Video Article

Large-scale Three-dimensional Imaging of Cellular Organization in the Mouse Neocortex

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Abstract

The mammalian neocortex is composed of many types of excitatory and inhibitory neurons, each with specific electrophysiological and biochemical properties, synaptic connections, and *in vivo* functions, but their basic functional and anatomical organization from cellular to network scale is poorly understood. Here we describe a method for the three-dimensional imaging of fluorescently-labeled neurons across large areas of the brain for the investigation of the cortical cellular organization. Specific types of neurons are labeled by the injection of fluorescent retrograde neuronal tracers or expression of fluorescent proteins in transgenic mice. Block brain samples, *e.g.,* a hemisphere, are prepared after fixation, made transparent with tissue clearing methods, and subjected to fluorescent immunolabeling of the specific cell types. Large areas are scanned using confocal or two-photon microscopes equipped with large working distance objectives and motorized stages. This method can resolve the periodic organization of the cell type-specific microcolumn functional modules in the mouse neocortex. The procedure can be useful for the study of three-dimensional cellular architecture in the diverse brain areas and other complex tissues.

Video Link

The video component of this article can be found at https://www.jove.com/video/58027/

Introduction

The mammalian neocortex is composed of a large number of cell types, each with the specific gene expression patterns, electrophysiological and biochemical properties, synaptic connections, and *in vivo* functions[1,2,3,4,5,6,7]. Whether these cell types are organized into repeated structures has been unclear. Cortical columns, including visual orientation columns and somatosensory barrels, have repeated structures, but their cellular organization remains unclear[8,9]. These are present in the specific cortical areas and are not a brain-wide system.

In neocortical layer 5, the large majority of neurons are classified into four major types. A major type of excitatory neurons, sub-cerebral projection neurons, projects axons to subcortical targets including the pons, spinal cord, and superior colliculus, and, therefore, represents the major cortical output pathway[10]. Cortical projection neurons, another major type of excitatory neurons, innervate the cortex[10]. Inhibitory neurons also contain two major classes: parvalbumin-expressing and somatostatin-expressing cells[11].

Recent analyses indicate that the four cell types are organized into repeated structures[12,13,14]. Both sub-cerebral projection neurons[12,13,14] and cortical projection neurons[14] organize into cell-type specific microcolumns with a diameter of 1–2 cells. Parvalbumin-expressing and somatostatin-expressing cells align specifically with microcolumns of sub-cerebral projection neurons but not with microcolumns of cortical projection neurons[14]. Microcolumns themselves periodically align to form a hexagonal lattice array[14] and are present in multiple cortical areas including visual, somatosensory, and motor areas in mouse brain[12,14] and in language areas of human brain[13]. Neurons in the individual microcolumn exhibit synchronized activity and have similar sensory responses[14]. These observations indicate that layer 5 cell types organize into a microcolumn lattice structure representing the first known brain-wide organization of repeating functional modules.

Microcolumns have a radius of approximately 10 µm and have a spatial periodicity of approximately 40 µm. In addition, the orientation of microcolumns is parallel to their apical dendrites and changes depending on their position in the cortex[14]. Therefore, the microcolumn system is difficult to analyze using conventional cortical slices with a typical thickness of a few tens of micrometers. In addition, the analysis of periodicity requires three-dimensional data from a wide-range of brain areas, and, therefore, the typical imaging area of confocal microscopy or *in vivo* 2-photon imaging is too narrow.

Recently, techniques have been developed to clear thick tissues[15,16]. Here we describe the application of these methods to obtain large-scale, three-dimensional images of the major cell types in mouse neocortical layer 5 that comprise the microcolumn system. Subcerebral projection neurons are labeled by the retrograde labeling or by the expression of the enhanced green fluorescent protein in *Crym-egfp* transgenic mice[12], and cortical projection neurons are labeled by either the retrograde labeling or by the tdTomato expression in *Tlx3-cre*/Ai9 mice[17]. Parvalbumin-

expressing and somatostatin-expressing cells are labeled by immunohistochemistry. The (Antibody Scale S) AbSca*l*e method[18] is used for the antibody staining experiments, while the (See Deep Brain) SeeDB method[19] is used for other experiments. These methods overcome the above-mentioned difficulties of the conventional imaging methods and reveal the accurate cellular organization of layer 5[14].

Protocol

All experimental procedures were approved by the RIKEN Wako Animal Experiments Committee and RIKEN Genetic Recombinant Experiment Safety Committee and performed according to the institutional guidelines of the animal facilities of the RIKEN Brain Science Institute.

1. Preparation of Imaging Chambers

1. **Imaging chamber**[19]
    1. Using silicone rubber sheets, prepare a chamber with a thickness of approximately 5 mm and floor plates of various thicknesses. Also, prepare Petri dishes with and without a glass bottom (**Figure 1A**).

2. **Slice spacer**
    1. Prepare spacers to hold samples, using silicone rubber sheets with a thickness of 0.5 mm (**Figure 1C**).

2. Tracer Injection

NOTE: Make injections into either the pons (2.1) or superior colliculus (2.2). Injection into the pons label sub-cerebral projection neurons in a wide brain region including the visual and motor areas, while injection into the superior colliculus labels sub-cerebral projection neurons in the visual area. For control experiments, inject saline instead of fluorescently-labeled cholera toxin subunit B. For the maintenance of sterile condition use sterilized equipment and plastic gloves cleaned with ethanol.

1. **Make injections into the pons of adult mice.**
    1. Draw 1 µL of fluorescently-labeled cholera toxin subunit B (25 µg/µL in PBS) into a 26G Hamilton syringe.
    2. Place an injector pump on the syringe.
    3. Place the syringe and pump on the tool holder of a manipulator placed on a stereotaxic instrument. Tilt the manipulator 12° posteriorly from the vertical axis.
    4. Anesthetize a male or female adult mouse (C57BL/6J or *Tlx3-cre*/Ai9) by injecting sodium pentobarbital intraperitoneally (60 mg/kg body weight) or by administering isoflurane (2–3%). Wait until the mouse makes no response when its tail is pinched with forceps, indicating that the mouse is fully anesthetized.
    5. Place the mouse on the stereotaxic instrument.
    6. Carefully remove hair using a razor blade to prevent infection and cut 10 mm of the scalp so that the bregma and lambda are visible. Administer 0.1 mL of 1% lidocaine using a pipette. Set the angle of the head by adjusting the vertical position of the mouthpiece on the stereotaxic instrument so that the bregma and lambda have the same z-level.
    7. Adjust the position of the manipulator by sliding it on the stereotaxic instrument so that the tip of the syringe is close to the bregma and record the position of the manipulator. Retract the syringe by moving the tool holder on the manipulator.
    8. Move the manipulator 5.4 mm posteriorly and 0.4 mm laterally. Advance the syringe so that the tip is close to the entry point on the skull. Retract the syringe and mark the entry point.
    9. At the marked position, drill a hole with a diameter of approximately 1 mm.
    10. Insert the syringe tip through the hole so that the tip depth is 6.9 mm more than that measured at the bregma.
    11. Inject 1 µL of tracers using the pump at 0.2 µL/min.
    12. Remove the syringe from the brain.
    13. If necessary, cover the exposed brain with small fragments of microfibrillar hemostat and instant adhesive.
    14. Rinse the exposed brain using saline delivered with a pipette to prevent infection and suture the scalp.
    15. Remove the mouse from the stereotaxic instrument. Allow the mouse to recover from anesthesia in an incubator at 30 ˚C, typically for 1 h. Do not leave the mouse unattended until it has regained sufficient consciousness to maintain sternal recumbency. Return the mouse to the company of other animals after it has fully recovered.
    16. Maintain the mouse for 3–7 days.

2. **Make injections into the superior colliculus of adult mice.**
    1. Prepare a glass pipette with a tip diameter of 30–50 µm.
    2. Connect the glass pipette to a Hamilton syringe through a plastic tube (**Figure 2A**).
    3. Fill the glass pipette, plastic tube, and Hamilton syringe with the paraffin liquid.
    4. Place an injector pump on the syringe.
    5. Place the glass pipette on the manipulator and tilt the manipulator 60° posteriorly from the vertical axis (**Figure 2B**).
    6. Perform 2.1.4–2.1.9. The position of the manipulator is 1.4 mm posterior, 0.5 mm lateral to the lambda.
    7. Place a small plastic paraffin film on the skull, then put approximately 1 µL of the tracer solution on it. Quickly advance the glass pipette and fill it with at least 0.5 µL of the tracer solution.
    8. Insert the glass pipette so that the tip depth is 3.0 mm from the brain surface.
    9. Inject 0.5 µL of tracers using the pump at 0.2 µL/min.
    10. Remove the glass pipette from the brain.
    11. Perform 2.1.13–2.1.16.

3. Fixation and Trimming

1. Inject sodium pentobarbital (60 mg/kg body weight) intraperitoneally into a mouse (C57BL/6J or *Tlx3-cre*/Ai9, with or without the tracer injection as described in step 2. Wait until the mouse makes no response when its tail is pinched with forceps, indicating that the mouse is fully anesthetized.
2. Euthanize the mouse humanely by perfusing the mouse transcardially[20] with 0.9% saline.
3. Fix the mouse[20] by perfusing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.5).
4. **Cut the scalp using a pair of scissors to expose the skull as described[20].**
    1. Cut the midline of the exposed skull using a pair of scissors. Remove the skull using forceps.
    2. If marking the position of the bregma and lambda is necessary, first remove one hemisphere of the skull. Insert thin tungsten needles into the brain at the positions of the bregma and lambda on the skull remaining on the brain, then remove the remaining skull.
    NOTE: The brain can be stored in PBS at 4 ˚C.
5. **To perform antibody staining of inhibitory neurons, cut the brain samples into slices.**
    1. Place the brain sample on a vibratome.
    2. Cut slices up to 500 µm thick in PBS at room temperature and proceed to step 5 (the AbSca*l*e method).
6. If antibody staining is unnecessary, trim the brain sample to blocks (up to 3 mm thick) using a razor blade (**Figure 3**) and proceed to step 4 (the SeeDB method).
NOTE: The brain can be stored in PBS at 4 °C after cutting or trimming. The SeeDB method is preferable when antibody staining is not necessary because it requires less time than the AbSca*l*e method.

4. Clearing without Antibody Staining (the SeeDB Method)

1. Transfer the sample using a spatula to a 50 mL plastic tube containing 20 mL of 0.5% α-thioglycerol and 20% (w/v) fructose and incubate it for 4 h with gentle shaking at room temperature.
2. Transfer the sample using a spatula to a 50 mL plastic tube containing 20 mL of 0.5% α-thioglycerol and 40% (w/v) fructose and incubate it for 4 h with gentle shaking at room temperature.
3. Transfer the sample using a spatula to a 50 mL plastic tube containing 20 mL of 0.5% α-thioglycerol and 60% (w/v) fructose and incubate it for 4 h with gentle shaking at room temperature.
4. Transfer the sample using a spatula to a 50 mL plastic tube containing 20 mL of 0.5% α-thioglycerol and 80% (w/v) fructose and incubate it for 12 h with gentle shaking at room temperature.
5. Transfer the sample using a spatula to a 50 mL plastic tube containing 20 mL of 0.5% α-thioglycerol and 100% (w/v) fructose and incubate it for 12 h with gentle shaking at room temperature.
6. Transfer the sample using a spatula to a 50 mL plastic tube containing 20 mL of 0.5% α-thioglycerol and 80.2% (w/w) fructose and incubate it for 24 h with gentle shaking at room temperature.
NOTE: Handle the sample carefully to keep deformations as small as possible. Do not incubate samples longer than indicated, as samples can quickly become opaque.
7. Embed the sample in an imaging chamber filled with the 80.2% fructose solution (**Figure 1B**). If necessary, fix the sample by putting small pieces of rubber adhesive around. If the chamber is too deep, put a floor plate in the chamber before placing the samples.
8. Place the Petri dish with a glass cover on the imaging chamber and put water in the dish, and image using confocal or two-photon microscopy with a water-immersion long working distance objective. Excitation wavelengths and emission filters are described in **Table 1**. If necessary, use a motorized stage.

5. Clearing with Antibody Staining (the AbSca*l*e Method)

1. Prepare reagents as described in **Table 2**.
2. Transfer the slices using a spatula to a 5 mL plastic tube containing 4 mL of Sca/eS0 solution and incubate them for 12 h with gentle shaking at 37 °C (**Figure 4B**).
3. Remove the solution from the tube using a pipette and add 4 mL of Sca/eA2 solution, and incubate the slices for 36 h with gentle shaking at 37 °C.
4. Remove the solution from the tube using a pipette and add 4 mL of Sca/eB4 solution, and incubate the slices for 24 h with gentle shaking at 37 °C.
5. Remove the solution from the tube using a pipette and add 4 mL of Sca/eA2 solution, and incubate the slices for 12 h with gentle shaking at 37 °C.
6. Remove the solution from the tube using a pipette and add 4 mL of PBS and incubate the slices for 6 h with gentle shaking at room temperature.
7. Carefully remove the slices to a 2 mL plastic tube using a spatula.
8. Incubate with primary antibodies (**Table 3**) in 1 mL of AbSca*l*e solution for 48-72 h at 37 °C (**Figure 4C**) with gentle shaking.
9. Carefully remove the slices to a 5 mL plastic tube using a spatula.
10. Incubate in 4 mL of AbSca*l*e solution for 2 h 2 times at room temperature with gentle shaking.
11. Carefully remove the slices to a 2 mL plastic tube using a spatula.
12. Incubate with fluorescently-labeled secondary antibodies (**Table of Materials**, 1:100) in 1 mL of AbSca*l*e solution for 48 h at 37 °C with gentle shaking.
13. Carefully remove the slices using a spatula to a 5 mL plastic tube containing 4 mL of the AbSca*l*e solution and incubate the slices for 6 h with gentle shaking at room temperature.
14. Remove the solution from the tube using a pipette and add 4 mL of the AbSca*l*e solution and incubate the slices for 2 h 2 times with gentle shaking at room temperature.

15. Remove the solution from the tube using a pipette and add 4 mL of 4% PFA and incubate the slices for 1 h with gentle shaking at room temperature.
16. Remove the solution from the tube using a pipette and add 4 mL of PBS and incubate the slices for 1 h with gentle shaking at room temperature.
17. Remove the solution from the tube using a pipette and add 4 mL of the Sca/eS4 solution, and incubate the slices for 12 h with gentle shaking at 37 °C.
18. Place the spacer on a glass slide and place the slices in the spacer and immerse the slices with Sca/eS4 solution. Seal the spacer with a cover glass (**Figure 1D**).
19. Put water on the cover glass and image using confocal or two-photon microscopy with a water-immersion long working distance objective. If necessary, use a motorized stage.
    NOTE: Check whether the deep parts of the sample are labeled similarly to the superficial parts to confirm the absence of a significant labeling bias.
    NOTE: Penetration of the tested antibodies is described in **Table 3**.

6. Cell Position Determination

1. For each position in the scanned images, calculate correlation values using a three-dimensional image filter[14] (**Figure 5A-5D**).
2. Determine the positions of the peaks of the correlation value (**Figure 5D**).
3. Investigate images around the peaks to locate cells (**Figure 5E**).

Representative Results

We labeled cortical projection neurons by expression of tdTomato in *Tlx3-cre*/Ai9 transgenic mice and visualized sub-cerebral projection neurons by injecting the retrograde tracer CTB488 into the pons. The left hemisphere of the brain was subjected to the SeeDB method and scanned using a two-photon microscope equipped with a water-immersion long working distance objective (25X, N.A. 1.1, working distance 2 mm) and a motorized stage. A stack of 401 images (512 x 512 pixels; pixel size = 0.99 μm) at z-step = 2.8 μm at each of 30 scanning positions was obtained. The cell bodies of the two cell types were visible over a wide range of brain areas (**Figure 6**), and optical sections showed periodic microcolumns (**Figure 7A**). In addition, the brains of *Crym-egfp* mice (maintained as heterozygotes with a C57BL/6J background) were cleared by the SeeDB method and imaged as above. The cell bodies and apical dendrites of EGFP-expressing sub-cerebral projection neurons were visible in optical sections (**Figure 7B**).

We also performed retrograde labeling of sub-cerebral projection neurons in C57BL/6J mice using CTB488. The fixed brain was cut into coronal slices with a thickness of approximately 500 μm and subjected to the AbSca*l*e method to label parvalbumin-expressing cells (Anti-parvalbumin, 1:1000; Anti-mouse IgG-fluorescently labeled,). Stack images (512 x 512 pixels, pixel size = 1.24 μm; z-step = 2.17 μm, 268 images/stack) were obtained using confocal microscopy with a water-immersion long working distance objective (20X, N.A. 1.0, working distance 2 mm). Sub-cerebral projection neurons and parvalbumin-expressing cells were both visible in the slices (**Figure 7C**).

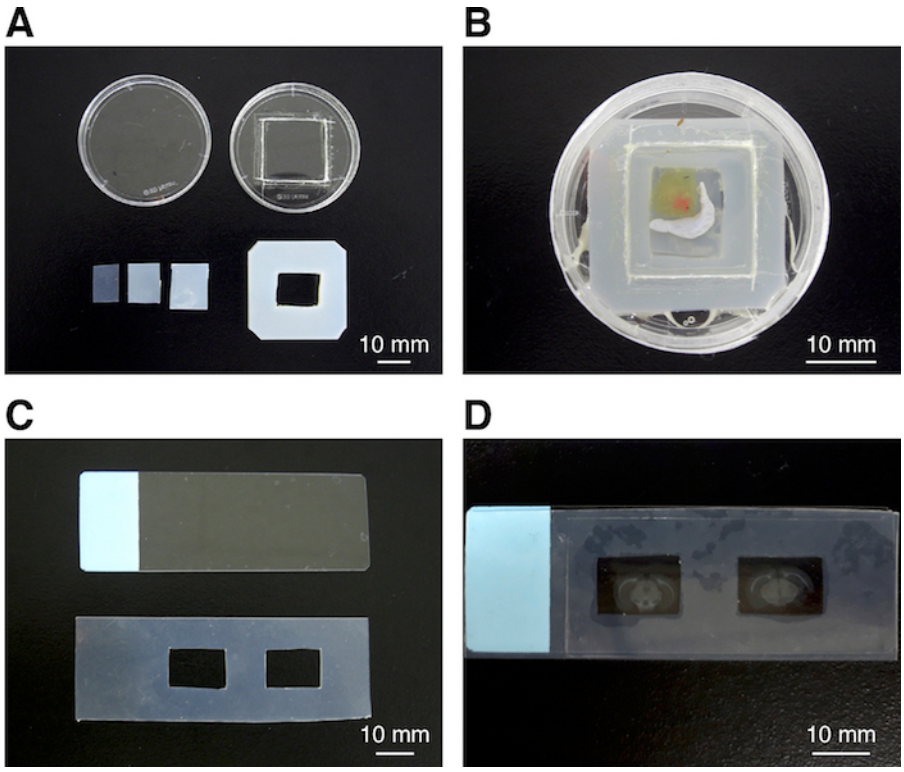

Figure 1: Imaging chambers and spacers. (**A**) Top: Handmade glass-bottomed Petri dish (right) and a Petri dish without a glass bottom (left). Bottom: A chamber (right) and floor plates (left) made of silicone sheets. (**B**) A mouse hemisphere subjected to the SeeDB method after injection of CTB555 into the superior colliculus is set into the chamber. The sample is fixed with a piece of reusable adhesive. (**C**) A glass slide (top) and a spacer made of a silicone sheet with a thickness of 0.5 mm (bottom). (**D**) Two coronal slices of the mouse brain were set into the spacer and covered with a cover glass. Please click here to view a larger version of this figure.

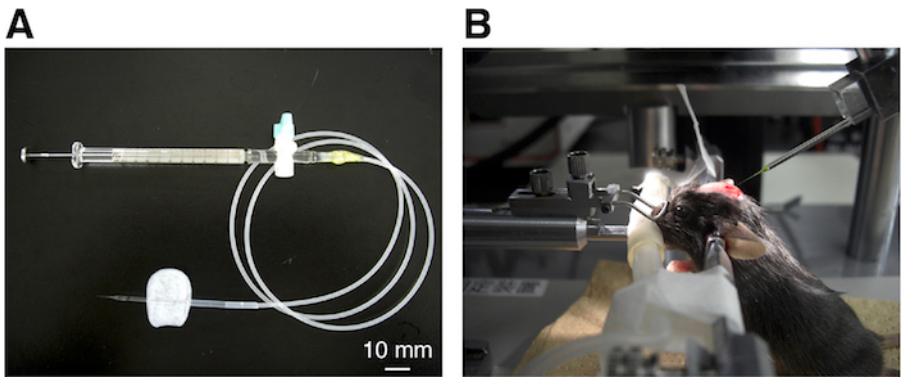

Figure 2: Tracer injection. (**A**) A glass pipette connected to a Hamilton syringe through a plastic tube. (**B**) A mouse is placed on a stereotaxic instrument. The glass pipette is tilted approximately 60° posteriorly from the vertical axis for injection into the superior colliculus. Please click here to view a larger version of this figure.

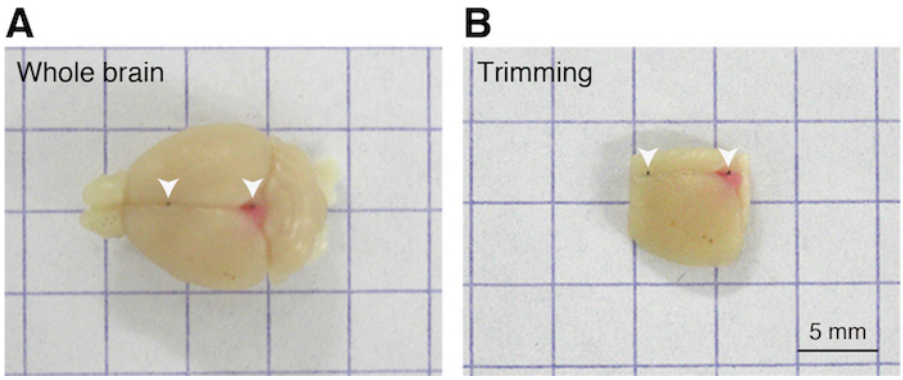

Figure 3: Trimming of brain samples. (**A**) Whole brain sample after injection of CTB555 into the superior colliculus and fixation. The bregma and lambda were marked with tungsten needles (arrowheads). (**B**) The same sample trimmed to obtain a hemisphere. Note that the tungsten needles remain (arrowheads). Please click here to view a larger version of this figure.

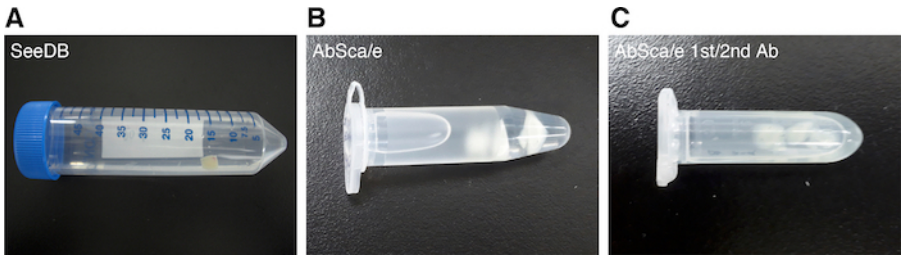

Figure 4:. Immersion of brain samples in solutions for the SeeDB and AbSca/e methods. (**A**) A hemisphere sample immersed in 0.5% α-thioglycerol and 20% (w/v) fructose for the SeeDB method. (**B**) Coronal slices immersed in Sca/eA2 solution for the AbSca/e method. (**C**) Coronal slices immersed in AbSca/e solution containing antibodies for the AbSca/e method. Please click here to view a larger version of this figure.

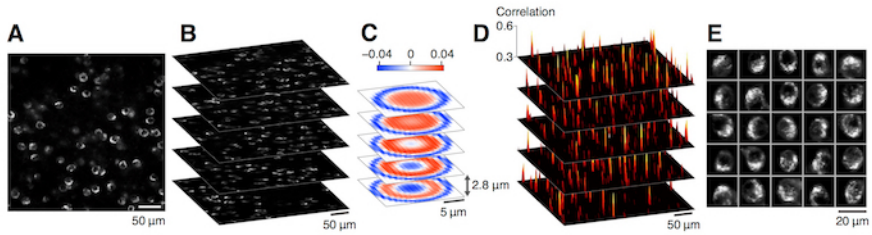

Figure 5: Determination of cell positions. Sub-cerebral projection neurons were labeled by injecting the retrograde tracer CTB488 into the pons at 5 weeks of age. The left hemisphere was subjected to the SeeDB method and scanned with two-photon microscopy (512 x 512 pixels, pixel size = 0.99 µm; z-step = 2.8 µm, 601 images/stack). (**A**) A single image. (**B**) Five images from a single image stack. (**C**) The image filter used to detect CTB-labeled sub-cerebral projection neurons. (**D**) Correlation values calculated for the five images in (B) using the filter in (C). (**E**) Examples of detected sub-cerebral projection neurons. Please click here to view a larger version of this figure.

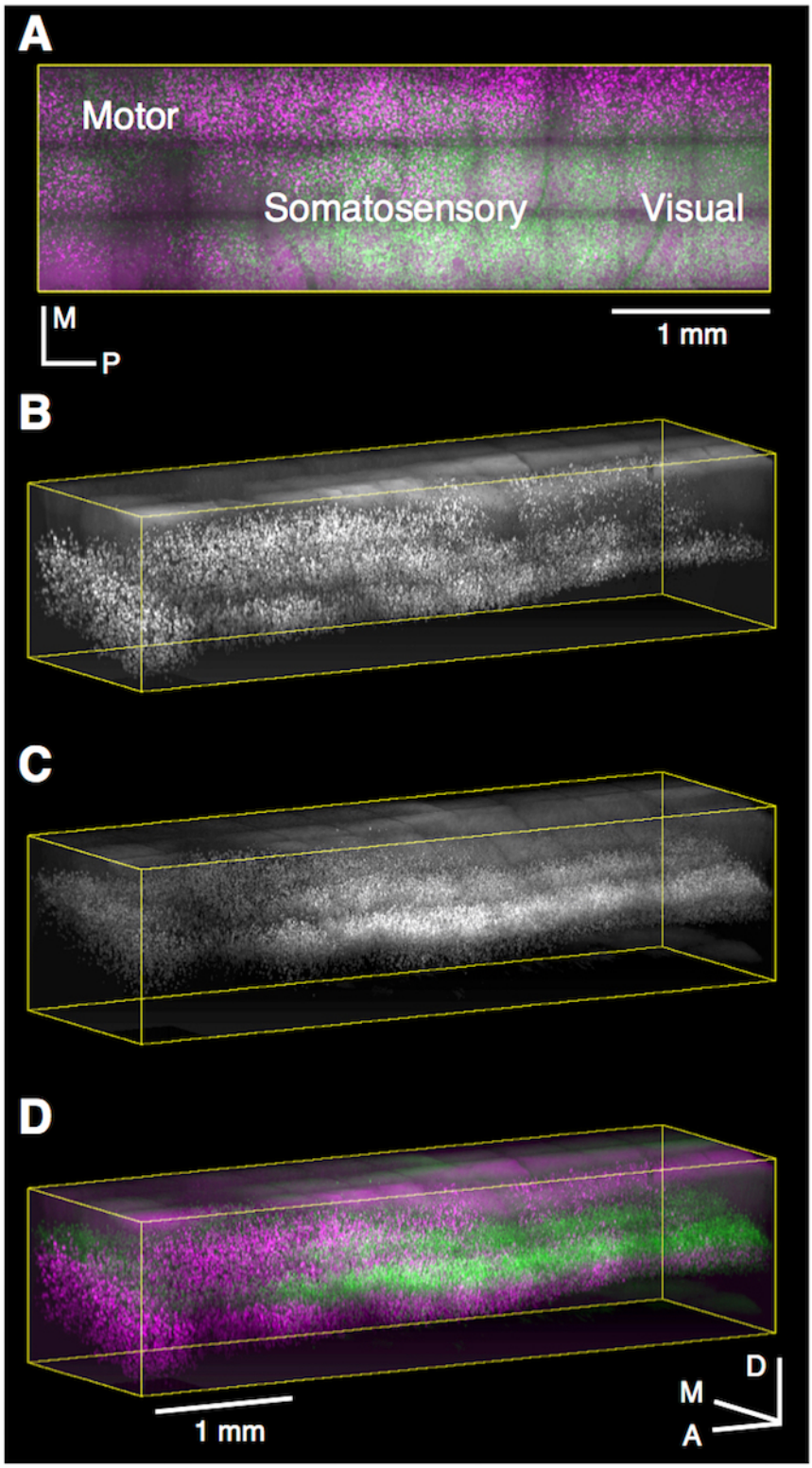

Figure 6: Representative results of large-scale imaging. Cortical projection neurons (green) were labeled by tdTomato-expression in *Tlx3-cre*/Ai9 transgenic mice, and sub-cerebral projection neurons (magenta) were visualized by injecting the retrograde tracer CTB488 into the pons at 7 weeks of age. The left hemisphere was subjected to the SeeDB method and the area 1,250 µm to 2,690 µm lateral and -3,400 µm to 1,230 µm anterior to the bregma was scanned using two-photon microscopy. (**A**) Top view. Approximate positions of cortical areas were shown. (**B-D**) Oblique view of CTB 488 fluorescence showing sub-cerebral projection neurons (B), tdTomato fluorescence showing cortical projection neurons (C), and the merged image (D). A: Anterior; P: Posterior; M: Medial; D: Dorsal. Please click here to view a larger version of this figure.

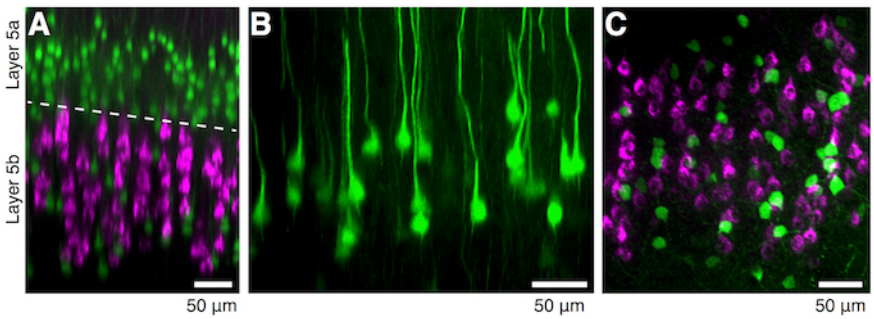

Figure 7: High magnification photographs of representative results. (**A**) An optical section of the image in **Figure 6D**. Image thickness = 20 µm. (**B**) EGFP-labeled sub-cerebral projection neurons in a heterozygous *Crym-egfp* mouse at 6 weeks of age. Image thickness = 30 µm. (**C**) Subcerebral projection neurons (magenta) were labeled by injecting CTB488 into the pons of C57BL/6J mice at postnatal day 49, and parvalbumin-expressing cells (green) were visualized by the AbSca*l*e method. Image thickness = 55 µm. Please click here to view a larger version of this figure.

Fluorophore	Ex. (nm)	Filter (nm)
EGFP	910	500-550 (FF03-525/50-25, Semrock)
tdTomato	1,000	578-633 (D605/55m,Chroma)
Alexa Fluor 488	750	500-550 (FF03-525/50-25, Semrock)
Alexa Fluor 555	750	563-588 (FF03-575/25-25,Semrock)
Alexa Fluor 594	750	601-657 (FF01-629/56, Semrock)
DAPI	700	400-480 (FF01-492/SP-25, Semrock)

Table 1: Excitation wavelengths and filters for two-photon microscopy.

Table 2: Reagents for the AbSca*l*e method. Please click here to download this file.

Antigen	Immunized animal	Product ID	Concentration	3 mm block	500 µm slice
NeuN	Mouse	MAB377	1:500	ND	+
NeuN	Rabbit	ABN78	1:500	ND	+
CTIP2	Rat	ab18465	1:100	L1-L6	+
Statb2	Mouse	ab51502	1:100	-	-
GAD67	Mouse	MAB5406	1:200	ND	+
GABA	Rabbit	A2052	1:100	-	-
Parvalbumin	Mouse	235	1:1000	ND	+
Parvalbumin	Goat	PV-Go-Af460	1:100	L1-L2/3	+
Parvalbumin	Mouse	P3088	1:1000	ND	+
Parvalbumin	Rabbit	ab11427	1:500	ND	-
Somatostatin	Rabbit	T-4103	1:1000	L1-L2/3	+
c-Fos	Rabbit	PC38	1:1000	ND	+

Table 3: Penetration of the tested antibodies. Results for 3 mm-thick blocks is shown as follows; L1-L6: penetration into the whole cortical thickness; L1-L2/3: penetration down to layer 2/3; -: no labeling; ND: not determined. Results for 500 µm-thick slices is shown as follows; +: uniform labeling; -: no or limited labeling.

Discussion

We have presented procedures to obtain large-scale three-dimensional images of the cell type-specific organization of the major cell types in mouse neocortical layer 5. Compared to the conventional slice staining, the method is more useful in determining the three-dimensional organization of the neocortex. The method enables image acquisition from the wider and the deeper brain regions compared to the typical *in vivo* 2-photon microscopy or conventional confocal microscopy and, thus, can allow the comprehensive analysis of the neocortical cellular organization.

A critical step of the method is the antibody penetration. A subset of antibodies shows poor penetration into thick specimens (**Table 3**), and, therefore, cannot be used for the AbSca*l*e method. To obtain uniform labeling with the antibodies used in this study, it was necessary to cut the

brain into 500 µm slices. The use of smaller antibody fragments, *e.g.,* Fab or F(ab')2 fragments, and/or other clearing methods[21,22,23,24,25,26,27,28] may improve the results.

Antibody antigen-binding specificity is usually characterized in thin tissue slices but might be different in thick tissues processed for clearing. To control for antibody specificity, we co-labeled tissues with tracer injection and marker gene expression in transgenic animals and also performed blocking experiments using antigen proteins and peptides[14]. These procedures and control experiments using mutant animals lacking the target antigens should be useful for confirming the specificity of antibodies.

A limitation of the method is that some deformation of the specimen is inevitable due to the handling of soft bulk samples. Obtaining *in vivo* images prior to fixation and using these images as references will help to correct such deformations.

The present procedures were designed for the analysis of neocortical layer 5. Recent analyses have identified many molecular markers that label neuronal cell types in other layers[4,5,6,7], and the application of these makers to the present method may enable identification of important cellular organization in other layers. In addition, it should be possible to perform similar analyses in brain regions other than the neocortex and in organs other than the brain, to investigate whether a precise cellular organization similar to microcolumns is present.

Disclosures

The authors have nothing to disclose.

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