Improved biocytin labeling and neuronal 3D reconstruction

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In this report, we describe a reliable protocol for biocytin labeling of neuronal tissue and diaminobenzidine (DAB)-based processing of brain slices. We describe how to embed tissues in different media and how to subsequently histochemically label the tissues for light or electron microscopic examination. We provide a detailed dehydration and embedding protocol using Eukitt that avoids the common problem of tissue distortion and therefore prevents fading of cytoarchitectural features (in particular, lamination) of brain tissue; as a result, additional labeling methods (such as cytochrome oxidase staining) become unnecessary. In addition, we provide correction factors for tissue shrinkage in all spatial dimensions so that a realistic neuronal morphology can be obtained from slice preparations. Such corrections were hitherto difficult to calculate because embedding in viscous media resulted in highly nonlinear tissue deformation. Fixation, immunocytochemistry and embedding procedures for light microscopy (LM) can be completed within 42–48 h. Subsequent reconstructions and morphological analyses take an additional 24 h or more.

INTRODUCTION

Biocytin was introduced as an intracellular marker in 1988 (ref. 1). In combination with simultaneous electrophysiological recordings, labeling of neurons via the intracellular injection of biocytin is now a widely used method that enables researchers to correlate the structural and functional properties of recorded cells[2]. Because biocytin is easily transported across gap junctions (a process called dye coupling), injection of biocytin into a single neuron can be used to assess the extent of gap junctional coupling between neurons. Dye coupling of biocytin has been observed, especially in immature and early postnatal tissue[3–6]. Another application of biocytin labeling has been the morphological classification of neurons. Both excitatory and inhibitory neurons in the neocortex, hippocampus and other brain regions are notoriously diverse, and they differ profoundly in their structural properties. A quantitative and reproducible approach to classifying neuronal cell types is by their morphology, often in combination with their electrophysiological properties or immunocyto- and histochemical staining patterns. Morphological features of neurons such as the axonal and dendritic projection patterns serve as the basis for a quantitative cluster analysis, which can then reveal different neuronal cell classes. Although the diversity of GABAergic interneurons has been accepted for a longer time and has been confirmed using quantitative methods[7–17], studies in recent years have demonstrated very clearly that excitatory neurons, such as those in the neocortex, are equally diverse[18–23]. This structural diversity translates directly into a functional diversity, and therefore knowledge of the neuronal morphology is important for understanding function. Furthermore, biocytin labeling has also been used extensively in paired or multiple recording studies from synaptically coupled neurons to reveal the identity of pre- and postsynaptic neurons and to determine the location of synaptic contacts[24–32] by the close proximity of a presynaptic axonal bouton and a postsynaptic dendritic shaft or spine. Three-dimensional (3D) models of the morphologically reconstructed synaptic connections can then be used for computational modeling[33,34].

Experimental design

Overview of the procedure. In this protocol, we present a method for biocytin labeling of neuronal tissue, which is used extensively in our laboratory[2,14–16,23,26,29,35–41]. In addition, we present a new detailed embedding procedure using the embedding medium Eukitt, which is now routinely used in the laboratory. This new embedding protocol improves the visibility of cytoarchitectonic features (including the lamination of brain regions, such as the neocortex or hippocampus, or cytoarchitectonic units, such as the barrels in rodent somatosensory cortex) so that additional labeling methods (e.g., cytochrome *c* oxidase staining[42,43]) are no longer required. This will be particularly helpful when the neuronal morphology is analyzed in the context of cytoarchitectonic modules such as cortical columns[14–16,44,45]. In this modified protocol, we also present a very gentle dehydration procedure that helps overcome the tissue distortion often seen in other procedures. Furthermore, we provide correction factors for reconstructions of neurons in brain slices so that a more realistic 3D neuronal geometry can be obtained from these preparations; this correction is now routinely used in the laboratory. Measurements of correction factors for spatial distortions were difficult to determine until now because the shrinkage of brain tissue embedded in a viscous medium, such as the commonly used Moviol, is nonlinear[44]. Finally, we describe some key steps in the computer-assisted 3D reconstruction of labeled neurons that can later be used for morphometric analyses or be processed further for electron microscopic examination. The flowchart in **Figure 1** details all procedures leading from biocytin staining to reconstruction and illustrates how the different steps of this protocol are interconnected.

Comparison of biocytin labeling with other labeling techniques. Biocytin labeling of neurons results in an extremely durable and strong staining; this is particularly true when it is coupled to an avidin–biotinylated horseradish peroxidase (HRP) complex (ABC) during the immunocytochemical processing and subsequently visualized using DAB as a chromogen, which forms a black reaction product[1,46]. The plant enzyme HRP is unlikely to cross-react with the

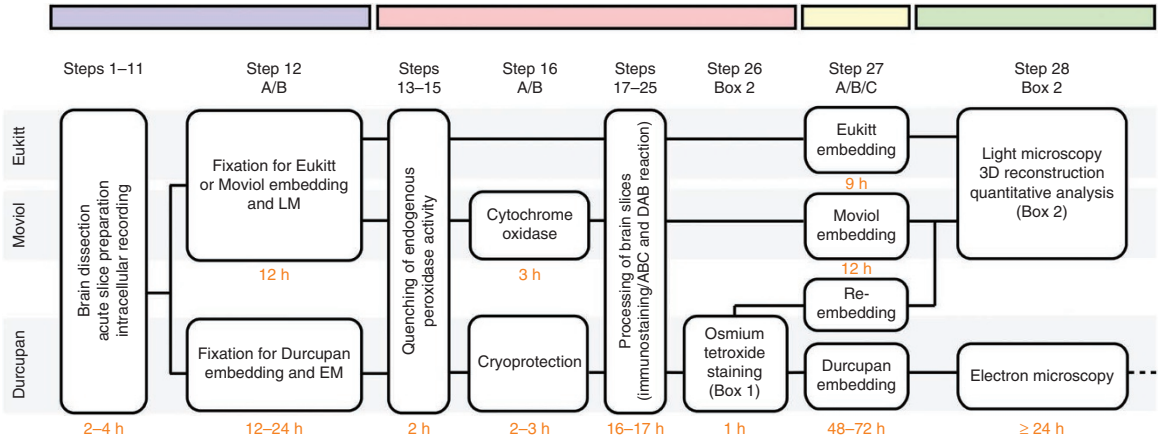

Figure 1 | Flowchart for biocytin labeling. This chart illustrates the common and distinct procedures for Eukitt and Moviol embedding and subsequent examination by LM, as well as for Durcupan embedding and subsequent EM. Timing is denoted in orange.

neuronal tissue; thus, biocytin-HRP labeling can be combined with other antibody staining. The turnover of the HRP substrates (here, hydrogen peroxide (H_2O_2) and DAB) results in the amplification of the detectable chromogenic end products and hence an enhanced staining. Therefore, this method results in labeled neurons whose staining highly contrasts with the otherwise unstained tissue, thus allowing a careful 3D reconstruction of the labeled structures, even years after the physiological recording has taken place. Notably, biocytin labeling allows the re-examination of tissue, e.g., to test a new scientific hypothesis or to verify the findings in a different context. As a substitute for biocytin, the biocytin derivate Neurobiotin[47], which has a lower molecular weight, may also be used.

Biocytin-DAB labeling has several advantages over fluorescence moieties that are also coupled with biocytin. Biocytin-DAB staining is not subject to photobleaching during examination with LM. In addition, it is much more sensitive and shows a higher contrast than most available fluorescent dyes, thus allowing, e.g., the identification of putative synaptic contacts. Further, it can be used in combination with ultrastructural examination of the tissue using transmission electron microscopy (EM). This is often done to verify the light-microscopically identified putative synaptic contacts from paired electrophysiological recording studies[23,26,27,29,39,48–54].

In contrast to the whole-mount biocytin labeling procedure described here, some laboratories use subsectioning of brain slices before the biocytin-DAB reaction[55]. Although this may help obtain a higher tissue penetration of the dye, it may result in the loss of tissue during the cutting procedure. In addition, this step renders neuronal 3D reconstructions more difficult and time-consuming—particularly for neurons with a high dendritic and axonal density—because an alignment of the subsections is necessary in order to obtain realistic 3D structures.

Shrinkage correction of histochemically processed tissue. To perform a light and subsequent electron microscopic analysis of the same biocytin-labeled neurons, embedding in the viscous medium Moviol[56] has been introduced because this medium allows for 3D morphological reconstructions with LM and a subsequent re-embedding in hard epoxy resins, such as Durcupan, which renders neurons suitable for ultrathin sectioning for ultrastructural examination by EM. However, the use of Moviol as an embedding medium has some serious disadvantages for LM reconstructions because it leads to nonlinear distortions of the neuronal tissue that are particularly strong in the *z* direction. Consequently, such cells show unrealistically compressed 3D morphologies. It is extremely difficult to correct for this type of shrinkage artifact. Therefore, reconstructions of neurons embedded in this medium do not represent the true 3D neuronal morphology. In a recent study[44], an attempt to quantify the 3D shrinkage in Moviol-embedded slices was made by comparing neuronal morphologies measured with two-photon microscopy in acute slices with the same biocytin-labeled structures after histochemical processing. Although this procedure gave meaningful results, it was quite laborious and would have to be redone if any of the variables in the embedding process (slice thickness, dimensions of the coverslip and so on) are altered.

Limitations of intracellular biocytin labeling. Intracellular biocytin labeling is an excellent technique to reveal the light microscopic structure of single and multiple neurons, especially when used in combination with electrophysiological recordings. It is thus an excellent technique to use for a correlated structural and functional analysis of neuronal microcircuits, as it allows the identification of both pre- and postsynaptic neuronal cell types. However, because of the extremely electron-dense reaction product, it does not permit a reliable resolution of subcellular structures such as pre- and postsynaptic densities with EM. For this, the reader is referred to other protocols that use less-electron-dense stains[57,58].

MATERIALS

REAGENTS

- Animals: the experimental animals include rats and mice **! CAUTION** All animal experiments must comply with all relevant ethics guidelines concerning the use of animals.

Reagents for brain dissection, acute slice preparation and recording

- ATP-magnesium salt (ATP-Mg^{2+}; Sigma-Aldrich, cat. no. A9187)
- Artificial cerebrospinal fluid (ACSF; see REAGENT SETUP)
- Ascorbic acid (Fluka, cat. no. 95209)

- Biocytin (Sigma-Aldrich, cat. no. B4261)
- Carbogen gas (95% O_2 and 5% CO_2; Linde AG, cat. no. 4040152)
- D(+)-Glucose (Sigma-Aldrich, cat. no. G7021)
- GTP sodium salt hydrate (GTP; Sigma-Aldrich, cat. no. G8877)
- Isoflurane (CP-Pharma, cat. no. B28H10A)
- Myo-inositol (Merck, cat. no. 1.04507)
- Magnesium chloride hexahydrate ($MgCl_2$; Merck, cat. no. 1.05833)
- Phosphocreatine disodium salt hydrate (Sigma-Aldrich, cat. no. P7936)
- Potassium chloride (KCl; Merck, cat. no. 1.04938)
- Potassium D-gluconate (Fluka, cat. no. 60245)
- Potassium hydroxide solution (KOH, 1 N; Sigma-Aldrich, cat. no. 31,937-6)
- Sodium chloride (NaCl; Merck, cat. no. 1.06404)
- Sodium dihydrogen phosphate monohydrate (NaH_2PO_4; Merck, cat. no. 1.06580)
- Sodium hydroxide solution (NaOH, 1 N; Sigma-Aldrich, cat. no. 31,951-1)
- Sodium hydrogen carbonate ($NaHCO_3$; Merck, cat. no. 1.06329)
- Sodium pyruvate ($NaC_3H_4O_3$; Sigma-Aldrich, cat. no. P2256)

Reagents for fixation and immunocytochemistry

- 3,3′-diaminobenzidine (DAB) tetrahydrochloride (in tablet form; Sigma-Aldrich, cat. no. D5905) **! CAUTION** It is a toxic compound; see H315-H318, European Union Commission Directive 2001/59/EC hazard codes and phrases 49-22-42/43-52/53. It may cause cancer after inhalation: the tablet form reduces the risk of contamination; in addition, it has an improved solubility.
- Ammonium nickel sulfate hydrate ($(NH_4)_2Ni(SO_4)_2$; Fluka, cat. no. 9885)
- Calcium chloride dihydrate ($CaCl_2$; Merck, cat. no. 1.02382)
- Cobalt chloride ($CoCl_2$; Sigma-Aldrich, cat. no. C2644)
- Cytochrome *c* oxidase (Sigma-Aldrich, cat. no. C2506)
- Disodium hydrogen phosphate monohydrate (Na_2HPO_4; Merck, cat. no. 1.06346)
- Glutaraldehyde (GAD, 25% (vol/vol); Polysciences, cat. no. 019090)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- Liquid nitrogen
- Osmium tetroxide (OsO_4; Sigma-Aldrich, cat. no. 19110) **! CAUTION** It is a toxic compound; see European Union Commission Directive 2001/59/EC hazard codes and phrases: 26/27/28-34.
- Paraformaldehyde powder (PFA; Sigma-Aldrich, cat. no. 15,812-7) **! CAUTION** It is a dangerous powder; see H228-H302-H315-H317-H318-H332-H335-H351, European Union Commission Directive 2001/59/EC hazard codes and phrases 20/22-37/38-40-41-43. It is suspected to cause cancer.
- Sodium dihydrogen phosphate monohydrate (NaH_2PO_4; Merck, cat. no. 1.06580)
- Sucrose (Sigma-Aldrich, cat. no. 16104)
- Triton X-100 (Sigma-Aldrich, cat. no. X100) **! CAUTION** It is a dangerous compound; see H302-H318, European Union Commission Directive 2001/59/EC hazard codes and phrases 22-41-51/53.
- Vectastain ABC (avidin-biotin complex) elite kit (Vector Laboratories, cat. no. PK6100)

Reagents for Eukitt embedding (LM)

- Chromium(III) potassium sulfate dodecahydrate (VWR, cat. no. 21095.293) **! CAUTION** It is a toxic compound; see H315, H319.
- Ethanol (Merck, cat. no. 8.18760)
- Eukitt embedding medium (poly(butyl methacrylate-co-methyl methacrylate); Fluka, cat. no. 03989)
- Gelatin (Fluka, cat. no. 04055)
- Xylene (Merck, cat. no. 1.08681)

Reagents for Moviol embedding (LM)

- Moviol 4–88 (Calbiochem, cat. no. 475904)
- Glycerol (Merck, cat. no. 1.04093)
- Tris-HCl (Tris(hydroxymethyl) aminomethane hydrochloride; Merck, cat. no. 1.08382)

Reagents for Durcupan embedding (EM)

- Durcupan (ACM Fluka, Sigma-Aldrich, cat. no. 44610)
- Ethanol (Merck, cat. no. 8.18760)
- Phosphoric tungsten acid (Merck, cat. no. 1.00583.0100) (optional)
- Propylene oxide (Sigma-Aldrich, cat. no. 56671)
- Uranyl acetate (1%; Sigma-Aldrich, cat. no. 73953) (optional). For a detailed list of reagents for Durcupan embedding and EM/ see appropriate protocols[26,57,58]

EQUIPMENT

Equipment for brain dissection, acute slice preparation and recording

- Borosilicate glass capillaries (Hilgenberg, cat. no. 1807502)
- Microtome (Microm HM650V; Microm International)
- Razor blades/Silver; Gillette Super (Procter & Gamble)
- Set of dissection instruments (scissors, forceps, spatula; Fine Science Tools)
- Slice chamber (custom-made) **▲ CRITICAL** Detailed descriptions of the patch-clamp setup including amplifiers, preamplifiers, cameras and a microscope with a motorized stage can be found in other protocols[32,59,60].

Equipment for fixation and immunocytochemistry

- Moist chamber to keep sections in humid atmosphere (custom-made)
- Multiwell plates (6 or 12 wells; BD Bioscience)
- Shaker (IKA, cat. no. KS 260 basic)
- Small vials (10 ml, 22 × 45; VWR)

Equipment for embedding procedures

- Adhesive, silane-coated Histobond microscope slides (Marienfeld Lab Glassware)
- Fine brush
- Coverslips (0.055–0.080 mm; Menzel-Gläser) **▲ CRITICAL** Use ultrathin coverslips to decrease additional tissue compression during embedding and to minimize the working distance between the fixated slice and microscope objective.
- Standard microscope slides to produce gelatinized slides (VWR, cat. no. 631-1553)
- Microcentrifuge tubes

Equipment for 3D reconstructions for LM

- Illustrator software for graphical reprocessing (Adobe Systems)
- Neurolucida and Neuroexplorer reconstruction and analysis software (Version 10.02, 32-Bit; Microbrightfield Biosciences) for personal computers
- Personal computer (Intel Core I7 870 with 4 GB RAM and a Nvidia GTX460 graphics card)
- Upright microscope (Olympus BX61; Olympus) with a computerized stage for digital 3D reconstructions of dendritic and axonal morphology (Microbrightfield Europe)
- Lens paper

REAGENT SETUP

Slicing solution for the dissection and slicing of brains from immature and juvenile animals Use an ACSF containing 125 mM NaCl, 2.5 mM KCl, 25 mM glucose, 1.25 mM NaH_2PO_3, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 3 mM Myo-inositol, 2 mM $NaC_3H_4O_3$ and 0.4 mM ascorbic acid. The osmolarity of the solution should be ~310 mOsm per liter. This solution has the normal Na^+ and K^+ concentration of a standard ACSF but a reduced Ca^{2+} and a high Mg^{2+} concentration to reduce synaptic transmission and possible excitotoxic effects. **▲ CRITICAL** The age of the animal is always crucial. Neurons in juvenile brain tissue are generally healthier and survive longer than neurons in mature brain tissue. Use appropriate solutions to minimize adverse effects on the experimental outcome. **▲ CRITICAL** Freshly prepare the solution before use and gas it with 95% O_2 and 5% CO_2. Excess solution can be retained for a further 24 h and stored at 4 °C.

Slicing solution for mature (>30 postnatal days and older) and adult animals Use a sucrose-based slicing solution (with reduced Ca^{2+} and increased Mg^{2+} concentration) to improve neuronal survival. This solution contains 206 mM sucrose, 2.5 mM KCl, 25 mM glucose, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 3 mM myo-inositol, 2 mM $NaC_3H_4O_3$ and 0.4 mM ascorbic acid. The osmolarity of the solution should be ~310 mOsm per liter. **▲ CRITICAL** Freshly prepare the solution before use and gas it with 95% O_2 and 5% CO_2. Excess solution can be retained for 24 h at 4 °C.

Extracellular recording solution During recording, use an extracellular ACSF with the following composition: 125 mM NaCl, 2.5 mM KCl, 25 mM glucose, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. The osmolarity of the solution should be ~310 mOsm per liter. **▲ CRITICAL** Prepare fresh solution before use and gas it with 95% O_2 and 5% CO_2. Excess solution can be retained for a further 24 h and stored at 4 °C.

Intracellular solution For whole-cell recording, use an intracellular biocytin solution containing 105 mM K-gluconate, 30 mM KCl, 10 mM HEPES, 10 mM phosphocreatine, 4 mM ATP-Mg^{2+} and 0.3 mM GTP. Adjust the pH to 7.3 with KOH. The osmolarity of the solution should be ~300 mOsm per liter. Add biocytin at a concentration of 3–5 mg ml^{-1} to label neurons during recording time. **▲ CRITICAL** Freshly prepare the solution before use and gas it with 95% O_2 and 5% CO_2. Discard excess solution after use.

ABC solution for LM Add 150 µl of component A, 150 µl of component B (components A and B are included in the Vectastain ABC kit) and 150 µl of 10% (vol/vol) Triton X-100 to 14.55 ml of phosphate buffer (PB) (1:1:1:97). **▲ CRITICAL** Freshly prepare this solution and keep it in the dark for about 30 min before use.

ABC solution for EM Add 150 µl of component A and 150 µl of component B (components A and B are included in the Vectastain ABC kit) to 14.7 ml of PB (1:1:98). Ensure that no membrane-permeabilizing detergent such as Triton X-100 is in the ABC solution when you are processing for EM. ▲ **CRITICAL** Freshly prepare this solution and keep it in the dark for about 30 min before use.

Cytochrome *c* oxidase solution This solution contains 60 ml of DAB (0.07% (wt/vol)), 24 mg of cytochrome *c* oxidase (0.04% (wt/vol)) and 2.4 g of sucrose. Heat this solution to 37 °C in the dark before use. ▲ **CRITICAL** Always freshly prepare this solution.

Cryoprotectant solution (10% (wt/vol), 20% (wt/vol), 30% (wt/vol)) For the 10, 20 and 30% (wt/vol) cryoprotectant solutions, add 1, 2 or 3 g sucrose, respectively, to 10 ml 100 mM PB. ▲ **CRITICAL** Always freshly prepare this solution. Discard excess solution after use.

DAB solution Add 2 µl of $CoCl_2$ (1% (wt/vol)) and 4 µl of $(NH_4)_2Ni(SO_4)_2$ (1% (wt/vol)) to 10 ml of 3,3′-DAB. ! **CAUTION** The solution is carcinogenic, and thus DAB must be used with utmost caution and should be handled under a fume hood. DAB waste needs to be inactivated with sodium hypochlorite (bleach) before disposal as a hazardous chemical. ▲ **CRITICAL** Always freshly prepare this solution.

Gelatin solution Mix 10 g of gelatin and 0.5 g of chromium (III) potassium sulfate dodecahydrate (i.e., chromium chloride) in 1 liter of pure water. Use gelatin solution for an additional coating of the microscope slides. ▲ **CRITICAL** Always freshly prepare this solution. Discard excess solution after use.

H_2O_2 solution (3% (wt/vol), stock solution) H_2O_2 solution is used for quenching of the residual endogenous peroxidase activity in the fixated tissue. This solution contains 2 ml of a 30% (wt/vol) H_2O_2 solution dissolved in 18 ml PB (100 mM). ▲ **CRITICAL** Freshly prepare this solution.

Moviol embedding medium Mix 6.0 g of glycerol, 2.4 g of Moviol 4–88, 12 ml of Tris-HCl (200 mM, pH 8.5) and 6 ml of pure water, stirring at 50–90 °C for at least 30 min. Afterward, centrifuge the mixed solution at 5,000*g* for 20 min at room temperature (RT; 20–25 °C). ▲ **CRITICAL** Prepare in advance and store in aliquots of 1 ml at −20 °C; aliquots can be used for about 12 months. When opened, Moviol can be stored at 4 °C and used for about 1 month.

PB (100 mM stock solution) Prepare two stock solutions, A and B. Solution A is 400 mM Na_2HPO_4; solution B is 400 mM NaH_2PO_4. Combine 200 ml of A and 50 ml of B, and then add 750 ml of pure water. Check that the pH is adjusted to 7.4. ▲ **CRITICAL** PB can be stored at 4 °C for 1–2 weeks.

Standard fixative solution for LM The standard fixative solution contains 4% (wt/vol) PFA in 100 mM PB, pH 7.4. Dissolve 20 g of PFA in 300 ml pure water and heat this mixture to ~60 °C. Add two or three drops of 1 M NaOH solution. Finally, add 125 ml PB (0.4 M) and adjust the final volume to 500 ml with pure water. ▲ **CRITICAL** Prepare this solution in advance. It can be stored for ~2 months at 4 °C.

Standard fixative solution for EM The standard fixative solution contains 1% (wt/vol) PFA and 2.5% (vol/vol) GAD in 100 mM PB (pH 7.4). Dissolve 5 g of PFA in 300 ml of pure water and heat up this mixture to ~60 °C. Add two or three drops of 1 M NaOH solution. Add 125 ml of PB (0.4 M) and adjust the volume to 495 ml with pure water. Finally, add 5 ml of GAD (25% (vol/vol)). ▲ **CRITICAL** Prepare this solution in advance. It can be stored for ~2 months at 4 °C.

EQUIPMENT SETUP

Coating slides with gelatin Immerse the microscope slides into the freshly filtered and warm gelatin solution and dry them for 2–4 d at RT or at 50 °C overnight in a compartment dryer. Microscope slides should be prepared in advance, preferably several days before the experiment. Freeze the object slides and store them at −20 °C until you are ready to use them.

PROCEDURE

Brain dissection, acute slice preparation and recording ● TIMING ~1.5 h

1| Anesthetize the animal (rat/mouse) under study (e.g., with isoflurane), decapitate it, rapidly remove the brain and transfer it immediately into ice-cold (4 °C) ACSF gassed with 95% O_2 and 5% CO_2.

▲ **CRITICAL STEP** Brain dissection and acute slice preparation, including subsequent electrophysiological measurements by whole-cell patch-clamp recordings, are not the main focus of this protocol and can be found described in more detail elsewhere[32,59–61]. Important and difficult steps are mentioned in brief below. Ensure that the appropriate slicing solution is used for immature/juvenile or more mature and adult animals.

▲ **CRITICAL STEP** All experimental procedures involving animals were carried out in accordance with the German animal welfare act and the guidelines of the Federation of Laboratory Animal Science Associations (FELASA). The appropriate permissions for killing animals for brain slice experiments were obtained from the Northrhine-Westphalian Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV).

? TROUBLESHOOTING

2| Prepare and cut the brain for the region of interest.

▲ **CRITICAL STEP** Completing Steps 1 and 2 should take <90 s, in order to keep the interruption of nutrient and oxygen supply to a minimum and to avoid anoxic shock to the cells.

3| Transfer the brain to the microtome chamber and trim the brain with a slice thickness of ~600 µm until the target area becomes visible. Thereafter, cut a series of acute brain slices from the region of interest with a thickness of 350 µm for adolescent and mature brain tissue. For young animals (in rodents, the first postnatal week), it is possible to obtain slices up to 800 µm thick, which helps preserve long-range axonal projections[62,63].

▲ **CRITICAL STEP** Ensure that the brain is cut at very low speed (8 ± 2 mm min^{-1}); use a high frequency (85 Hz) and amplitude (0.9 mm).

4| Transfer each slice to an incubation chamber filled with ACSF and gassed with 95% O_2 and 5% CO_2. Timing for Steps 3 and 4 must be about 30 min.

■ **PAUSE POINT** Slices must be stored in the incubation chamber at RT for a further 30 min before use in order to improve slice viability.

5| For physiological recordings, transfer brain slices to the recording chamber of the patch-clamp setup.
▲ **CRITICAL STEP** Continuously superfuse the brain slices with an extracellular solution at relatively high speed (~5 ml min^{-1}) and control the bath temperature (about 32–35 °C) to maintain optimal recording conditions.

6| Perform a whole-cell voltage clamp recording on the neurons of your choice using patch pipettes pulled from borosilicate glass capillaries. Patch pipettes should be advanced slowly in order to avoid tissue distortion.
▲ **CRITICAL STEP** Adjust the tip size of the patch pipette to the soma size of the patched neuron. Use low-resistance patch pipettes (3–6 MΩ) for neurons with large soma sizes (e.g., pyramidal cells with diameters exceeding 10 μm). Use high-resistance patch pipettes (7–10 MΩ) for neurons with small cell bodies (e.g., granule cells in the cerebellum or olfactory bulb or neocortical spiny stellate neurons with a diameter <10 μm). Patch pipettes with an inappropriate tip size can result in a failure to form a GΩ seal before and after recording (in particular, when it lasts >1 h) or in the retraction of the neuronal cell body from the slice preparation (see Step 8). This will seriously compromise the subsequent immunostaining and visualization.
When moving the pipette into a brain slice, apply positive pressure very gently to avoid spilling biocytin-containing solution into the extracellular space. Try to quickly obtain a GΩ seal with the patch pipette onto the membrane of the neuron that is to be filled with biocytin.
? TROUBLESHOOTING

Intracellular filling ● TIMING 0.5–2 h
7| During electrophysiological recordings, let the biocytin diffuse out of the patch pipette into the patched neuron; no de- or hyperpolarizing current injection is necessary to facilitate the diffusion or prevent pipette clogging. In general, a recording/labeling time of 15–20 min is sufficient to completely fill the axon and dendrites of large neurons such as pyramidal cells in neocortical layers 5 and 6. However, even after prolonged electrophysiological recordings (>2 h), excellent biocytin stainings can be obtained, which can still be used for EM[29,64].
? TROUBLESHOOTING

8| After a sufficiently long filling time, retract the electrode, when possible, under visual control.
▲ **CRITICAL STEP** Pipette movements should be very slow to maintain the quality of the seal and thus the quality of the staining. If the detachment of the patch pipette from the cell membrane is difficult to achieve, we occasionally use high-current injection to destabilize the neuronal cell membrane. This was successful in a few cases; however, biocytin labeling was mostly suboptimal when we used this procedure.
? TROUBLESHOOTING

9| Leave the brain slice for another 10–15 min in the recording chamber to wash out excess biocytin from the extracellular space.

10| Afterward, take the brain slice carefully from the recording chamber of the patch-clamp setup and transfer it into a small vial.

11| Completely remove any excess extracellular solution.
▲ **CRITICAL STEP** Any remaining extracellular solution may affect the subsequent fixation.
? TROUBLESHOOTING

Fixation of acute brain slices
12| For fixation of acute brain slices, follow options A or B, depending on the goal of the experiment. Use option A for processing for Eukitt embedding or Moviol embedding and subsequent LM; use option B for processing for Durcupan embedding and subsequent EM.
(A) Fixation for Eukitt embedding or Moviol embedding (LM) ● TIMING ~12 h
(i) Fix brain slices at 4 °C in 4% (wt/vol) PFA dissolved in PB (100 mM, pH 7.4) for at least 12 h. Subsequently, transfer the slices into a fixative-free PB solution.
■ **PAUSE POINT** Slices can be kept for several days at 4 °C in the fixative-free PB solution.
(B) Fixation for Durcupan embedding and EM ● TIMING ~24 h
(i) Fix brain slices at 4 °C in 1% (wt/vol) PFA and 2.5% (vol/vol) GAD dissolved in PB (100 mM, pH 7.4).
▲ **CRITICAL STEP** Transfer the brain slices to a fixative-free PB solution after a maximal fixation period of 12–24 h.
■ **PAUSE POINT** Slices can be kept for several days at 4 °C in the fixative-free PB solution.

Quenching of endogenous peroxidase activity ● TIMING ~2 h
13| Rinse the brain slices extensively with 100 mM PB six to eight times each for about 10 min per wash.

14| Incubate each brain slice in about 2 ml of H_2O_2 solution (3% (wt/vol) H_2O_2) for 20–30 min to block any endogenous peroxidase activity. This results in the intensive development of oxygen bubbles.

15| Repeat Step 13 until no further oxygen bubbles are visible, thereby indicating that the intracellular peroxidase activity is quenched.
▲ **CRITICAL STEP** Quenching for immature brain tissue must be longer than that for mature brain tissue because of a higher endogenous peroxidase activity.
? TROUBLESHOOTING

16| If you are processing slices for Moviol embedding and LM, either carry out option A to obtain a similar quality of embedding as with Eukitt or proceed directly to Step 17. If you are intending to perform Durcupan embedding and EM, follow option B before proceeding with Step 17. If you are performing Eukitt embedding and LM, proceed immediately to Step 17.
(A) Additional cytochrome *c* oxidase ● TIMING ~3 h
(i) Completely remove the PB.
▲ **CRITICAL STEP** When you use Moviol as the embedding medium, it is necessary to perform an additional histochemical processing using cytochrome *c* oxidase to identify cytoarchitectonic features such as the barrel structure of layer 4 and the general six-layered structure of the somatosensory cortex. The cytochrome *c* oxidase staining technique has been described in detail previously[42,43,65], and is therefore mentioned only briefly here.
(ii) Transfer the brain slices into cytochrome *c* oxidase solution and incubate them at 37 °C for 1–2 h in the dark.
(iii) Stop the immunoreaction by transferring the slices into phosphate-buffered solution when individual barrels are clearly distinguishable from the background.
(iv) Rinse all slices at least six to eight times for 10 min per wash.
■ **PAUSE POINT** Slices can be kept in 100 mM PB at 4 °C for a few (3–5) days.
(B) Cryoprotection for EM ● TIMING approximately 2–3 h
(i) Cryoprotect the brain slices by transferring them into 10% (wt/vol) sucrose solution for 45 min.
(ii) Transfer the slices into 20% (wt/vol) sucrose solution and incubate them for at least 45 min.
(iii) Transfer the slices into 30% (wt/vol) sucrose solution and incubate them for at least 45 min, until the slices sink to the bottom of the well.
(iv) Transfer the slices into microcentrifuge tubes and freeze them in liquid nitrogen.
(v) Thaw the frozen slices immediately afterward by transferring them into 100 mM PB at RT.
(vi) Rinse the brain slices in 100 mM PB four times for 10 min per wash.
■ **PAUSE POINT** The slices can be kept in 100 mM PB at 4 °C for a few (3–5) days.

ABC reaction for biocytin-labeled brain slices ● TIMING ~15 h
17| Thoroughly remove all excess PB.

18| For LM analysis (i.e., slices prepared as described in Step 12A), incubate brain slices in 1% (vol/vol) avidin–biotinylated HRP complex (ABC) solution containing 0.1% (vol/vol) Triton X-100 at RT for 1 h in complete darkness. For analysis by EM (i.e., slices prepared as described in Step 12B), incubate the brain slices in 1% (vol/vol) ABC solution.
▲ **CRITICAL STEP** ABC solution for subsequent EM should not contain any detergents, e.g., Triton X-100.

19| For both LM and EM analysis, incubate slices for a further 60 min on a shaker at 4 °C and for at least 12 h in the ABC solution. Subsequent steps are valid for both LM and EM analysis.

20| The next day, incubate the slices in the ABC solution for 1 h at RT.

21| Rinse the slices with 100 mM PB six to eight times for 10 min per wash.

DAB reaction ● TIMING ~1.5 h
22| Incubate each slice on a shaker for 30 min at 4 °C in 2 ml of a nickel- and cobalt-intensified DAB solution.

23| Start the chromogenic reaction by adding 6.5 µl of H_2O_2 solution (3% (wt/vol)) to the DAB solution and incubate the slices for a further 30–60 s until the biocytin staining of neuronal structures becomes visible. If a slower reaction time is desired (e.g., to better control the staining intensity), reduce the H_2O_2 concentration accordingly (range 0.01–0.5% (wt/vol)), thereby prolonging the reaction time.

Box 1 | OsO_4 staining of brain slices for EM ● TIMING ~1 h

(i) Incubate the brain slices that have been processed for EM at RT in a sucrose buffer containing 0.4–0.5% (wt/vol) OsO_4 for 30 min to enhance contrast.
▲ CRITICAL STEP Pipette one drop at a time carefully on the slices.
(ii) Transfer the slices to fresh multiwell plates and rinse them three times in 100 mM PB for 10 min per wash.
(iii) Proceed with the main PROCEDURE from Step 27C.

24| Stop the immunoreaction immediately by transferring the slices into 100 mM PB.
▲ CRITICAL STEP Do not incubate the brain slices too long in DAB solution; otherwise, processed brain slices become too dark.
? TROUBLESHOOTING

25| Rinse all slices at least six to eight times with 100 mM PB for 10 min per wash.
■ PAUSE POINT Keep brain slices in 100 mM PB at 4 °C for up to 1 d.

OsO_4 staining of brain tissue for EM ● TIMING ~1 h

26| If you intend to carry out EM on Durcupan-embedded slices, treat the slices as described in **Box 1**. If you intend to carry out LM on Eukitt-or Moviol-embedded slices, proceed immediately to Step 27.

Embedding procedures

27| Carry out option A for Eukitt embedding for LM, option B for Moviol embedding for LM and option C for Durcupan embedding for EM. The method for Durcupan embedding is briefly outlined; more details can be found in previous publications[26,57,58,66,67].

(A) Eukitt embedding method (LM) ● TIMING ~9 h

(i) Use a fine brush to transfer the brain slices to gelatinized microscope slides.
▲ CRITICAL STEP Eukitt is a hydrophobic, homogeneous, fast-drying embedding medium and is not as durable as Moviol. Therefore, it should be prepared shortly before use.
▲ CRITICAL STEP Chromium chloride should be added to the gelatin solution to obtain microscope slides that are clean and free of streaks.
? TROUBLESHOOTING
(ii) Thoroughly remove all excess PB.
(iii) Dry the brain slices in a moist chamber for at least 3–6 h.
▲ CRITICAL STEP The humidity in the moist chamber must be at least 80%.
? TROUBLESHOOTING
(iv) Air-dry the slices at RT for another 10 min.
(v) Dehydrate each slice, using ten dehydration steps, for 10 min each, with increasing ethanol concentrations (vol/vol) (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, twice in 100%).
▲ CRITICAL STEP Small increments in the alcohol concentration during dehydration are indispensable for optimal tissue preservation and to avoid distortions of neuronal structures (see 'corkscrew' effect, **Fig. 2**).
? TROUBLESHOOTING
(vi) Incubate each slice for a further 10 min in xylene.
(vii) Cover the slices with one or two drops of Eukitt embedding medium and coverslip them with ultrathin coverslips.
(viii) Air-dry the slices for at least 20 min at RT.
■ PAUSE POINT Embedded slices can be stored permanently in a box at RT.

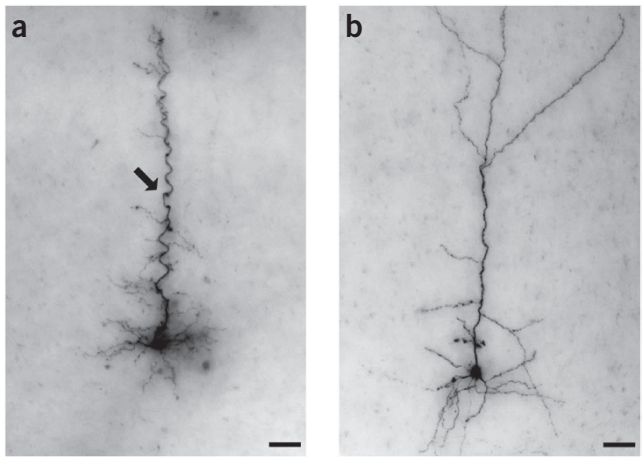

Figure 2 | 'Corkscrew' effect as a result of overly rapid dehydration. (**a**,**b**) Extended focus images of biocytin-filled pyramidal neurons embedded in Eukitt after a rapid (**a**) and a slow dehydration procedure (**b**) are shown. Note that the apical dendrite of the pyramidal neuron shown in **a** has a tortuous, corkscrew-like appearance (arrow), which results from a rapid dehydration in ethanol/xylene. The pyramidal neuron in **b** shows smooth dendrites as a result of a careful dehydration in slowly increasing ethanol concentrations; no corkscrew artifacts are observed in these conditions. Scale bars, 100 µm.

Box 2 | Suggestions for 3D, light-microscopic reconstructions of neurons

The processed and embedded specimen containing biocytin-labeled neurons can be examined by LM. Perform 3D reconstructions of the labeled neurons with the aid of the Neurolucida software[72]. This software allows manual reconstructions of neurons in all three dimensions and generates reconstruction data files in the Neurolucida format for a quantitative morphological analysis. In addition, ASCII files of the reconstructions can be generated, which are used in neuronal simulation environments such as NEURON[73].

Tissue embedded in Eukitt also permits the reconstruction of cytoarchitectonic features such as neocortical layer borders and barrel fields. For this, use the contour mode of Neurolucida. The borders of each layer should be traced as open contours; cytoarchitectonic units such as barrels should be traced as closed contours. The color intensity and contrast of a specimen is substantially higher in Eukitt than in Moviol (**Figs. 3** and **4**), revealing a continuous staining of neuronal structures with clear transitions and mostly without interruptions (**Fig. 5**).

Neurons embedded in a solid medium such as Eukitt undergo an almost linear shrinkage that is different for the *x*, *y* and *z* dimensions. We have determined this shrinkage to be 91.8 ± 6.2% in the *x* and 85.8 ± 2.8% in the *y* dimension, indicating that the *x* and *y* shrinkage is almost identical. In contrast, the shrinkage in the *z* dimension is 47.1 ± 0.4%, which is substantially greater. Therefore, the morphological reconstructions appear compressed (**Fig. 6**).

▲ **CRITICAL STEP** To correct for the tissue shrinkage, multiply the *x* and *y* dimensions of the reconstruction with a factor of 1.1 and the *z* dimension with a factor of 2.1 to obtain realistic neuronal morphologies.

A similar shrinkage correction is not possible for viscous embedding media such as Moviol[44]. The tissue is more compressed at the slice surface than at its bottom after the addition of a coverslip. Furthermore, the pressure exerted by the objective is transmitted via the immersion oil to the specimen adding an additional pressure artifact.

For a quantitative analysis of the morphological structures reconstructed with Neurolucida, use the Neuroexplorer software. We recommend using the Illustrator software for subsequent graphical reprocessing.

Some general suggestions regarding computer-aided reconstructions of neurons in brain slice preparations are given below.

1. Before beginning a reconstruction or continuing with the one started earlier, clean the microscope slide thoroughly with lens paper to remove dust, debris and traces of previously applied immersion oil. It may be necessary to use ethanol to clean the slide/coverslip surface completely.
2. Adjust the optics for best illumination of the stained neurons and optimal contrast. Center the condenser diaphragm and set up Köhler illumination[59,74]. Try to use the microscope objective with the highest possible magnification (×60 or ×100 oil-immersion objectives). Take care to ensure that lower-power objectives are not contaminated with the immersion oil in order to avoid lengthy cleaning procedures.
3. Commence the reconstruction by setting an easily recognizable reference point, preferentially on a structure close to the soma of the neuron that is to be reconstructed. We normally use the part where the axon joins the neuron cell body.
4. Next, outline the somata in the plane of focus, and continue by tracing first the dendritic processes and then the axon in the neuron-tracing mode of Neurolucida.
5. Use a shallow depth of field for soma near cell structures, where the density of processes is high, to facilitate the resolution of distinct but very close structures. This will help avoid the erroneous reconstruction of dendritic and axonal processes.
6. Increase the depth of field when tracing more distal or deeper neuronal processes with a considerable density.
7. When tracing, always take care that the brain slice and its superimposed reconstruction remain aligned.
8. Increase the structural resolution of the reconstructions by setting data points at relatively small intervals. This will increase the size of the reconstruction data file but will provide more morphological details.
9. Check the *z* axis value frequently to avoid strong leaps in the *z* direction, which may result in distorted reconstructions and unrealistic morphologies.
10. Neurolucida allows to set both bi- and trifurcating nodes as branching points in axonal or dendritic structures. However, many simulation programs (such as NEURON) cannot deal with trifurcating nodes. Therefore, use bifurcating nodes only; trifurcations should be represented by two bifurcating nodes.
11. It may be easier to reconstruct some of the dendritic and axonal processes by starting at their respective ends. The polarity of these structures can be reversed once the connection point to the reset of the dendritic and axonal tree has been identified.
12. Neurolucida jumps automatically back to previously set branch points (nodes). Always check that there is no shift in the *z* dimension of these branching points.
13. The slicing procedure always results in some cut dendritic and axonal processes. Such cut, incomplete endings are visible as stained blobs and should be marked.
14. If desired, outline the lamination of brain regions (e.g., in the neocortex, hippocampus or cerebellum) or the borders of cytoarchitectonic units such as cortical barrels in the somatosensory cortex at low (×4 or ×10) magnification. This should be done in the contour mode of Neurolucida.
15. When the reconstruction of a cell or of multiple cells is completed, turn on the grid mode in Neurolucida and check the tracing for completeness by focusing in every grid box from the slice surface to its bottom. This way, you may be able to detect missed dendritic or axonal collaterals.
16. Finally, correct for shrinkage by multiplying the *x*, *y* and *z* dimensions with the appropriate correction factors (**Fig. 6**).

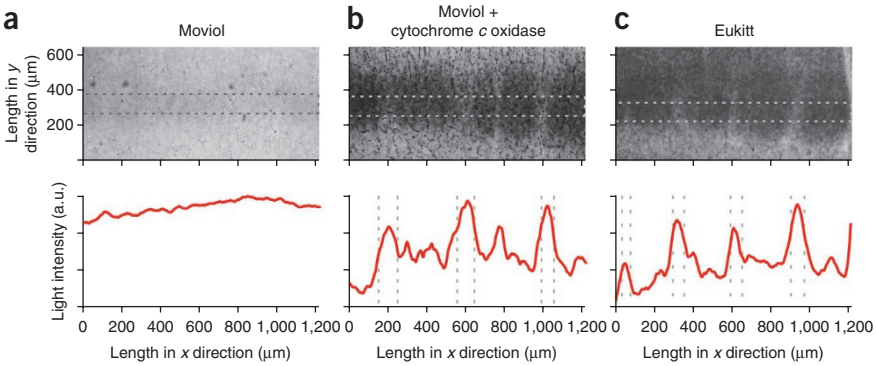

Figure 3 | Optical effects in the barrel field of neocortical layer 4 due to different embedding media. (**a**–**c**) Upper images give an overview of a barrel region in a thalamocortical brain slice embedded in Moviol and visualized by transmission bright-field microscopy (**a**), in Moviol with additional cytochrome *c* oxidase staining (**b**) and in Eukitt (**c**). Lower images show horizontal light intensity profiles recorded in the regions marked by dashed lines in the corresponding upper panels. The light intensity per cortical length (red lines) is given in arbitrary units; dark structures have a low light intensity. (**a**) Moviol-embedded slices have a linear light intensity due to a homogeneous color intensity and low contrast. The barrels at the level of layer 4 are barely visible. (**b**,**c**) For cytochrome *c* oxidase–processed slices embedded in Moviol, and for Eukitt-embedded slices, barrel regions are clearly visible. In the bright-field images (**b**,**c**), barrels can be identified as evenly spaced light hollows separated by narrow dark stripes representing the barrel borders/septae[2,70,71]; septae are visible as peaks in the light intensity profiles (**b**,**c**). When compared with Eukitt-embedded slices, cytochrome *c* oxidase–processed slices show an enhanced barrel size for the layer-2/3 and layer-5 directions and a very high background staining. a.u., arbitrary units.

(B) Moviol embedding method (LM) ● **TIMING ~12 h**

(i) Use a fine brush to mount the brain slices on adhesive, silane-coated Histobond objective slides.

▲ **CRITICAL STEP** Moviol can be prepared in advance and is durable for 1 month at 4 °C or for 6–12 months at −20 °C.

(ii) Remove all excess PB with a clean tissue.

(iii) Cover each slice with about 250 µl of Moviol; cover with ultrathin coverslips, taking care to avoid bubbles.

(iv) Air-dry all specimens at RT for at least 12 h (or overnight).

■ **PAUSE POINT** Embedded slices can be stored permanently (>10 years) in a box at RT.

(C) Durcupan embedding method (EM) ● **TIMING 48–72 h**

(i) Use a fine brush to transfer the brain slices processed for EM to standard object slides and fix them with coverslips.

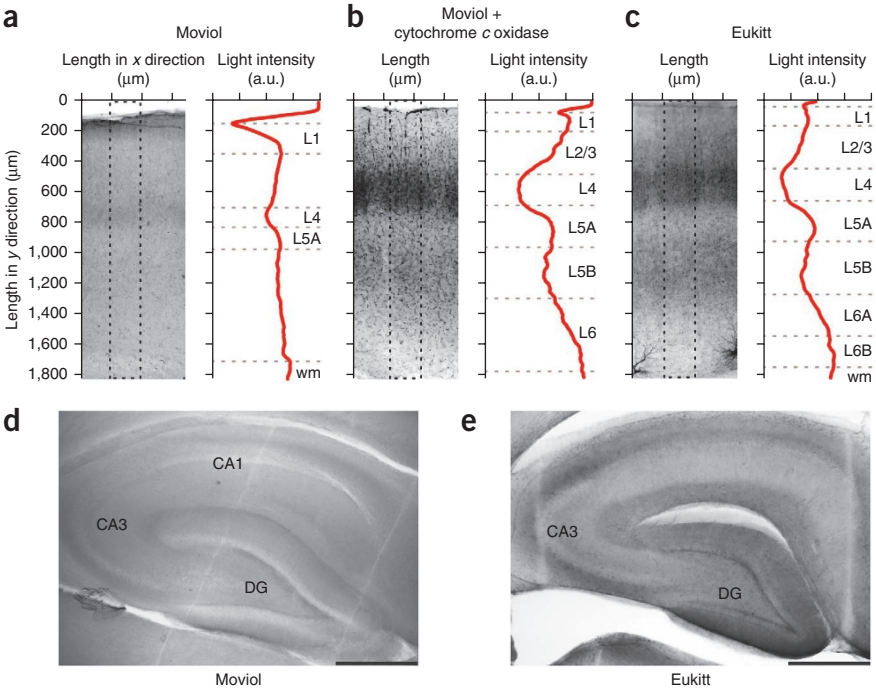

Figure 4 | Visibility of cortical lamination in different embedding media. (**a**–**c**) The left image of each pair shows an overview of a thalamocortical barrel-related brain slice section visualized by transmission bright-field microscopy embedded in Moviol (**a**), in Moviol with additional cytochrome *c* oxidase staining (**b**) and in Eukitt (**c**). Note the homogeneous shading and the low contrast in the Moviol-embedded slice. An additional histochemical staining for cytochrome *c* oxidase is necessary to visualize cytoarchitectonic structures in Moviol-embedded slices; in contrast, neocortical lamination is readily discernible in the Eukitt-embedded slice without any additional histochemical processing. The right-hand image of each pair shows vertical light intensity profiles through all layers shown in the corresponding left-hand image. The regions in which the intensity profiles were recorded are outlined by dashed lines. The light intensity per cortical length (red lines) is given in arbitrary units (a.u.); dark structures have a low light intensity. (**a**) Slices embedded in Moviol have a linear light intensity so that layer borders are not clearly discernible. Layers 1, 4 and 5A are barely visible. (**b**,**c**) For cytochrome oxidase–processed slices embedded in Moviol and for Eukitt-embedded slices, the borders between cortical layers and sublaminae are visible as changes in light intensities. Slices embedded in Eukitt reveal a much better shading and sharp contrast than cytochrome oxidase–processed slices, especially at the borders between layers 2/3 and 4 and the borders between layers 4 and 5. Above all, neocortical lamination is less blurred and shows clearer transitions. (**d**,**e**) These advantages are also visible in other brain regions, as it can be seen here for the hippocampus, which is shown after Moviol embedding (**d**) and Eukitt embedding (**e**) respectively. CA, Cornu ammonis area; DG, dentate gyrus; wm, white matter. Scale bars (**d**,**e**), 250 µm.

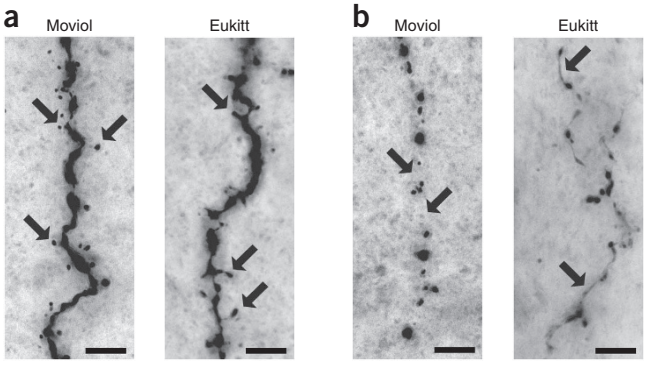

Figure 5 | Resolution of subcellular structures in slices embedded in Moviol and Eukitt. (**a**,**b**) Extended focal images of distal dendritic (**a**) and axonal (**b**) structures are shown. (**a**) Biocytin-labeled dendrites in Moviol-embedded slices appear to be discontinuous and do not have clear transitions (arrows) to their dendritic spines compared with those in Eukitt-embedded slices. (**b**) Axon collaterals in Moviol-embedded slices appear often incompletely filled and cannot be traced continuously but only by their axonal boutons (which appear as dots here), especially in more mature tissue. In Eukitt-embedded slices, biocytin labeling of axon collaterals generally shows a continuous staining, which simplifies the neuronal tracing. Scale bars, 5 µm.

(ii) Dehydrate slices in four dehydration steps of increasing ethanol concentrations (vol/vol) (30%, 50%, 60%, 70%) each for 10 min.

(iii) Optional step: to enhance contrast, incubate the slices for 1 h in a 70% (vol/vol) ethanol solution containing 1% (wt/vol) uranyl acetate and 1% (vol/vol) phosphoric tungsten acid.

(iv) Remove the coverslips and dehydrate the slices in six additional dehydration steps of increasing ethanol concentrations (vol/vol) (70%, 80%, 90%, 95%, twice in 100%) each for 10 min.

(v) Subsequently, incubate the slices twice, for 2–5 min each time, in propylene oxide.

(vi) Flat-embed each brain slice in a Durcupan/propylene oxide mixture (the ratio is 1:1) for 30 min.

(vii) Transfer the slices into pure Durcupan for 12 h (or overnight).

(viii) The next day, mount the slices on microscope slides and embed them with Durcupan.

(ix) Dry the slices on a heater (~50 °C) and remove all excess Durcupan. Allow the slices to polymerize for 2 d at 60 °C.

■ **PAUSE POINT** Slices can be stored for prolonged periods of time (2 years or more) before EM processing.

(x) Process slices for EM as described previously[26,57,58,68,69].

LM analyses of Eukitt- and Moviol-embedded slices ● **TIMING** ≥**24 h**

28| If you intend to carry out computer-assisted reconstruction of neurons in Moviol- or Eukitt-embedded slices, proceed as described in **Box 2**. See also **Figures 3–6**.

? TROUBLESHOOTING

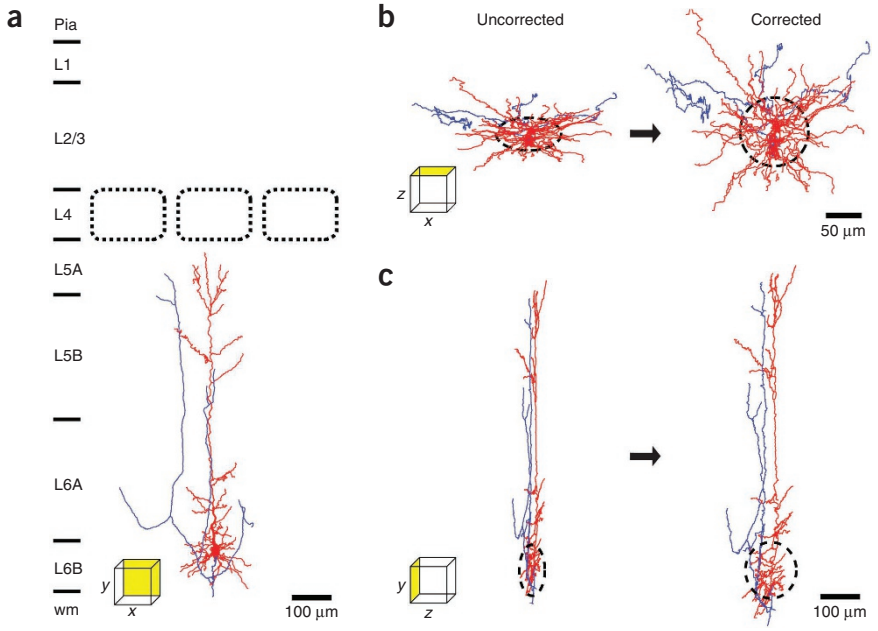

Figure 6 | Shrinkage correction of biocytin-labeled neurons in brain slices. (**a**) Three-dimensional reconstruction of a L6B pyramidal neuron of the barrel cortex shown in the *x*–*y* plane. (**b**,**c**) The same neuron is shown after rotation into the *y*–*z* plane (**b**) and into the *x*–*z* plane (**c**) before and after shrinkage correction. Dendrites are shown in red and axons in blue, respectively. The cubes indicate the direction of rotation. Neocortical barrel fields in layer 4 (**a**) are indicated by closed dashed lines. (**b**,**c**) Note that the shrinkage is particularly prominent in the *z* dimension and less strong in the *x* and *y* dimensions. In the case of pyramidal neurons, this results in an ellipsoid basal dendritic field (**b**; uncorrected reconstruction). To recover the original 3D cell morphology, a shrinkage correction was performed, leading to an almost circular dendritic field (**b**; corrected reconstruction) that is in accordance with observations from *in vivo* fillings. Pia, pia mater; wm, white matter.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	The viability of slices is poor and the fraction of dead neurons is high	Inadequate ACSF was used	For more mature animals (older than 30 postnatal days), use a sucrose-based slicing solution to improve neuronal survival
6	High background staining and understained neurons	Inappropriate patch pipettes were used during recording; biocytin spill	Adjust the tip size of the pipettes to soma size: use high-resistance pipettes (7–10 MΩ) for small somata (e.g., spiny stellates, <10 μm) and low-resistance patch pipettes (3–6 MΩ) for neurons with large somata (e.g., pyramidal neurons, ≥10 μm)
7	Incomplete staining of dendritic and axonal structures	Insufficient labeling time during recording	A labeling time of at least 15–20 min is required to obtain an adequate diffusion of biocytin and completely stained dendrites and axon
8	Resealing after biocytin filling is not achieved; increased background staining	The pipette size is too large	Reduce the pipette size (resistance of 7–10 MΩ) for cells with a diameter ≤10 μm. For cells with larger diameters, use low-resistance pipettes (3–6 MΩ)
11	The brain slices are not fully fixed	Inadequate tissue fixative solution was used	Be sure to remove all excess extracellular solution within the vials and check the composition of fixative solutions
15	High background staining (in particular, stained erythrocytes in blood vessels)	Incomplete quenching of the endogenous peroxidase activity (particularly common for immature tissue)	Increase incubation time in H_2O_2 solution; younger tissue needs longer quenching times than more mature tissue
24	The processed slices are very dark	The immunoreaction was stopped too late	Incubate slices only briefly in the DAB solution (~30–60 s). It is absolutely necessary that the chromogenic reaction is performed under permanent visual control and stopped as soon as the neuronal structures are clearly visible. Multiple washing steps are required to remove DAB entirely in order to stop the chromogenic reaction
	The stained neuron is only weakly visible	Insufficient filling or chromogenic reaction was stopped too early	Try a brief staining in PB containing 0.1% (wt/vol) OsO_4 to enhance contrast of the stained tissue. When the slice turns dark, remove it immediately from this solution and wash it repeatedly in PB
27A(i)	The gelatinized microscope slides for Eukitt embedding have a streaky surface and are not useful for embedding	Chromium chloride was not added to the gelatin solution	Add appropriate amounts of chromium chloride to the gelatin solution to obtain slides that are free of streaks
	The slices detach from slides during transfer, drying or dehydration	Gelatinization does not ensure adherence of slices	Increase the time of gelatinizing the slides, filter the gelatin solution more thoroughly to prevent contamination and check the temperature of the gelatin solution
27A(iii)	The tissue structure is ruptured	The tissue may be too dry	Control the humidity in the moist chamber and ensure that it is at least 80%

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
27A(v)	Neuronal structures have a corkscrew-like appearance	Slice dehydration was too fast or insufficient dehydration steps were performed	Perform dehydration with smaller increments (i.e., slowly increasing ethanol content by 10% or less), especially in the beginning of the dehydration process. Ensure that the slices remain for about 10 min within each ethanol beaker
28	Reconstruction of thin axons or dendrites is not possible	Thin dendritic and axonal processes are insufficiently or weakly stained or are located deep in the section	Increase the depth of field of the microscope by closing the aperture diaphragm
	No reconstruction at 100-fold magnification is possible (Eukitt)	Either Eukitt embedding medium or the coverslip is too thick	Use only one or two drops of Eukitt medium for a flat surface and use ultrathin coverslips to minimize the working distance. Try to reconstruct the cell at a lower magnification (×60, ×40) or (as a last resort) re-embed the slice in new Eukitt embedding medium
	Inaccurate 3D models and distortions in the *z* direction (Moviol)	The selected magnification for neuronal tracing was too high and the coverslips experience pressure. This will distort neuronal dimensions in the *z* direction, particularly those of deep structures	Reduce the magnification of the objective. If necessary, use either a ×60 oil-immersion objective or a normal ×40 objective to avoid pressure artifacts

● TIMING

Steps 1–6, brain dissection, acute slice preparation and recording: ~1.5 h
Steps 7–11, intracellular biocytin filling: 0.5–2 h
Step 12A, fixation for Eukitt/Moviol and LM: ~12 h
Step 12B, fixation for Durcupan embedding and EM: ~24 h
Steps 13–15, quenching of endogenous peroxidase activity: ~2 h
Step 16A, additional cytochrome oxidase histochemistry: ~3 h
Step 16B, cryoprotection for EM: approximately 2–3 h
Steps 17–21, ABC reaction: ~15 h
Steps 22–25, DAB reaction: ~1.5 h
Step 26 and **Box 1**, OsO_4 staining of brain slices for EM: ~1 h
Step 27A, Eukitt embedding method (LM): ~9 h
Step 27B, Moviol embedding method (LM): ~12 h
Step 27C, Durcupan embedding method (EM): 48–72 h
Step 28 and **Box 2**, morphological analysis and reconstruction (LM): ≥24 h

ANTICIPATED RESULTS

If the above-mentioned steps for biocytin-labeled neurons are followed, a reliable staining of neuronal structures for light and electron microscopic examination can be obtained. For LM analysis, we strongly recommend using Eukitt as the embedding medium because biocytin-labeled structures show a higher contrast. Furthermore, the lamination and cytoarchitectonic units of different brain regions are clearly visible without having to resort to additional staining protocols. When this improved embedding procedure is used in combination with a slow dehydration procedure with small increases in the ethanol concentration, corkscrew-like distortions of the dendritic and axonal arbors can be avoided. Finally, the correction factor for tissue shrinkage provided in this protocol will lead to the recovery of realistic neuronal geometries, thus leading to accurate 3D models of the neuronal structure and its dendritic and axonal projection pattern. These reconstructions can then be used for neuronal modeling and simulation studies.

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