Supporting Information

Zhou et al. 10.1073/pnas.1310233110

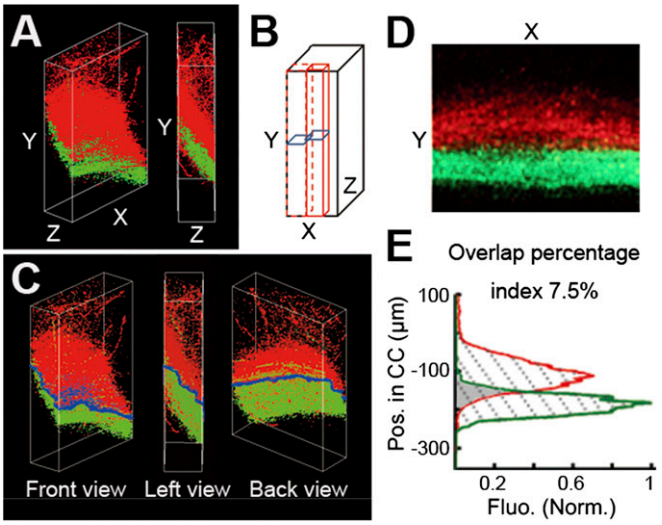


Fig. S1. Analysis of axon segregation. (*A*) Rescaled image in two different views. (*B*) The corpus callosum (CC) was divided into square columns along the dorsal–ventral (D–V) (*y*) axis. (*C*) The segregation surface for red and green axons within each square column is depicted in blue and is shown in three different views. (*D*) The fluorescence intensities along the anterior–posterior (*z*) axis were summed after the image within each column was realigned and shifted along the *y*-axis to flatten the segregation surface into a plane. (*E*) The fluorescence intensities along the *x*-axis then were summed to obtain the fluorescence profile along the D–V (*y*) axis. Fluo.(Norm), normalized fluorescence; Pos., position.

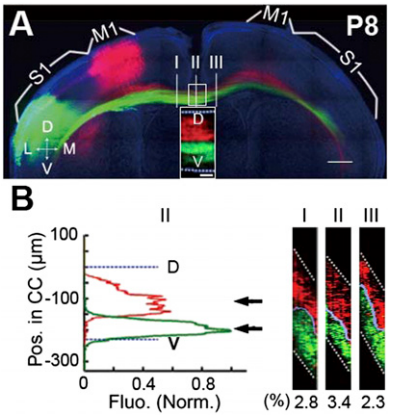


Fig. S2. Axons from two distinct M1 and S1 cortical regions showed a lower percentage of overlap near the midline. (*A*) A 200-μm coronal section from a P8 mouse brain with M1 and S1 neurons labeled with mCherry and EGFP, respectively. Segregation of axons at three different locations (marked I, II, III) were analyzed before and after midline crossing. (Scale bar, 500 μm.) (*Inset*) Projection of a realigned image of axons for section II in *A* (scale bar, 50 μm) for quantitative analysis of axon segregation within the CC (*Materials and Methods*). Dashed white lines indicate dorsal and ventral surfaces of the CC. (*B*) (*Left*) Fluorescence profiles of labeled axons at section II at various positions along the D–V axis of the CC. The profile represents the normalized fluorescence intensity of axons (*Materials and Methods*). The percentage of overlap between two populations of axons was calculated as described in *Materials and Methods*. Arrows indicate the position of the mean fluorescence intensity. (*Right*) A sagittal view of three sections before and after midline crossing in the CC (thickness: 92–139 μm), with percentages of overlap shown below. D, dorsal; L, lateral; M, medial; V, ventral.

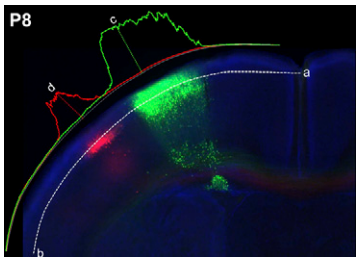


Fig. S3. Analysis of the average somal position of labeled neurons. A 200-μm coronal section from a P8 mouse brain with EGFP and mCherry labeling neurons of two adjacent cortical regions. The white dashed line (drawn with ImageJ software) crosses the layer II/III cortical regions along the cortical surface from medial to lateral, starting from midline (a) and ending at the position (b) where the cortical surface is parallel to the midline. In practice, the line width was 300 pixels including most of the layer II/III cortical regions. Plots of the green and red fluorescence along the path are shown above the cortex. The thin white line below the profiles represents the base line. The green (c) and red (d) dashed lines indicate the center of mass for green and red fluorescence profiles, respectively. The arc distance from the midline to the center of mass (i.e., to c and d, respectively) was defined as the averaged somal position of labeled axons. The arc distance from c to d represents the averaged somal distance between the two populations of labeled neurons. The averaged somal position and averaged somal distance were normalized further by dividing the arc distance from a to b, which varied in the different cortical sections and was used to eliminate individual difference.

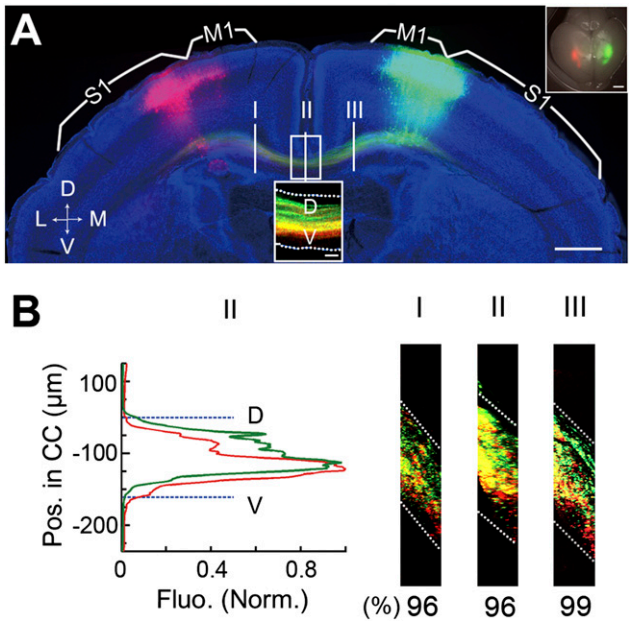


Fig. S4. Axons from homotopic regions of both hemispheres are colocalized within the same region of the CC. (*A*) A 200-μm coronal section from a P8 mouse brain with homotopic regions of each hemisphere labeled with mCherry and EGFP, respectively. Segregation of axons at three different locations (marked I, II, III) was analyzed before and after midline crossing. (Scale bar, 500 μm.) (*Inset Lower Center*) Higher-resolution realigned image of CC axons in section II used for quantitative analysis of axon segregation within the CC (*Materials and Methods*). (Scale bar, 50 μm.) Dotted white lines indicate dorsal and ventral surfaces of the CC. (*Inset Upper Right*) Image of the whole-mount cortex. (Scale bar, 500 μm.) (*B*) (*Left*) Fluorescence profiles of labeled axons in the area marked as box II in A projected at the same location along the D–V axis of the CC. The profile represents the normalized fluorescence intensity of CC axons (*Materials and Methods*). The percentage of overlap between two populations of axons was calculated as described in *Materials and Methods*. (*Right*) Sagittal view of three sections, with the percentage of overlap shown below.

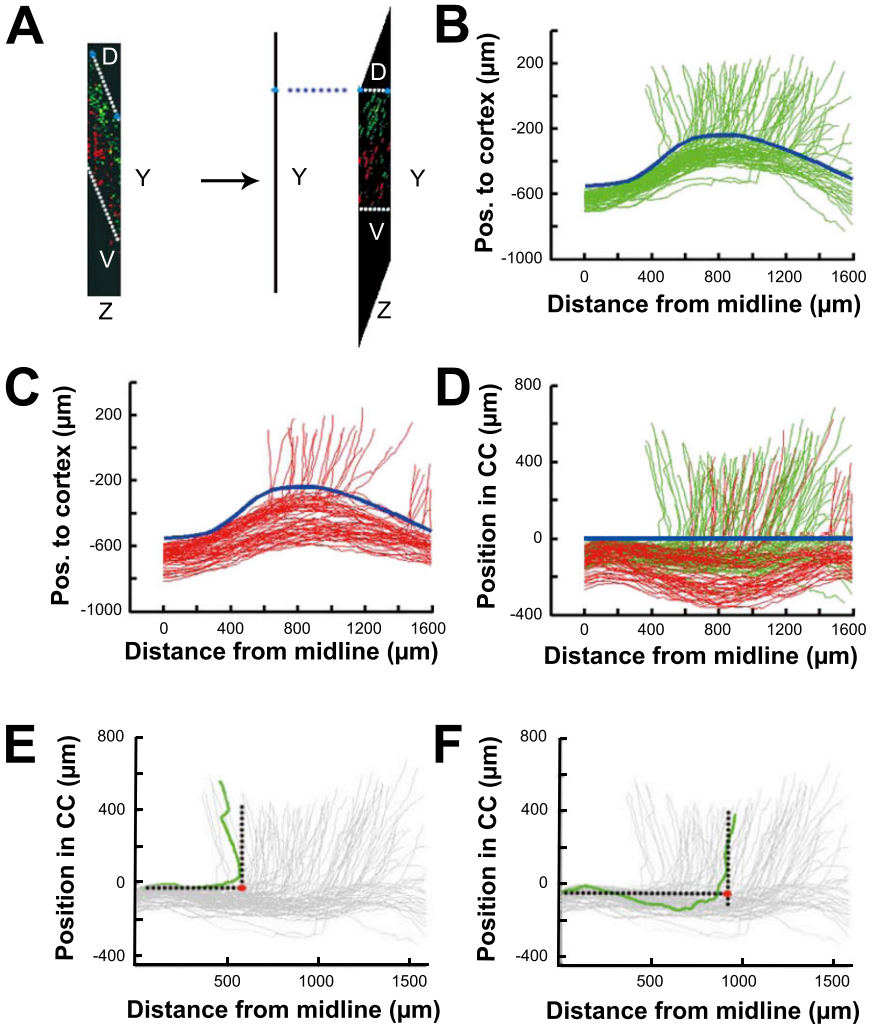


Fig. S5. Analysis of tracing data. (*A*) (*Left*) Sagittal view of axons within the CC at the midline. White dashed lines represent the dorsal and ventral CC surfaces. The blue dots represent the dorsal CC border (also visible in *B*–*D*). (*Right*) To compare axon tracings in 3D, we adjusted the dorsal CC surface along the rostral–caudal (*z*) axis to a plane; thus the two blue lines overlapped when projected in the coronal (*x*–*y*) plane. (*B* and *C*) All axon tracings in 3D were projected onto a coronal plane. Blue lines represent the adjusted dorsal surface of the CC. The starting position of the axon at the midline was defined as position 0 of the x-axis. (*D*) To determine the distance from the midline, the CC border was further straightened to a line (blue line in *D*), so the positions of axons along the medial–lateral axis could be compared. The adjusted CC border was defined as position 0 of the *y*-axis. The starting position of the axon at the midline was defined as position 0 of the *x*-axis. (*E* and *F*) The turning point of the axon was defined by the intersection point (red circle) of the two lines representing the axon's original direction and projected direction. The axon's D–V position was its starting point at the midline.

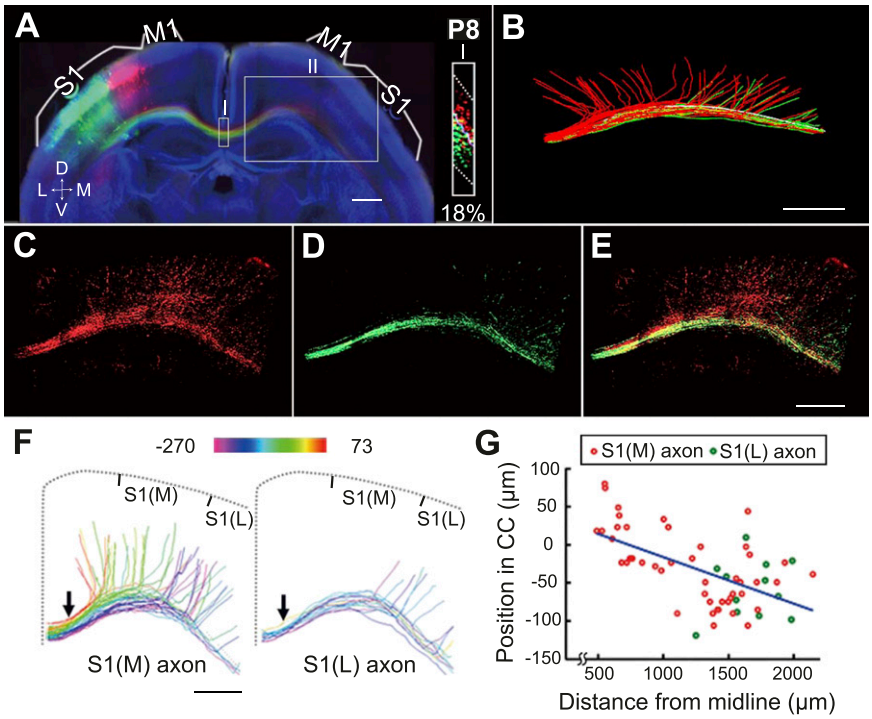


Fig. S6. Dorsally located axons in CC project medially after midline crossing. (*A*) A coronal section from a P8 mouse brain double-labeled with mCherry and EGFP in two adjacent S1 regions. (*Inset*) High-resolution image of a sagittal view of area of box I (percentage of overlap, 18%). (*B*) Tracing of axon projections to the contralateral cortex (from midline to S1, box II in *A*). The white line indicates the dorsal surface of the CC. (*C–E*) High-magnification images of box II in *A*. (Scale bars, 500 μm for all panels.) (*F*) Axons from M1 and S1 were color-coded by their positions within the CC (at the locations marked by arrows). The color scale shows the coding of D–V position linearly from −270 to 73 μm. Position 0 is defined by the CC boundary, as revealed by Hoechst staining (*Materials and Methods*). (*G*) Correlation between the axon's D–V position in the CC (the location of axon's starting point at the midline) and the axon's turning point along the CC (defined by the distance from the midline). See Fig. S5 and *Materials and Methods* for a detailed description. $R^2 = 0.33$, $P = 2 \times 10^{-6}$. S1(L), lateral region of S1; S1(M), medial region of S1.

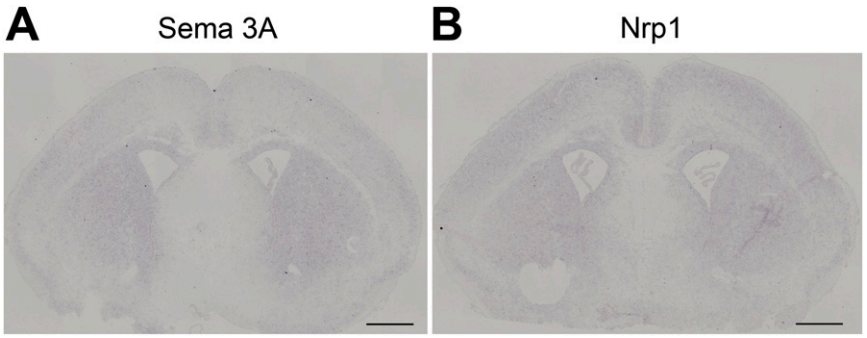


Fig. S7. Hybridization signals were specific for semaphorin3A (Sema3A) and neuropilin-1 (Nrp1). Coronal brain sections (12 μm) from P0 WT mice, showing in situ hybridization of sense probes for Sema3A (*A*) and Nrp1 (*B*) mRNAs obtained under the incubation conditions shown in Fig. 3. No signals were observed compared with anti-sense probes (Fig. 3). (Scale bars, 500 μm.)

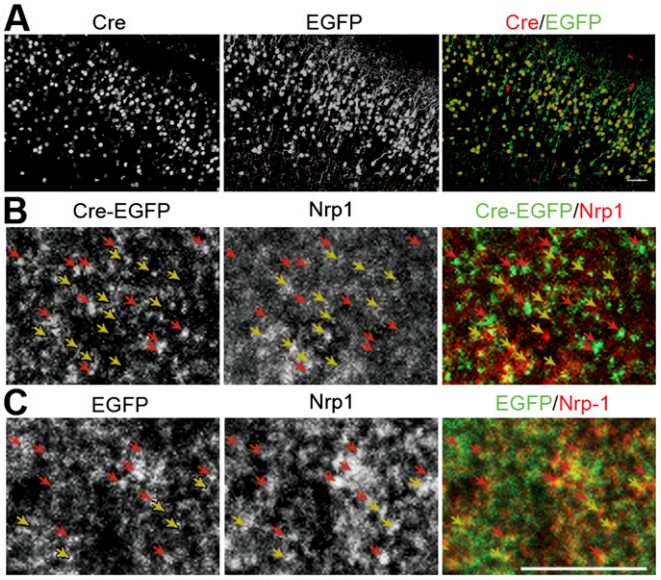


Fig. S8. Effectiveness and specificity of Cre-recombinase (Cre) expression in vivo. (*A*) A 50-μm coronal brain section from a P8 *Nrp1*$^{M1-/-}$ mouse with Cre and EGFP labeling of M1. Immunostaining for Cre and EGFP showed colocalization in cell bodies, indicating the effectiveness of Cre expression in vivo. (Scale bar, 50 μm.) (*B*) Coronal sections (12-μm) from P3 *Nrp1*$^{M1-/-}$mice in which CC axons were immunostained with EGFP and Nrp1 in M1. The immunostaining of EGFP showed the Cre and EGFP expression in M1. Most axons with EGFP staining showed low levels of Nrp1 staining (red arrows; positions are the same in all three images). Most axons with Nrp1 staining showed low levels of EGFP staining (yellow arrows). (*C*) Coronal sections (12-μm) from P3 floxed *Nrp1* mice with EGFP expression only in M1. Data are presented as in *B*. EGFP-stained axons also showed strong Nrp1 staining. (Scale bar, 5 μm for *B* and *C*.)

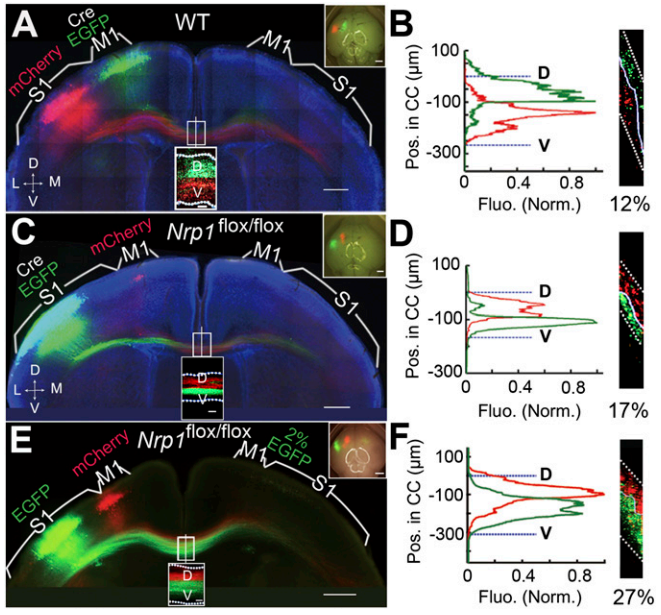


Fig. S9. The disruption of axon order was specific for Nrp1$^{M1-/-}$ mouse brains. (*A* and *B*) Coronal sections (200-μm) from P8 *Nrp1* $^{WT/WT}$ mice with Cre and EGFP expression in M1 and mCherry expression in S1 presented as in Fig. S4 *A* and *B*. The D–V order within the CC was normal. (*C* and *D*) A 200-μm coronal section from a P8 *Nrp1* $^{flox/flox}$ mouse with mCherry expression in M1 and Cre/EGFP in S1. Data are presented as in *A* and *B*. No disruption of axonal order was observed in the CC. (*E* and *F*) A 200-μm coronal section from a P8 *Nrp1*$^{M1-/-}$ mouse expressing mCherry in M1 and EGFP in S1 in one hemisphere and the empty vector together with the low-level expression of EGFP (that labeled the cell bodies but not the axons) in the contralateral homotopic M1. Data are presented as in *A* and *B*. (Scale bars, 500 μm in *A*, *C*, and *E*.)

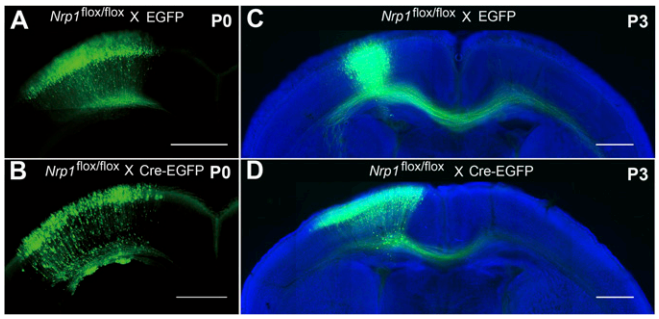


Fig. S10. Sema3A/Nrp1 signaling does not affect axon guidance. (*A* and *B*) Two coronal sections from P0 *Nrp1*$^{flox/flox}$ mice expressing EGFP (*A*) and Cre/EGFP (*B*) in the cortex. (*A*) Substantial axon projections entered CC tract, as in WT mice. (*B*) Some migration defects were observed in Nrp1 conditional KO mice, (*C* and *D*) Substantial CC axon growth was observed at P3 in both WT (*C*) and Nrp1 conditional KO (*D*) mice. (Scale bars, 500 μm.)

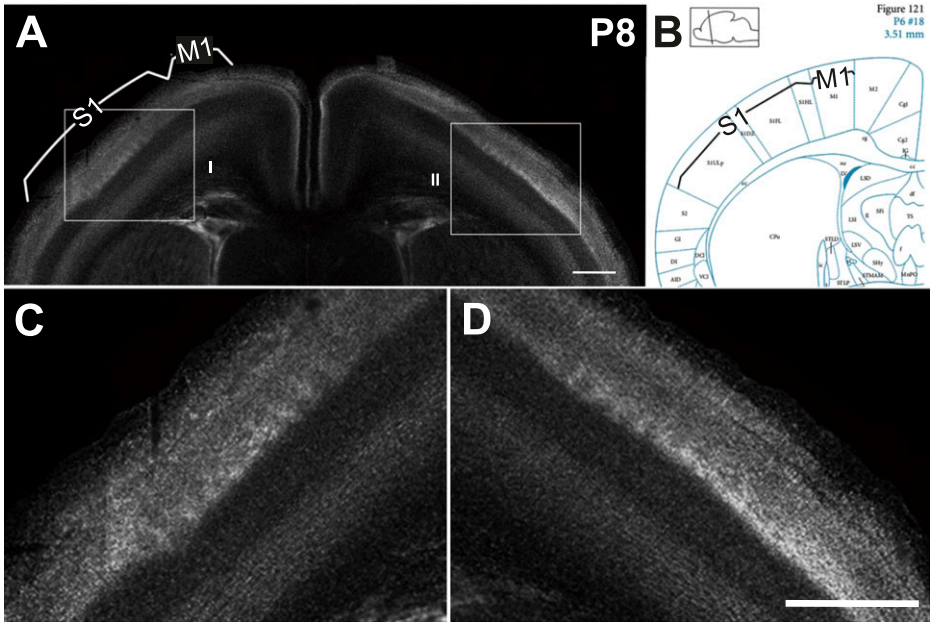
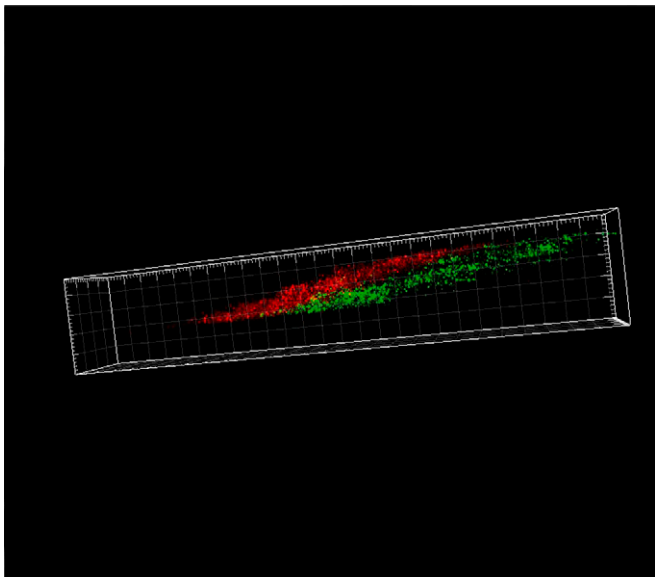
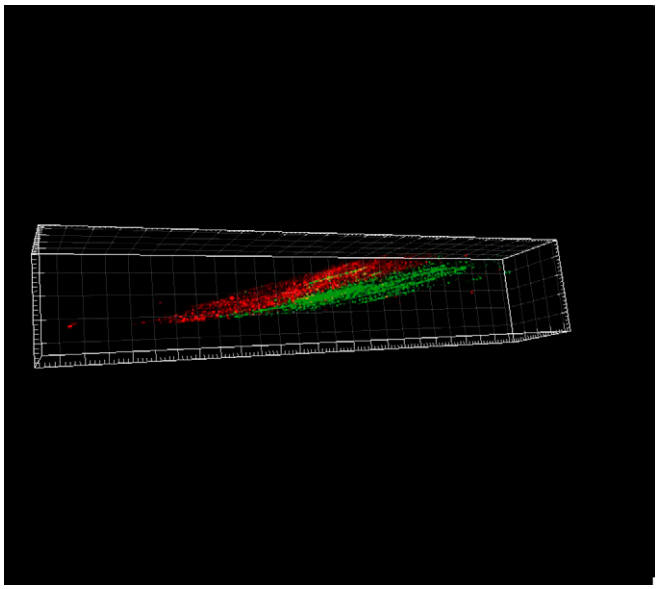


Fig. S11. Identification of cortical regions. (*A*) A coronal brain section of a P8 mouse counterstained with Hoechst. Primary somatosensory cortex (S1) was identified by the presence of barrel structures (anatomically distinguishable barrels which are separated from each other by septa), and primary motor cortex (M1) was identified as a more medial area adjacent to S1. Boxed areas I and II in the SI are shown in high resolution in *C* and *D*, respectively. (*B*) Sections were compared with a developing mouse brain atlas to verify the position of each area. (*C* and *D*) High-resolution images of area shown in box I (*C*) and box II (*D*) in *A* showing obvious barrel structures in S1. (Scale bars, 500 μm in *A* and *D*. *C* and *D* have the same resolution.)



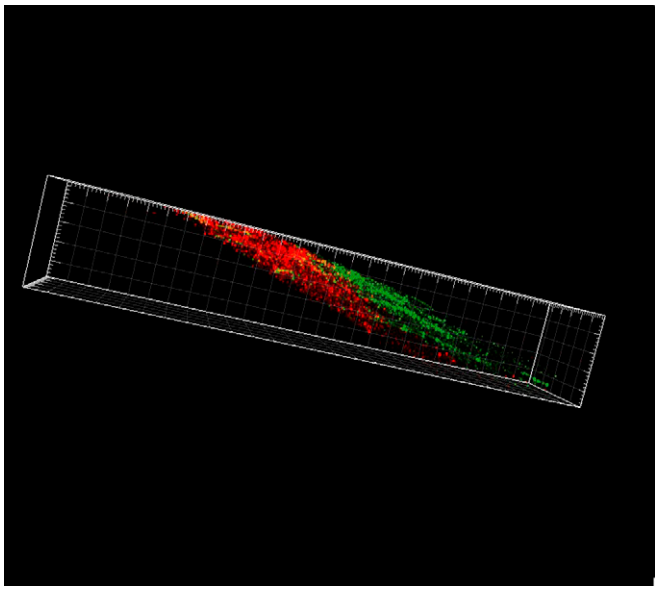
Movie S1. This movie demonstrates that labeled M1 (red color) and S1 (green color) axons located in different regions of the CC are segregated from each other. This movie corresponds to position I in Fig. 1 *A* and *B*.

Movie S1



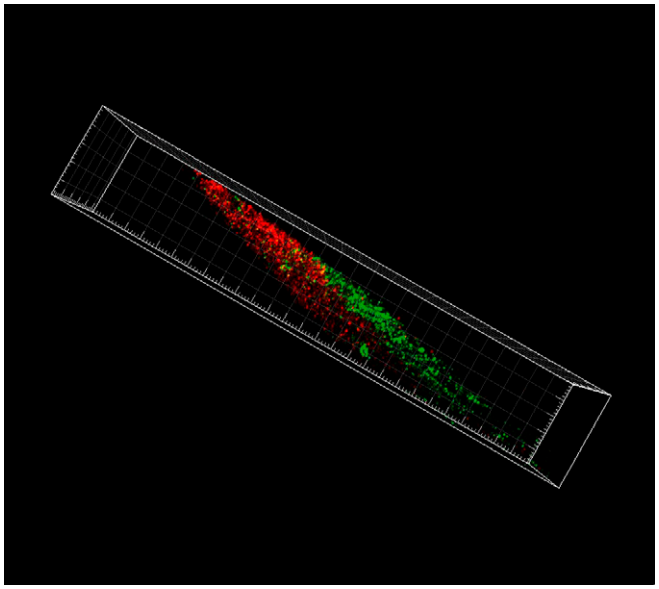
Movie S2. This movie demonstrates that labeled M1 (red color) and S1 (green color) axons located in different regions of the CC are segregated from each other. This movie corresponds to position II in Fig. 1 *A* and *B*.

Movie S2



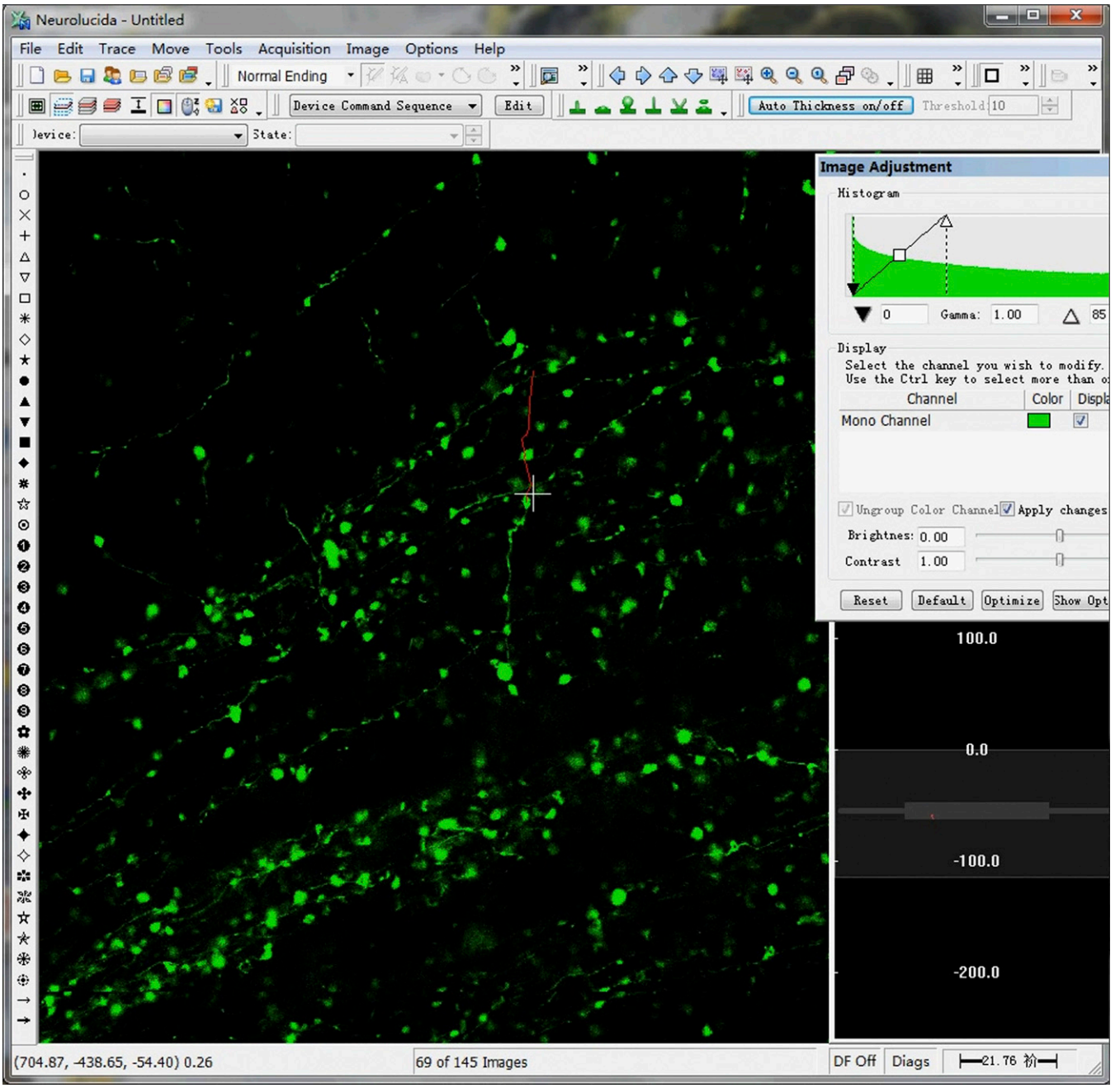
Movie S3. This movie demonstrates that labeled M1 (red color) and S1 (green color) axons located in different regions of the CC are segregated from each other. This movie corresponds to position III in Fig. 1 *A* and *B*.

Movie S3



Movie S4. This movie demonstrates that labeled M1 (red color) and S1 (green color) axons located in different regions of the CC are segregated from each other. This movie corresponds to position IV in Fig. 1 *A* and *B*.

Movie S4



Movie S5. This movie demonstrates how single-axon projections within the CC were traced manually using Neurolucida 9.0 software. The stitched 3D images were loaded into the Neurolucida 9.0, and only one section was presented at a time. The section change was shown in the window of lower right corner, and the image contrast of each section was adjusted as shown in the window in the upper right corner. The red line demonstrates how a projected axon was traced. We traced axons starting from the hemisphere contralateral to the cell bodies and then tracing back to the midline, representing only part of the entire projection.

Movie S5