layers of the CA2 region and horizontally in SO and SP of the CA2 and CA3 regions. One horizontal dendrite also reached the CA1 region. The axon (in red) extended to the CA3 and CA1 regions. Bb: The biocytin-filled (AMCA staining) basket cell was PV-immunopositive (FITC staining) and CB-immunonegative (Texas-Red staining). Bc: Representative trace of voltage responses to hyperpolarizing and depolarizing current injection of a CA2 basket cell with wide dendritic and axonal arbor. Bd: Composite EPSP averages show brief train facilitation apparent during responses to trains of three spikes. Examples of other types of interneurons recorded in CA2 can be found in previous studies^{25, 30}.

Figure 2: 3D cell body reconstruction. (A) 3D tracing of the cell body. View of the different contours traced at different z positions whilst focusing through the cell body. (B) 3D view of the different contours. (C) 3D view of the cell body at a different angle.

Figure 3: Morphometry analyses of 3D neuronal reconstructions. (A) 3D reconstruction of a CA2 narrow arbor basket cell. Each dendritic branch is represented by a color (green, blue, red and pink) and axon is in light blue. (B) Dendrogram of the basket cell representing the number of dendritic branches and length of each segment contained within spheres concentric with the soma and at 100 μ m intervals from the soma. The colors on the dendrogram correspond to those of the dendrites in A. (C) Example of morphometric analysis performed on CA2 pyramidal cells (adapted from a previous study²³). The number of dendritic branches was plotted against the distance from the soma of CA2 pyramidal cells.

Figure 4: Examples of good (A) and poor (B) HRP staining. (A) Biocytin recovery revealed a very well filled interneuron in CA2. The cell body (CB) is darkly stained and has clear outlines. The dendrites are beaded and displayed some spines (represented by red stars). The axonal arbor (A) is dense and presents small boutons. (B) Example of a poor dendritic and axonal staining of 2 pyramidal cells in CA2. The staining of the CB is faint with no clear outline. Poor staining is often associated with shorter electrophysiological recordings resulting in the presence of very few biocytin-filled branches.

Video 1: 3D neuronal reconstruction of a CA2 basket cell with restricted dendritic arbor (also referred to as CA2 narrow arbor basket cell) with its soma in stratum pyramidale, dendrites

spanning all layers and axon in CA2 stratum pyramidale and adjacent stratum oriens and radiatum. Very few branches reached the proximal CA3 stratum oriens and CA3 stratum pyramidale. This cell was filled with biocytin following electrophysiological recordings and sections were processed with avidin-HRP following the protocol described here. Due to slicing, only the axon within the depth of the slice was recovered, though the dendrites are intact. Dendrites are in dark pink and axon in white. Layer and region boundaries have been added at the beginning of the video. 3D reconstruction by Georgia Economides- 3D video by Svenja Falk. The video was recorded with a neuron reconstruction software as stated in Step 2.17 and edited with a video editing software. Link to the video: <http://uclsop.net/interneuron-reconstruction/ca2-basket>. Other examples of 3D reconstructions of CA1 interneurons can be found in a previous study³⁰.

Table 1. Table of solutions.

DISCUSSION:

Electrophysiological recordings *in vitro* (**Figure 1 Ac,d** and **Bc,d**) combined with histochemical and immunohistochemical procedures enable the detailed morphology, calcium binding protein content and identity of adult cortical interneurons recorded to be revealed. In the CA2 region, this technique allowed the study of the local circuitry for the first time and revealed subclasses of interneurons that had not been previously described in CA1 or CA3: wide dendritic and axonal arbor basket cells (**Figure 1B**), bistratified cells and SP-SR interneurons.

The protocol described here has been optimized to preserve the ultrastructure of the neurons and obtain excellent recovery of both dendritic (including spines) and axonal arbors. Critical steps includes the use of the double fixation technique to enhance contrast for light microscopy²⁶ and the addition of glutaraldehyde and picric acid solution to the fixative solution to enhance antibody penetration and preserve the neuronal ultrastructure²⁷. Gentle freeze-thaw permeabilization gives better preservation of fine structure, while osmication and resin embedding reduce z-plane shrinkage²⁸. In addition, the visualization of very fine structures (fine axons with small boutons for example) is improved by incubating the sections with H₂O₂ and NaBH₄ to reduce background staining. Contrast can also be increased with the addition of NiCl₂ to the HRP reaction.

The histological procedure detailed here offers excellent results in terms of reproducibility and reliability. However, the duration of the electrophysiological recordings will determine the quality of the biocytin/fluorescence staining, with shorter recordings usually associated with poor axonal staining. The choice of recording protocols (intracellular recordings using sharp electrodes vs. whole-cell patch clamping) may also influence biocytin retention and preservation of fine anatomy.

While the difficulties encountered in preserving fine structure during histological processing described here and the time taken to reconstruct at 100X magnification (1-4weeks depending on the complexity of the axon) are appreciated, this method gives an accurate representation of dendritic and axonal diameters. The use of less demanding protocols to reveal biocytin-

labelling is understandable, however, these often preclude clear visualization of fine axonal branches. Detergents, to promote entry of Avidin-HRP to reveal the biocytin and antibodies, are often necessary in thick sections, but can disrupt fine structure. Neuroscientists search constantly for semiautomatic methods of reconstruction, but, for now and for axons especially, biocytin-HRP with manual reconstruction remains the gold standard³¹.

Highly detailed neuronal reconstructions, especially accurate drawings of axonal boutons and nodes, the presence or absence of myelin and more generally the drawing of complete axonal arbor, with the representation of accurate axon-diameter changes along its length, provide further information for accurate identification of a distinct type of interneurone. Although many interneurons may not fit exactly into a specific class, the technique described above provides correlated data on neuronal electrophysiological properties, the short-term plasticity associated with a specific type of connection and detailed neuronal reconstructions, allowing the wiring diagram, in the CA2 region, for example, to be studied in detail.

Fine, detailed structure is often simplified in computational models. While understandable, this results in the loss of the information that could prove critical in the future. Analysis of detailed 3D reconstructions with parallel synaptic data will allow the addition of further criteria for interneuronal classification. Data can be deposited in public repositories and used by modellers to explore the outcome of sporadic changes in axon diameter and myelination on action potential propagation computationally.

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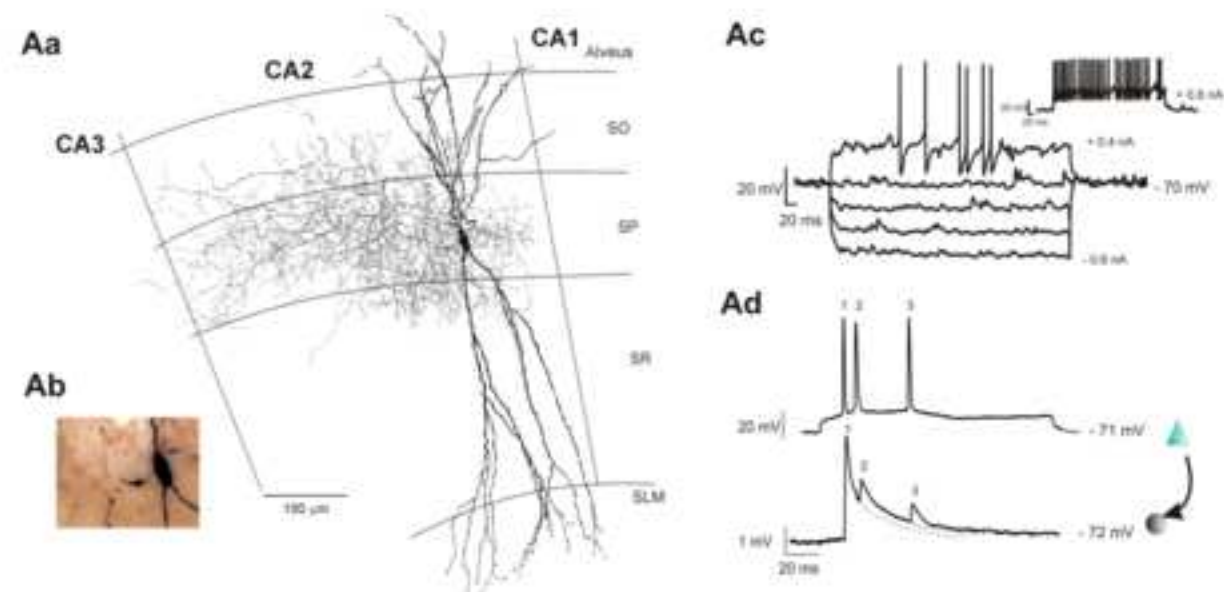
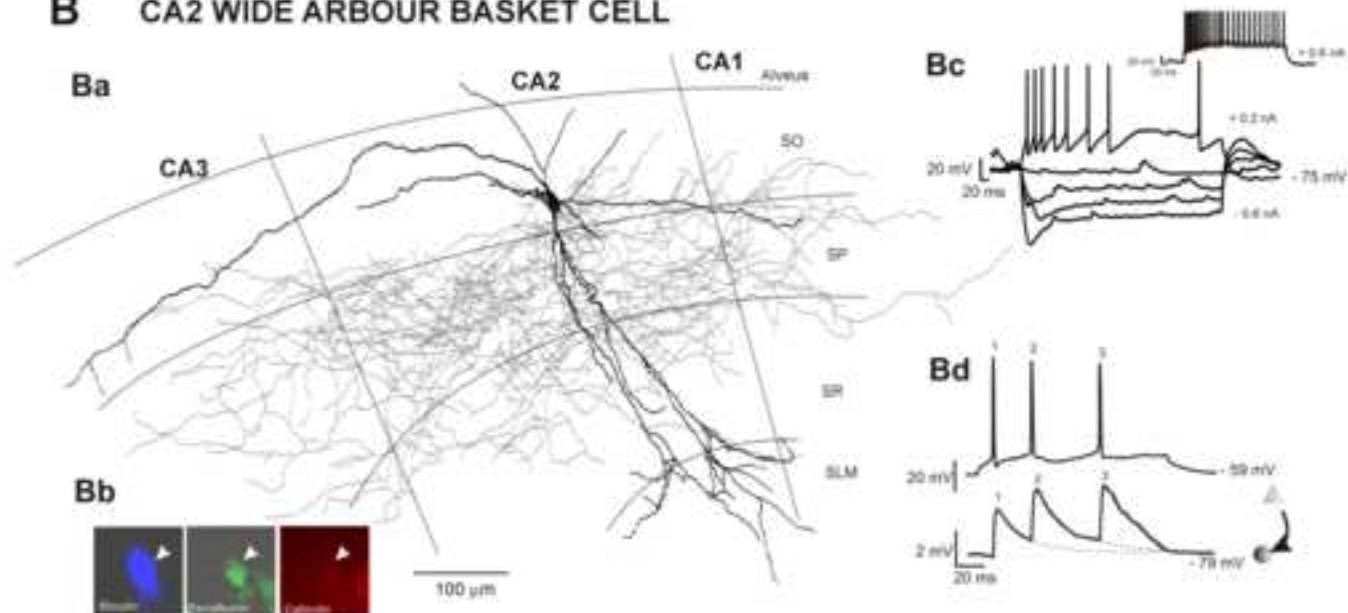
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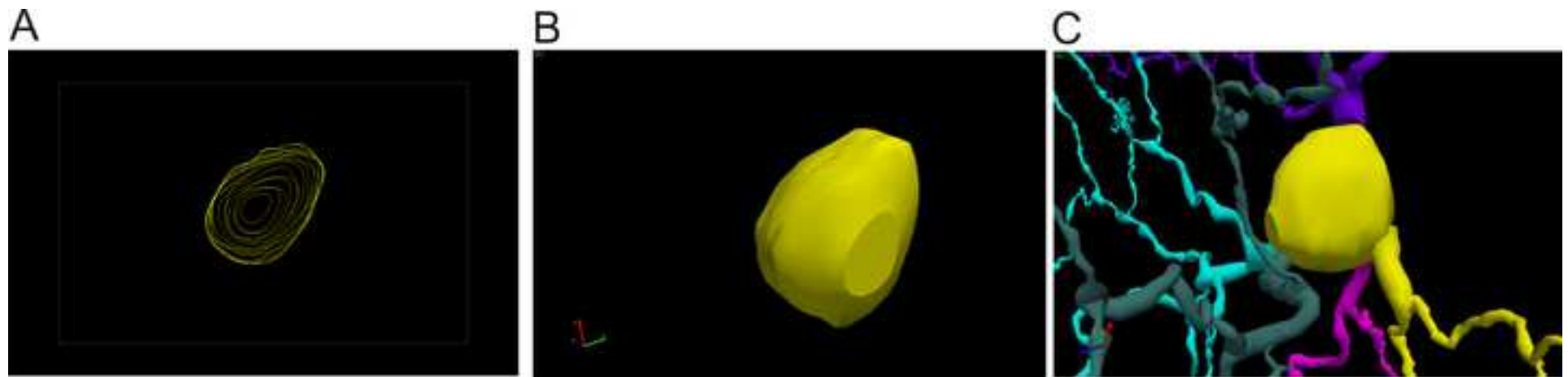
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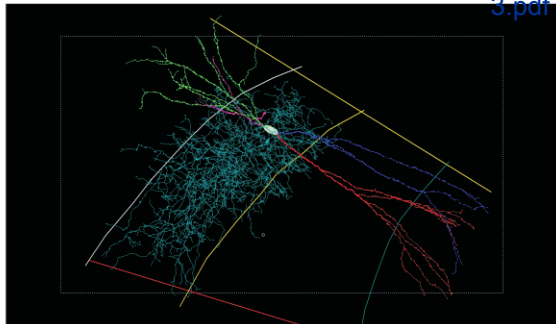
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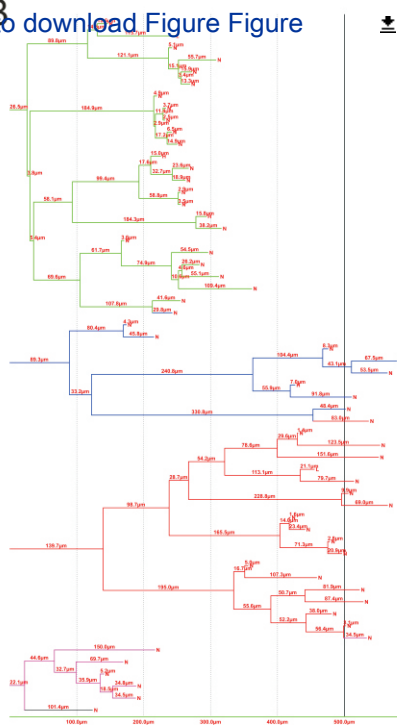
A CA2 NARROW ARBOUR BASKET CELL**B CA2 WIDE ARBOUR BASKET CELL**



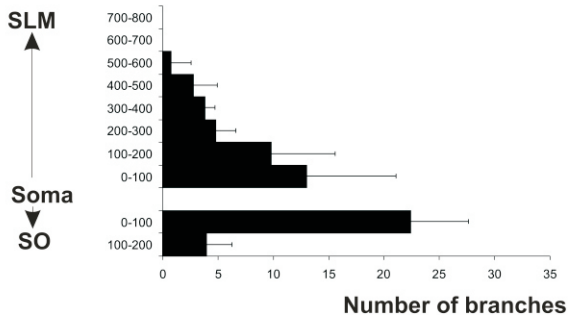
A Figure



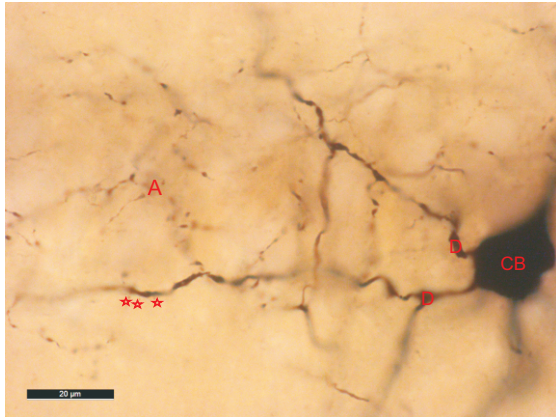
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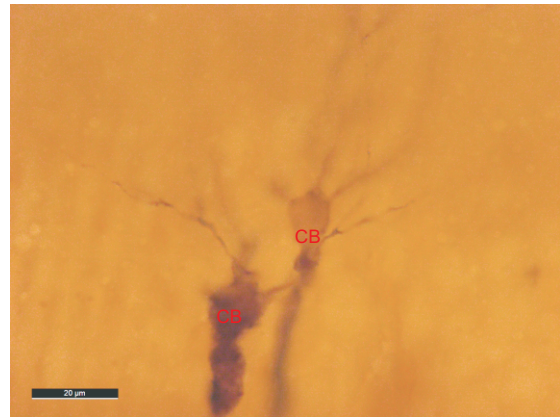
C



A



B



Solutions used**Fixation solution****0.1M Phosphate Buffer pH 7.6****Phosphate buffered saline (PBS) pH 7.4/7.5****TRIS buffer pH 7.5****Buffered glutaraldehyde and paraformaldehyde fixative solution****ABC solution****Durcupan epoxy resin:**

Composition/Instructions

4% paraformaldehyde, 0.2% saturated picric acid solution, 0.025% glutaraldehyde solution in 0.1 M phosphate buffer

Add 100 mL of stock 1 M Phosphate Buffer to 900 mL of distilled water

Add 10 mL of 0.1M phosphate buffer, 0.2 g of KCl and 8.76 g of NaCl to 990 mL of distilled water

Dissolve 5.72 g of Tris Hydrochloride and 1.66 g of Tris Base in 50 mL of distilled water. Then make up to 1 L with distilled water.

4% paraformaldehyde, 0.2% saturated picric acid solution, 0.025% glutaraldehyde solution in 0.1 M Phosphate buffer.

Solution to be made at least 30 min before use from the ABC kit. Add 1 drop of solution A and 1 drop of solution B to 2.5 mL of PBS.

To make 20 pots: 20 g of component A, 20 g of component B, 0.6 g of component C and 0.4 g of component D-
Protect the balance from spills by covering the plate with a circle of filter paper. Carefully weigh the reagents into a tripour beaker in the proportions stated above. Mix thoroughly by vigorously stirring using two wooden sticks for at least 5 min. The mixture should become a uniform density dark brown colour. Place the beaker into the oven at ~50 °C for a maximum of 10 min to remove as many air bubbles as possible. NOTE: The resin will start to cure if you leave the beaker in the oven longer than 10 min. Decant the resin out into plastic pots or 5 mL syringes, date them and store in the -20 °C freezer ready for use.

(PB)

Name of Material/ Equipment

Avidin-7-amino-4-methylcoumarin-3-acetic acid (Avidin-AMCA) antibody- 20.8 µg/mL

Biocytin ≥98% (TLC)

3,5 diaminobenzidine (DAB) tablet, To prepare 5 mL

Durcupan epoxy resin A

Durcupan epoxy resin B

Durcupan epoxy resin C

Durcupan epoxy resin D

Ethanol, puriss. p.a., absolute, ≥99.8%

Gelatin

Glutaraldehyde solution, grade I, 25 % in H₂O

Glycerol, ≥99%

Goat serum

Goat anti-mouse fluorescein isothiocyanate (FITC)- 14.3 µg/mL

Goat anti-rabbit Texas Red (TR)- 3.3 µg/mL

Hydrogen peroxide, 30% solution

Immersion oil, viscosity 1.250 cSt (lit.)

Nickel chloride (NiCl₂ · 6H₂O)

Osmium tetroxide, for electron microscopy, 4% in H₂O

Paraformaldehyde, reagent grade, crystalline

Picric acid, moistened with water, ≥98%

Phosphate buffer 1 M

Propylene oxide (C₃H₆O), 99%

Sodium tetrahydroborate

Sucrose

Trizma Hydrochloride, ≥99.0%

Trizma base, ≥99.9%

Vectashield Antifade Mounting Medium, , refractive index 1.45

Vectastain Elite ABC HRP kit

Equipment used

Vibratome

C4A Cupped aluminium planchettes

Leica DMR microscope

X-Cite 120PC Q fluorescence light source

Leica DFC450 digital microscope camera

Rapidograph technical drawing pen 0.18mm

0.18mm rapidograph nib

Rapidograph technical drawing pen 0.25mm

0.25mm rapidograph nib

Neurolucida system including PC workstation, stage, camera and joystic for XYZ stage control

Neurolucida software version 2017

CorelDRAW graphics Suite X5

Video editing software

Glass vials 14 mL

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Sigma	D4293
Sigma	44611
Sigma	44612
Sigma	44613
Sigma	44614
Sigma	32221
Sigma	48723
Sigma	G5882
Sigma	G5516
Sigma	G9023
Sigma	F2653
Invitrogen	T2767
Sigma	H-1009
Sigma	I0890
Sigma	N5756
Sigma	75632
Sigma	P6148
Sigma	197378
Sigma	P3619
Alfa Aesar	30765
VWR	27885.134
Fisher scientific	S/8600/53
Sigma	T5941
Sigma	T6066
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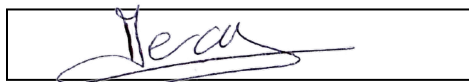
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4. The highlighted protocol steps are over 2.75 page limit. Please adjust the highlighting.

The highlighting has been adjusted. Highlighted protocol can be found below:

1. Determination of calcium binding protein or protein content of interneurons and biocytin visualization following electrophysiological recordings and biocytin filling.

1.2 In a fume cupboard, carefully pick up the brain slice with a paintbrush and roll it onto a small piece of fine quality filter paper. Place another piece of moistened filter paper onto the slice and place the two pieces of wet filter paper in a small plastic pot containing 5-10 mL of fixative solution and store in the fridge overnight at 4 °C.

1.4 Replace the fixative solution with 2 mL of 0.1 M PB. Place the tissue in a petri-dish (9cm D- 1.4 cm H) ensuring that it lies flat with no folds or creases and remove the excess buffer using a dry paintbrush.

1.5 Cover the tissue with the warm gelatin solution and place the petri-dish onto a frozen block to quickly cool the solution.

1.7 Move the dish of setting gelatin to the fridge and leave at 4 °C for 30-60 min.

1.8 In a fume cupboard, cut out a small block (~ 1x1 cm) containing the gelatin-embedded tissue of the dish using a scalpel blade, lift the block using a small spatula and carefully place it in the same but fresh fixative solution used to fix the slices for at least 30 min at 4 °C.

1.9 Wash the gelatin block in 5 mL of 0.1 M PB three times, dry it using a piece of paper tissue and stick the block side up (*i.e.* with the tissue at the top) onto a vibratome chuck using superglue.

1.10 Remove excess glue with a piece of filter paper and use a scalpel blade to cut the corners of the block off, leaving a diamond shape.

1.11 Section the slice at 50 μ m thickness using a vibratome and place each section carefully in a glass vial containing 10 % sucrose.

1.12 Carefully pick up a section from the vial, place it flat into a petri-dish lid. Using a dissecting microscope and a fresh scalpel blade, remove the gelatin from around the section and return the section to a vial containing 2ml of fresh 10 % sucrose

1.13 Cryo-protect the sections in 0.1 M PB-based sucrose-glycerol solution at room temperature by incubating them for 10 min in 10 % sucrose solution, 20 min in 20 % sucrose-6 % glycerol solution twice and finally 30 min in 30 % sucrose-12 % glycerol solution twice under constant agitation.

1.14 Place the sections flat onto a small rectangle of tin foil using a paintbrush. Remove any excess liquid from the sections and carefully fold the tin foil into a parcel.

1.15 Hold the tin foil close to the surface of liquid nitrogen without touching the surface for 30 s and then allow the sections to thaw completely for approximately 30 s. Repeat the freeze-thaw another two times.

1.16 Remove all sections with a paintbrush and place them in a glass vial containing 2 mL of 0.1M PB under constant agitation to wash off excess sucrose.

1.17 Remove the PB with a Pasteur pipette and incubate the sections in 2 mL of 1 % aqueous H₂O₂ for 30 min. Wash sections in 2 mL of 0.1M PB 3 x 5 min.

1.18 Remove the 0.1 M PB with a Pasteur pipette and add 1 % sodium borohydride (NaBH₄) in 0.1 M PB Do not cap the vial, as NaBH₄ solution gives off hydrogen gas.

1.19 Remove the sodium borohydride with a Pasteur pipette and wash the sections thoroughly in 2 mL of 0.1 M PB 5x 5 min.

1.20 Replace the 0.1M PB with 10% normal goat serum (NGS) in 0.1M PB for 30 min.

1.21 Remove the goat serum and incubate the sections overnight at 4 °C in a mixture of mouse monoclonal and rabbit polyclonal antibodies made up in ABC solution.

1.22 Incubate the sections for 2 h in the dark in a mixture of fluorescently-labelled secondary antibodies (Table of reagents).

1.23 Mount the sections onto slides in mounting medium and cover with a coverslip.

1.24 Take images of fluorescence labelling at 40X magnification. (Representative images in Fig. 1Bb).

1.27 Perform the avidin-HRP reaction by firstly incubating the sections in ABC for at least 2 h to amplify the HRP reaction product.

1.29 Wash the sections with PBS three times for 10 min and then with Tris buffer twice for 10 min. Remove the Tris buffer after the last wash.

1.30 Quickly add one drop of 8 % NiCl₂ solution to the DAB solution, pipette the solution in and out to mix and quickly add 1 mL of this solution over the sections. Incubate the sections in the DAB/NiCl₂ solution for 15 min.

1.31 Add 10 μ L of 1 % H₂O₂ to the DAB solution. Allow the reaction to proceed in the dark under constant agitation for about 1 to 2 min and monitor the labelling of the filled cells with a dissecting microscope.

- 1.32 Stop the reaction by removing the DAB/NiCl₂/H₂O₂ solution and wash the sections with Tris buffer twice for 5 min.
- 1.33 In a fume cupboard, place a small circle of filter paper into a Petri-dish and dampen it with a little 0.1 M PB. Lift the sections one at a time from the glass vial using a paintbrush, place them carefully flat upon the paper.
- 1.34 Cover the sections with another moistened circle of filter paper and remove excess buffer by gently touching tissue paper to the surface.
- 1.35 Apply 8-9 drops of 1% osmium tetroxide in 0.1 M PB to the top paper, cover the dish and retain in the fume hood for at least 30 min, but no more than 1 h.
- 1.36 Open the petri-dish and lift the top filter paper. Lift the sections carefully one at a time with a paintbrush, place them in a glass vial and rinse them in distilled water twice.
- 1.38 Place each section flat onto a glass slide and coverslip the sections. Transfer the slide into a Petri dish, place an empty glass vial over the coverslip to retain it in place and cover with 50% absolute alcohol. After 15 min, remove the slide from the solution and remove the sections from the slide. Place the sections back on the slide and then place the slide in a solution of 70% alcohol for 15 min. Repeat the same process with 95% and finally 100% alcohol solution.
- 1.39 Transfer the sections to a glass vial containing a solution of 100 % alcohol on a shaker in a fume cupboard. Replace the alcohol solution with a solution of propylene oxide (C₃H₆O) and wash three times for 5 min. Following the last wash, keep ~2mL of propylene oxide in the vial and add resin (1:1 ratio). Ensure that the resin is dissolved and keep the sections under constant agitation for 30 min.
- 1.40 Place each section in an aluminium planchette containing epoxy resin using a wooden stick and incubate overnight.
- 1.41 Place the planchette over a hot plate for approximately 10 min. Pick up each section with a wooden stick and place them on a clean slide. Keep the orientation of each section consistent using a dissecting microscope. Place a coverslip over the sections. Place the slide in the oven for 48 h at 56 °C for curing.

2. 3D neuronal reconstructions using a Neurolucida software

- 2.1 Place a slide on the stage and secure with stage clip and open the neuron reconstruction software. Click on 'Acquire' tab and select 'live image'.
- 2.3 Use a low-magnification objective to focus on the 'home' section containing the cell body. When the cell body is in focus, click in the center to mark the reference point.
- 2.5 To trace the soma in 3D using a 100X objective, select 'Contour' from the 'Trace' tab and select the 'Cell Body' contour. Use the joystick to move the focus to the very top of the cell body. Place points by clicking around the perimeter of the part of the cell body that is currently in focus. Right-click and select 'Close Contour' to finish this first outline. Focus down a little, and repeat. Repeat this process at different z positions until the bottom of the cell body is reached (Figure 2).
- 2.7 To trace the dendritic arbor, select 'Dendrite' or 'Apical Dendrite' in the 'Neuron' menu. First trace a short, initial segment for each dendrite. Trace along each dendrite using the joystick to move across the section and the mouse scroll wheel to adjust the diameter of the cursor to match the diameter of the dendrite.
- 2.9 When a node in the tree is reached, right-click and select 'Bifurcating Node' or 'Trifurcating Node' from the drop-down menu.

2.10 When the end of a branch has been reached, select an ending from the drop-down menu in the 'Neuron' menu. Select the correct ending type, i.e. 'High Ending' or 'Low Ending' to facilitate matching across sections.

2.12 To identify matching points between dendrites in a section that matches the completed section, Click on 'Move' tab and select 'Match points'. Select the number of points needed to be matched (three or more points is preferred) and then press OK. Click on the ending of a completed branch and then click on the branch it will match to on the live microscope image. Do this for each match point. Repeat this process at higher magnifications at 1000X to ensure accurate matching.

2.14 Once all the dendrites of each section have been traced, trace the axon using the same process (steps 2.2.6-2.2.13) by selecting 'Axon' from the 'Neuron' menu.

5. Step 1.7: What's the size of the small block? [Done](#)

6. 1.31: Issecting microscope? [Typo has been rectified.](#)

7. 2.14: Please write this step in imperative tense. [Done](#)

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