

Journal of Visualized Experiments

Biocytin recovery and 3D reconstructions of filled hippocampal CA2 interneurones. --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58592R2
Full Title:	Biocytin recovery and 3D reconstructions of filled hippocampal CA2 interneurones.
Keywords:	Immunohistochemistry, immunofluorescence protocol, HRP protocol, neuronal reconstructions, Neurolucida, Camera Lucida, neuronal anatomy, dendrites, axon
Corresponding Author:	Audrey Mercer University College London School of Pharmacy London, London UNITED KINGDOM
Corresponding Author's Institution:	University College London School of Pharmacy
Corresponding Author E-Mail:	a.mercer@ucl.ac.uk
Order of Authors:	Georgia Economides Svenja Falk Audrey Mercer
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US\$4,200)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	London

TITLE:

Biocytin Recovery and 3D Reconstructions of Filled Hippocampal CA2 Interneurons

AUTHORS & AFFILIATIONS:

Georgia Economides^{1*}, Svenja Falk^{1*}, Audrey Mercer¹

¹Department of Pharmacology, University College London, UK

* These authors contributed equally to this work.

Corresponding author:

Audrey Mercer

Email address: a.mercer@ucl.ac.uk

KEYWORDS:

Immunohistochemistry, immunofluorescence protocol, HRP protocol, neuronal reconstructions, Neurolucida, Camera Lucida, neuronal anatomy, dendrites, axon

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.

ACKNOWLEDGMENTS:

This research has received funding from Novartis Pharma (Basel), the Medical Research Council, the Biotechnology and Biological Sciences Research Council (BBSRC- BB/G008639/1), the Physiological Society, the European Union's Horizon 2020 Framework Programme for Research and Innovation under the Specific Grant Agreement No. 720270 (Human Brain Project SGA1) and under the Specific Grant Agreement No. 785907 (Human Brain Project SGA2).

DISCLOSURES:

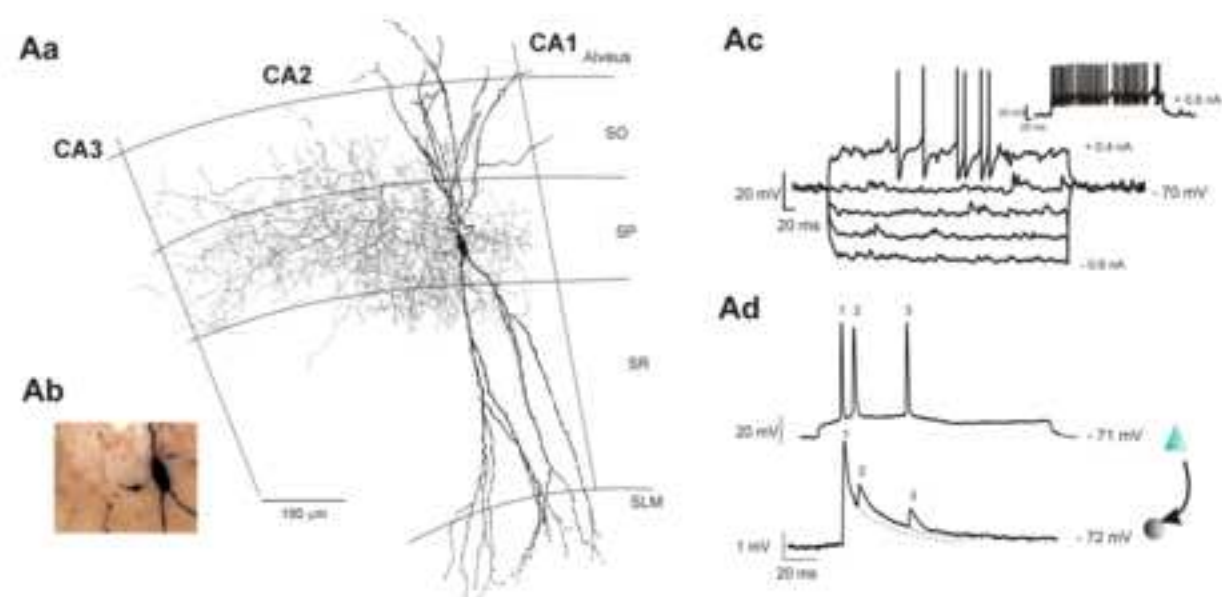
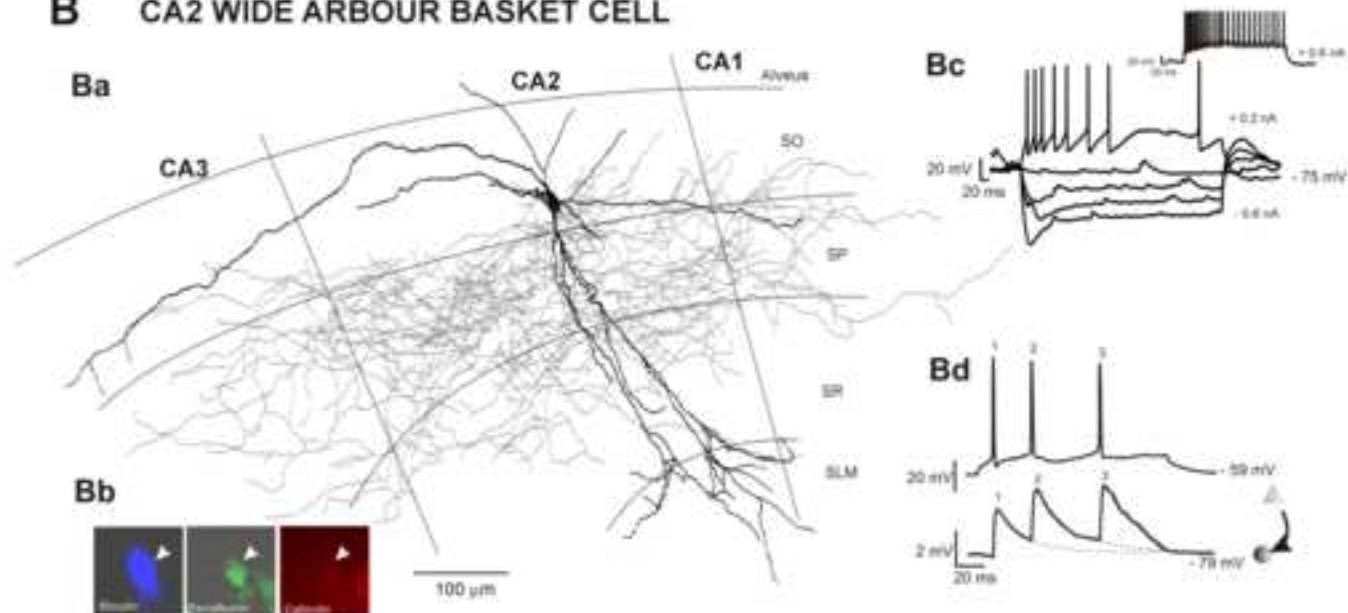
The authors have nothing to disclose.

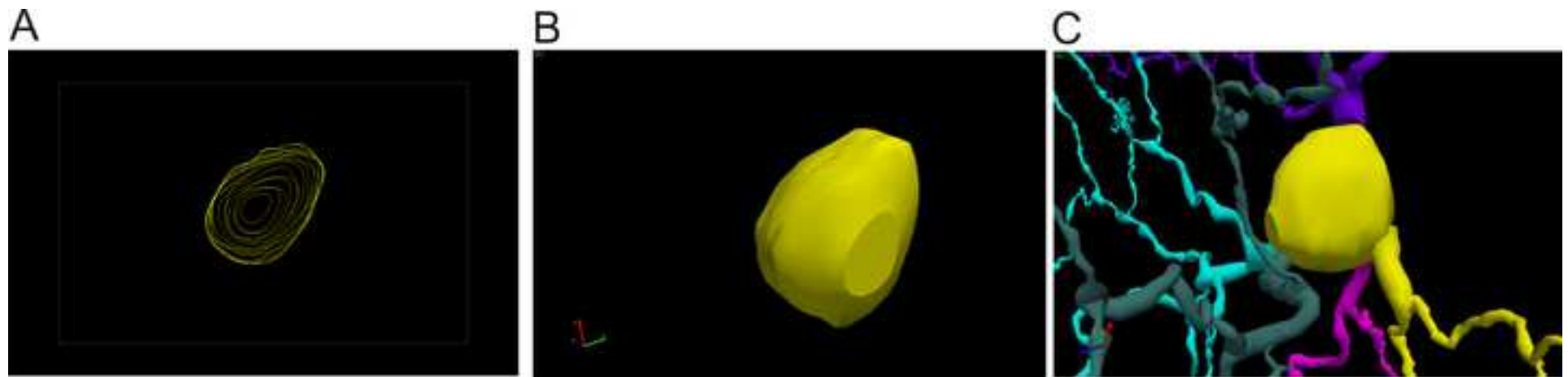
REFERENCES:

1. Klausberger, T., Somogyi, P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science*. **321**, 53–57 (2008).
2. DeFelipe, J., *et al.* New insights into the classification and nomenclature of cortical GABAergic interneurons. *Nature Reviews Neuroscience*. **14** (3), 202–16 (2013).
3. Bezaire, M.J., Soltesz, I. Quantitative assessment of CA1 local circuits: knowledge base for interneuron-pyramidal cell connectivity. *Hippocampus*. **23**, 751–785. (2013).
4. Katona, L., *et al.* Behavior-dependent activity patterns of GABAergic long-range projecting neurons in the rat hippocampus. *Hippocampus*. **27**, 359–377 (2017).
5. Ali, A.B., Thomson, A.M. Facilitating pyramid to horizontal oriens-alveus interneurone inputs: dual intracellular recordings in slices of rat hippocampus. *Journal of Physiology*. **507**, 185–199 (1998).

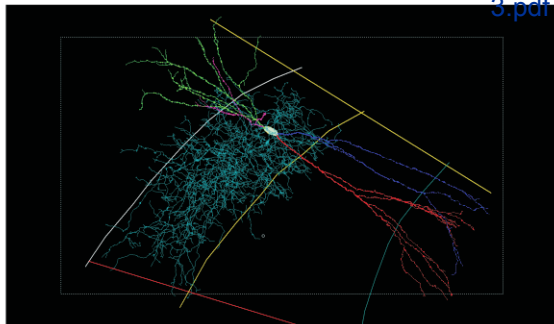
- 568 6. Ali, A.B., Thomson, A.M. Synaptic alpha 5 subunit-containing GABAA receptors mediate
569 IPSPs elicited by dendrite-preferring cells in rat neocortex. *Cerebral Cortex*. **18**, 1260–1271
570 (2008).
- 571 7. Ali, A.B., Bannister, A.P., Thomson, A.M. Robust correlations between action potential
572 duration and the properties of synaptic connections in layer 4 interneurons in neocortical
573 slices from juvenile rats and adult rat and cat. *Journal of Physiology*. **580**, 149–169 (2007).
- 574 8. Pawelzik, H., Bannister, A. P., Deuchars, J., Ilia, M., Thomson, A.M. Modulation of
575 bistratified cell IPSPs and basket cell IPSPs by pentobarbitone sodium, diazepam and Zn²⁺: dual
576 recordings in slices of adult rat hippocampus. *European Journal of Neuroscience*. **11**, 3552–3564
577 (1999).
- 578 9. Pawelzik, H., Hughes, D. I., Thomson, A. M. Physiological and morphological diversity of
579 immunocytochemically defined parvalbumin- and cholecystokinin-positive interneurons in CA1
580 of the adult rat hippocampus. *Journal of Comparative Neurology*. **443**, 346–367 (2002).
- 581 10. Thomson, A. M., Lamy, C. Functional maps of neocortical local circuitry. *Frontiers of*
582 *Neuroscience* **1**, 19–42 (2007).
- 583 11. Kepecs, A., Fishell, G. Interneuron cell types are fit to function. *Nature*. **505** (7483), 318–
584 26 (2014).
- 585 12. Wilent, W.B., Nitz, D.A Discrete Place Fields of Hippocampal Formation Interneurons.
586 *Journal of Neurophysiology* **97**:4152-4161 (2007).
- 587 13. Hirsch, J.A., Martinez, L.M. Laminar processing in the visual cortical column. *Current*
588 *Opinion in Neurobiology*. **16** (4), 377-84 (2006).
- 589 14. Kay, K., Sosa, M., Chung, J.E., Karlsson, M.P., Larkin, M.C., Frank, L.M. A hippocampal
590 network for spatial coding during immobility and sleep. *Nature*. **531**, 185–190 (2016).
- 591 15. Yu, J. Y., *et al.* Distinct hippocampal-cortical memory representations for experiences
592 associated with movement versus immobility. *eLife*. **6**, e27621, (2017).
- 593 16. Markram, H., *et al.* Reconstruction and Simulation of Neocortical Microcircuitry. *Cell*
594 **163**, 456–492 (2015).
- 595 17. Van Geit, W., *et al.* BluePyOpt: Leveraging Open Source Software and Cloud
596 Infrastructure to Optimise Model Parameters in Neuroscience. *Frontiers in Neuroinformatics*.
597 **10**, 17 (2016).
- 598 18. Gal, E., *et al.* Rich cell-type-specific network topology in neocortical microcircuitry.
599 *Nature Neuroscience*. **20**, 1004–1013 (2017).
- 600 19. Thomson, A. M., West, D. C., Wang, Y., and Bannister, A. P. Synaptic connections and
601 small circuits involving excitatory and inhibitory neurons in layers 2–5 of adult rat and cat
602 neocortex: triple intracellular recordings and biocytin labeling in vitro. *Cerebral Cortex*. **12**, 936–
603 953 (2002).
- 604 20. Mercer, A., West, D. C., Morris, O. T., Kirchhecker, S., Kerkhoff, J. E., Thomson, A. M.
605 Excitatory connections made by presynaptic cortico-cortical pyramidal cells in layer 6 of the
606 neocortex. *Cerebral Cortex*. **15**, 1485–1496 (2005).
- 607 21. West, D. C., Mercer, A., Kirchhecker, S., Morris, O. T., Thomson, A. M. Layer 6 cortico-
608 thalamic pyramidal cells preferentially innervate interneurons and generate facilitating EPSPs.
609 *Cerebral Cortex*. **16**, 200–211 (2006).

22. Bannister, A.P., Thomson, A.M. Dynamic properties of excitatory synaptic connections involving layer 4 pyramidal cells in adult rat and cat neocortex. *Cerebral Cortex*. **17**, 2190–2203, (2007).
23. Mercer, A., Trigg, H.L., Thomson, A.M. Characterization of neurons in the CA2 subfield of the adult rat hippocampus. *Journal of Neuroscience*. **27** (7), 7329-38 (2007).
24. Mercer, A., Eastlake, K., Trigg, H.L., Thomson, A.M. Local circuitry involving parvalbumin-positive basket cells in the CA2 region of the hippocampus. *Hippocampus*. **22**(1), 43-56 (2012a).
25. Mercer, A., Botcher, N.A., Eastlake, K. and Thomson, A.M. SP-SR interneurons: a novel class of neurones of the CA2 region of the hippocampus. *Hippocampus*. **22** (8), 1758-1769 (2012).
26. Sabatini, D.D., Bensch, K., Barnett, R.J. Cytochemistry and electron microscopy: The Preservation of Cellular Ultrastructure and Enzymatic Activity by Aldehyde Fixation. *Journal of Cell Biology*. **17**, 19-58 (1963).
27. Somogyi, P., Takagi, H. A note on the use of picric acid-paraformaldehyde-glutaraldehyde fixative for correlated light and electron microscopic immunocytochemistry. *Neuroscience*. **7**, 1779-1783 (1982).
28. Hughes, D.I., Bannister, A.P., Pawelzik, H., Thomson, A.M. Double immunofluorescence, peroxidase labelling and ultrastructural analysis of interneurons following prolonged electrophysiological recordings in vitro. *Journal of Neuroscience Methods*. **101**,107-116 (2000).
29. Botcher, N.A, Falck, J.E., Thomson, A.M., Mercer, A. Distributions of interneurons in the CA2 region of the rat hippocampus. *Frontiers in Neuroanatomy*. **8**, 104 (2014).
30. Mercer, A., Thomson, A.M. Cornu Ammonis Regions–Antecedents of Cortical Layers? *Frontiers in Neuroanatomy*. **11**, 83 (2017).
31. Blackman, A.V., Grabuschnig, S., Legenstein, R. and Sjöström, P.J. A comparison of manual neuronal reconstruction from biocytin histology or 2-photon imaging: morphometry and computer modeling. *Frontiers in Neuroanatomy*. **8**, 65 (2014).

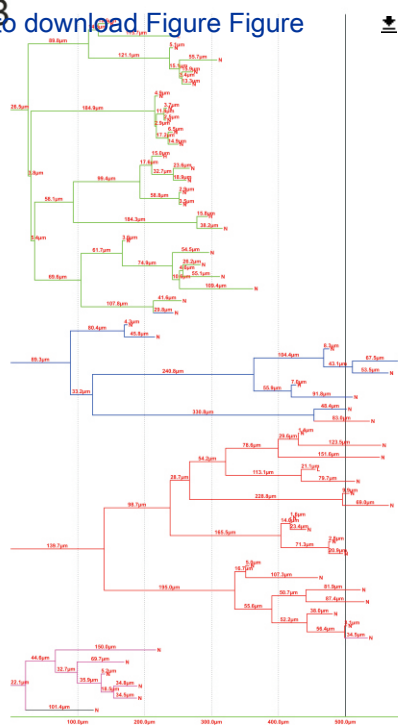
A CA2 NARROW ARBOUR BASKET CELL**B CA2 WIDE ARBOUR BASKET CELL**



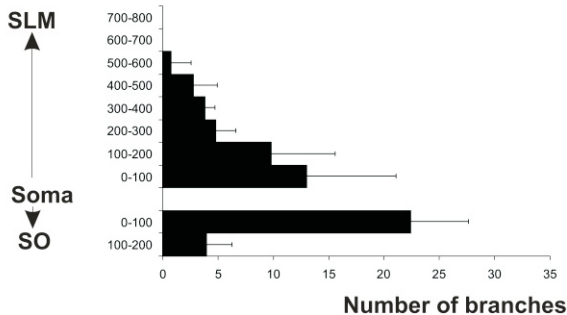
A Figure



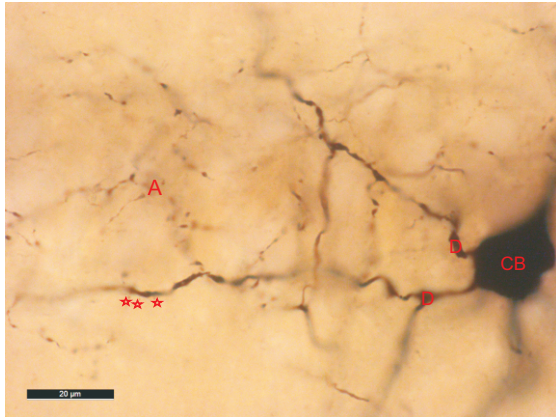
B [Click here to download Figure Figure 3.pdf](#)



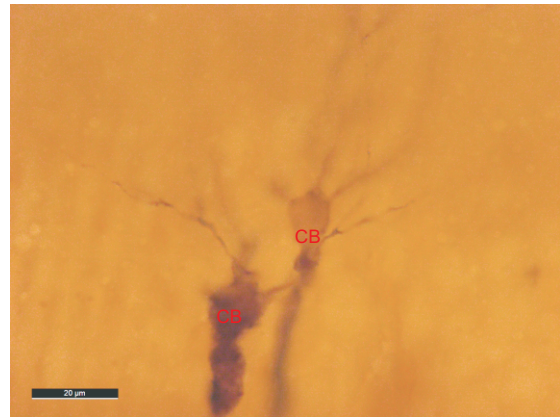
C



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

Company	Catalog Number
Vector laboratories	A2008
Sigma	B4261
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
Vector laboratories	H-1000

Vector laboratories

PK6100

Agar Scientific

GA-MA & ASSOCIATES, INC.

Leica Microsystems

Excelitas Technologies

Leica Microsystems

London graphics centre

London graphics centre

London graphics centre

London graphics centre

Microbrighfield (MBF)

Bioscience

Microbrighfield (MBF)

Bioscience

Corel

Adobe Premiere Pro

Fisher scientific

Comments/Description

--

--



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Biocytin recovery and 3D reconstructions of filled hippocampal CA2 interneurons

Author(s):

Georgia Economides, Svenja Falk, Audrey Mercer

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☐

Standard Access

☒

Open Access

Item 2: Please select one of the following items:

☒

The Author is **NOT** a United States government employee.

☐

The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐

The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJoVE Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:

Audrey Mercer

Department:

Department of Pharmacology

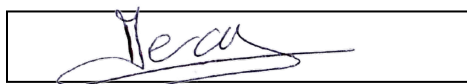
Institution:

UCL School of Pharmacy

Title:

Dr

Signature:



Date:

27/07/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear editor,

Please find below the revisions made to the manuscript.

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

[This has been done.](#)

2. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Examples of commercial language in your manuscript include Durcupan, etc.

[All commercial language has been removed.](#)

3. Please ensure that the references appear as the following:
Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi: DOI (YEAR).
For more than 6 authors, list only the first author then et al.

[The reference list has been updated.](#)

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](#)

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Jul 25, 2018

This Agreement between Dr. Audrey Mercer ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4381841090050
License date	Jul 04, 2018
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Hippocampus
Licensed Content Title	Local circuitry involving parvalbumin-positive basket cells in the CA2 region of the hippocampus
Licensed Content Author	Audrey Mercer, Karen Eastlake, Hayley L. Trigg, et al
Licensed Content Date	Sep 29, 2010
Licensed Content Volume	22
Licensed Content Issue	1
Licensed Content Pages	14
Type of use	Journal/Magazine
Requestor type	Author of this Wiley article
Is the reuse sponsored by or associated with a pharmaceutical or medical products company?	no
Format	Electronic
Portion	Figure/table
Number of figures/tables	2
Original Wiley figure/table number(s)	Figures 4 and 5
Will you be translating?	No
Title of new article	Biocytin recovery and reconstructions in 2D and 3D of filled hippocampal CA2 interneurons.
Publication the new article is in	Journal of Visualized Experiments
Publisher of new article	MyJove Corp
Author of new article	Georgia Economides, Svenja Falk, Audrey Mercer
Expected publication date of new article	Sep 2018
Estimated size of new article (pages)	15
Requestor Location	Dr. Audrey Mercer 29-39 Bunswick Square

Publisher Tax ID

Total

Terms and Conditions

London, WC1N 1AX
United Kingdom
Attn: Dr. Audrey Mercer

EU826007151

0.00 GBP

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at <http://myaccount.copyright.com>).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.
- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order**, is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.
- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. **For STM Signatory Publishers clearing permission under the terms of the [STM Permissions Guidelines](#) only,**

the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts, You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto
- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.
- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE

POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.
- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of

process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The [Creative Commons Attribution License \(CC-BY\)](#) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The [Creative Commons Attribution Non-Commercial \(CC-BY-NC\)License](#) permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The [Creative Commons Attribution Non-Commercial-NoDerivs License](#) (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library
<http://olabout.wiley.com/WileyCDA/Section/id-410895.html>

Other Terms and Conditions:

v1.10 Last updated September 2015

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.