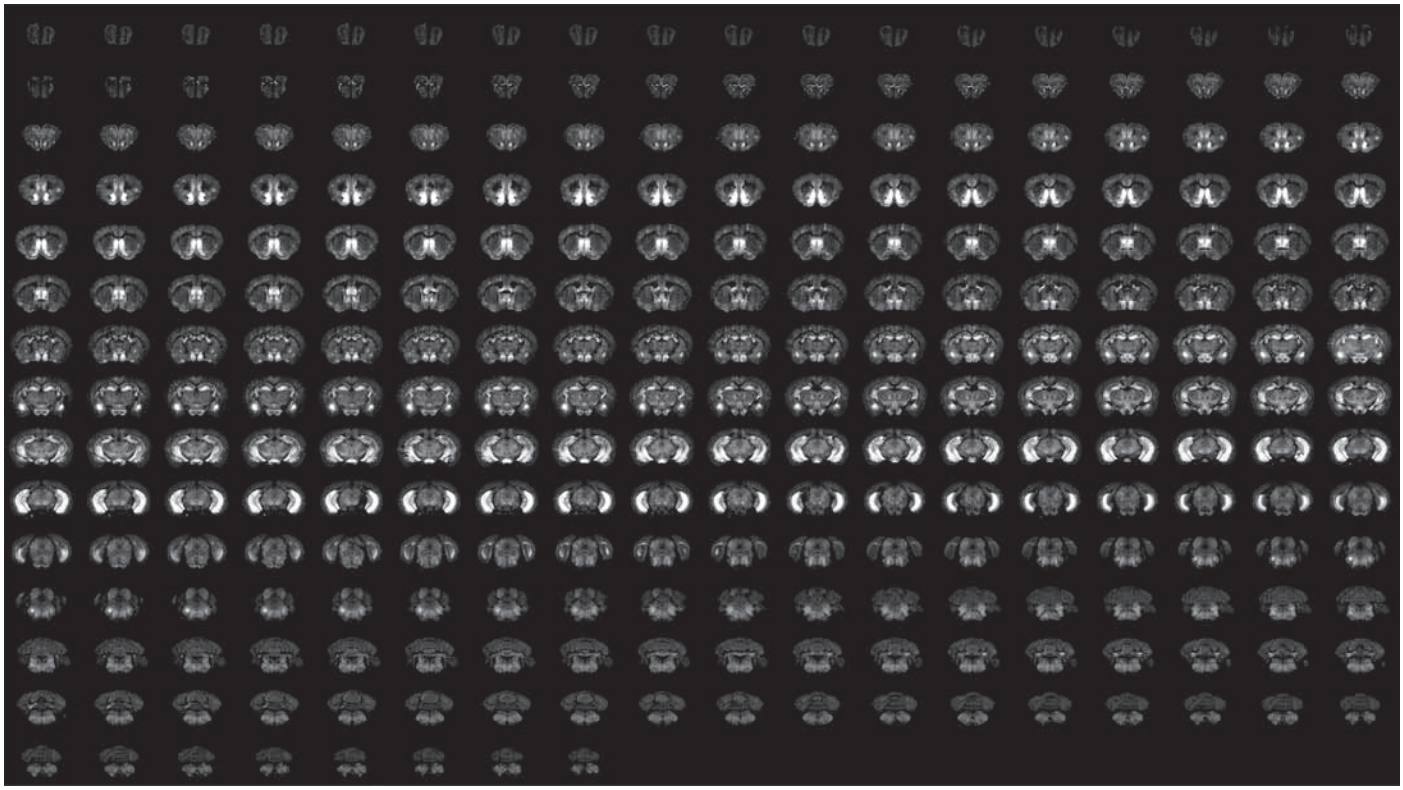
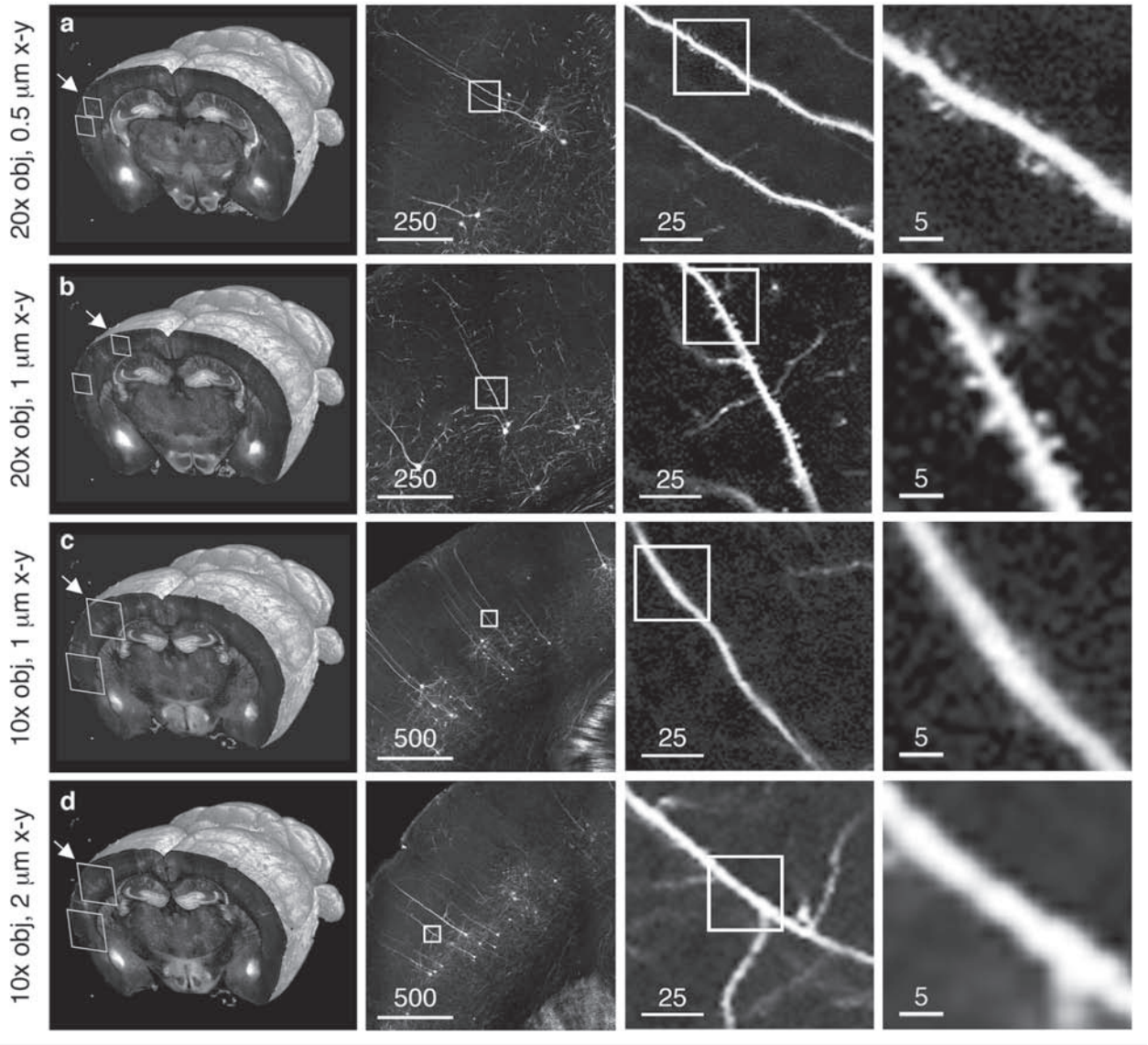
Supplementary Figure 1. 2D montage of a GFPM STP-tomography dataset.



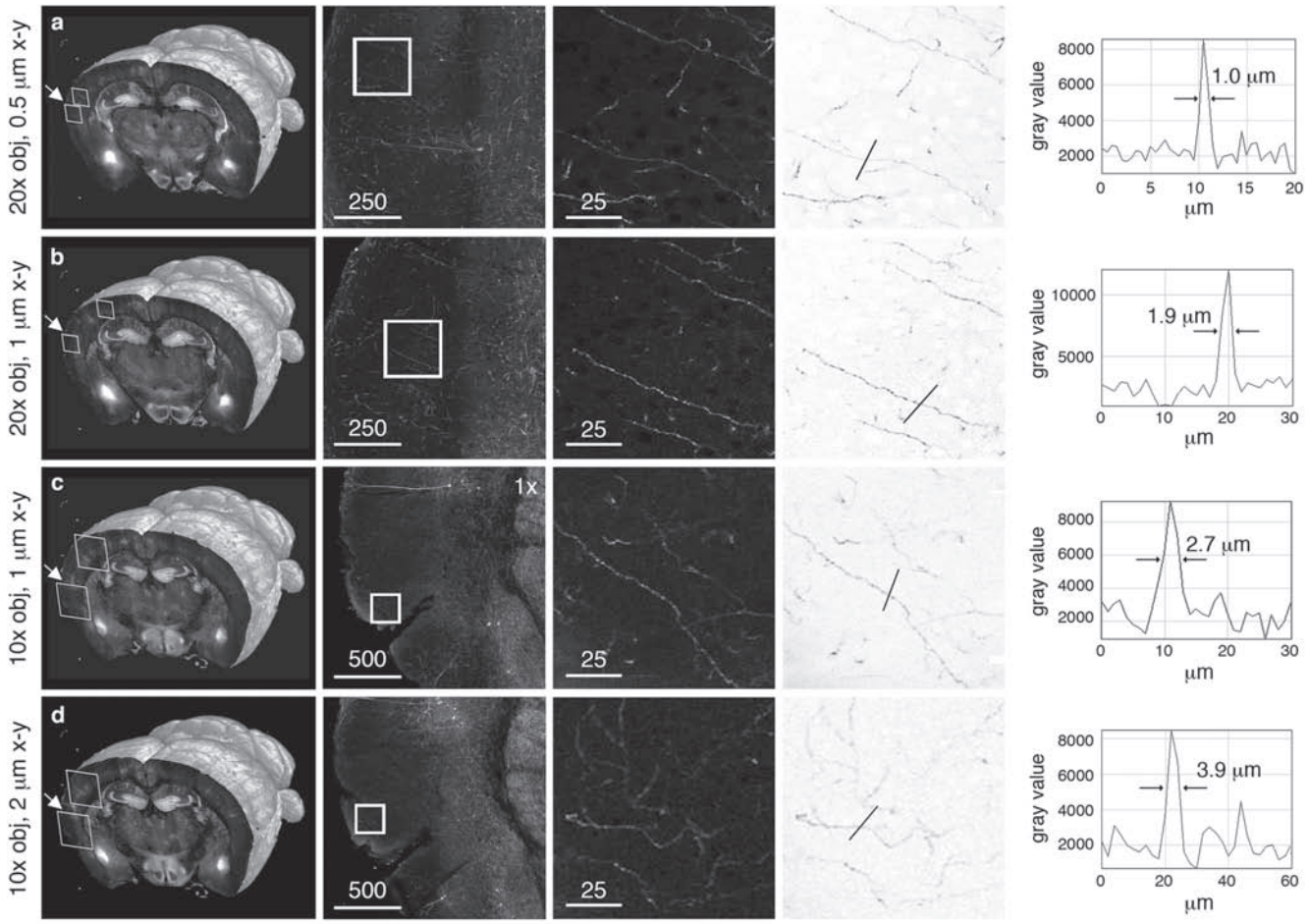
Supplementary Figure 1. The brain was imaged as 260 coronal sections, mostly with 20x objective at XY sampling of 1 µm. In the mid-region, we tested XY sampling resolution of 0.5, 1, and 2 µm with 20x and 10 objectives, as shown in Supplementary Figs. 2 and 3.

Supplementary Figure 2. XY sampling resolutions for imaging dendritic spines.



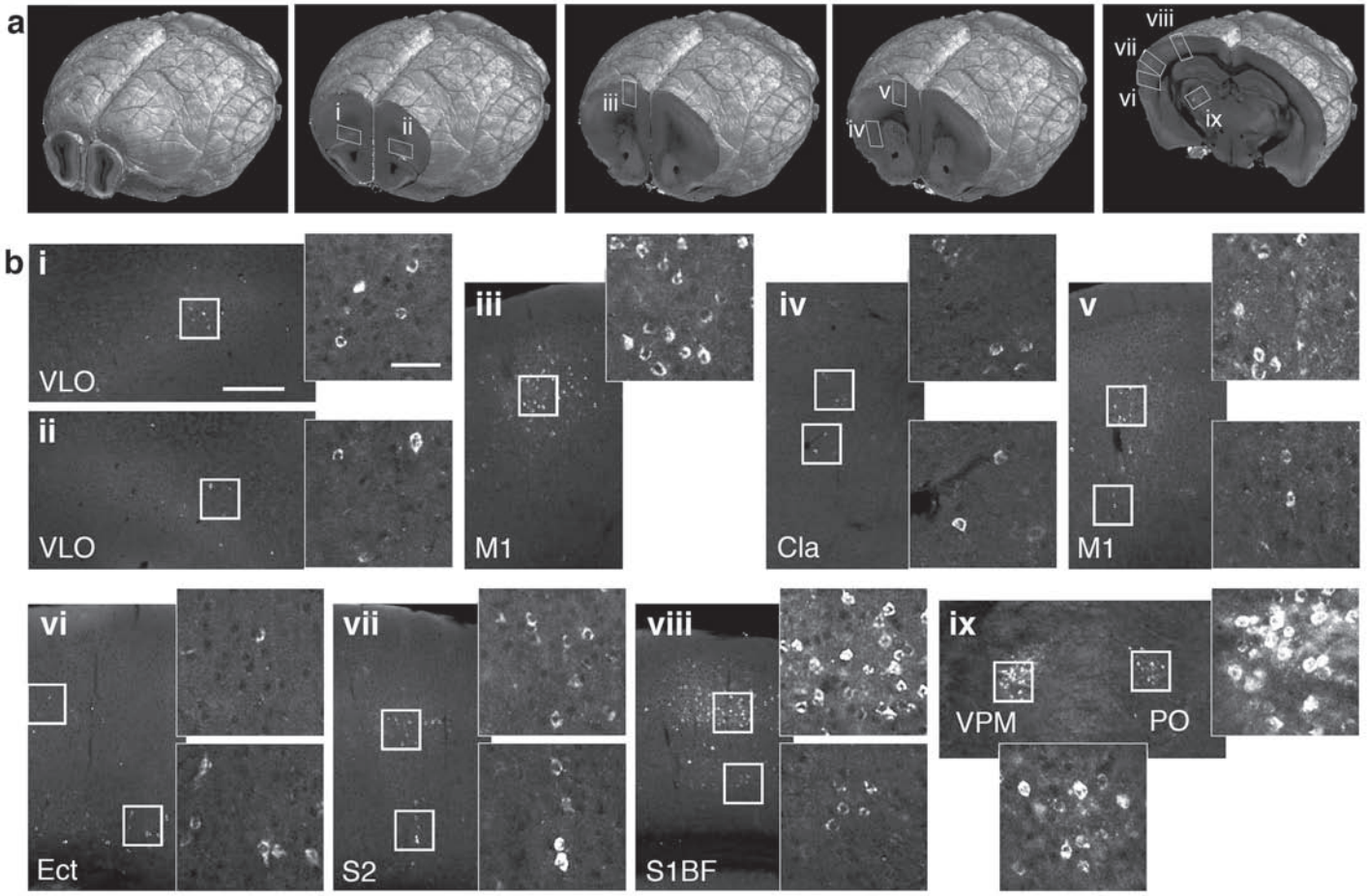
Supplementary Figure 2. GFPM mouse brain was imaged with a 20x objective at (a) 0.5 µm and (b) 1 µm XY resolution or with a 10x objective at (c) 1 µm and (d) 2 µm XY resolution. The scale bar numbers are in microns. Note that row (a) (20x, 0.5 µm) is the same as shown in Fig. 1. The arrowheads in the left panels point to the regions magnified in the right panels.

Supplementary Figure 3. XY sampling resolutions for imaging axons.



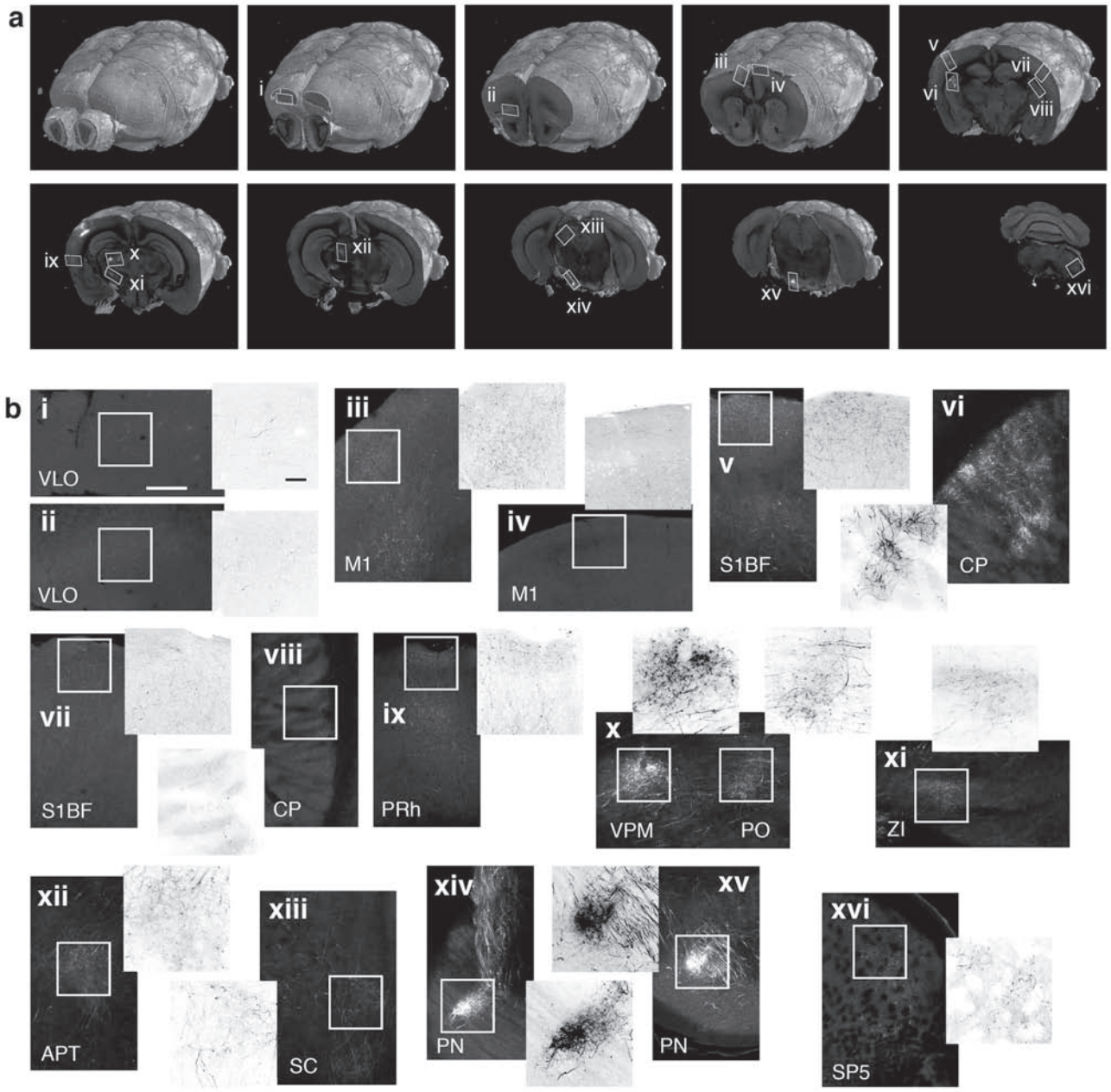
Supplementary Figure 3. Regions comprising only axons (marked by arrowheads) were selected in the same datasets as shown in Supplementary Fig. 2. The scale bar numbers are in microns. The inverted grayscale images of axon fibers contain black bars indicating the cross-sections used to evaluate the resolution for imaging GFP-labeled axons in the plot profiles shown in the most right panels (the plot profiles were measure with ImageJ on tif 16 bit images with no digital zoom). Mean values (±SEM) from five plot profile measurements for each condition were (µm) : 1.2±0.1 (a), 1.9±0.2 (b), 2.7±0.3 (c), and 3.9±0.3 (d) (note that the back aperture was less filled for the large 10x lens).

Supplementary Figure 4. Retrograde tracing by CTB-Alexa-488.



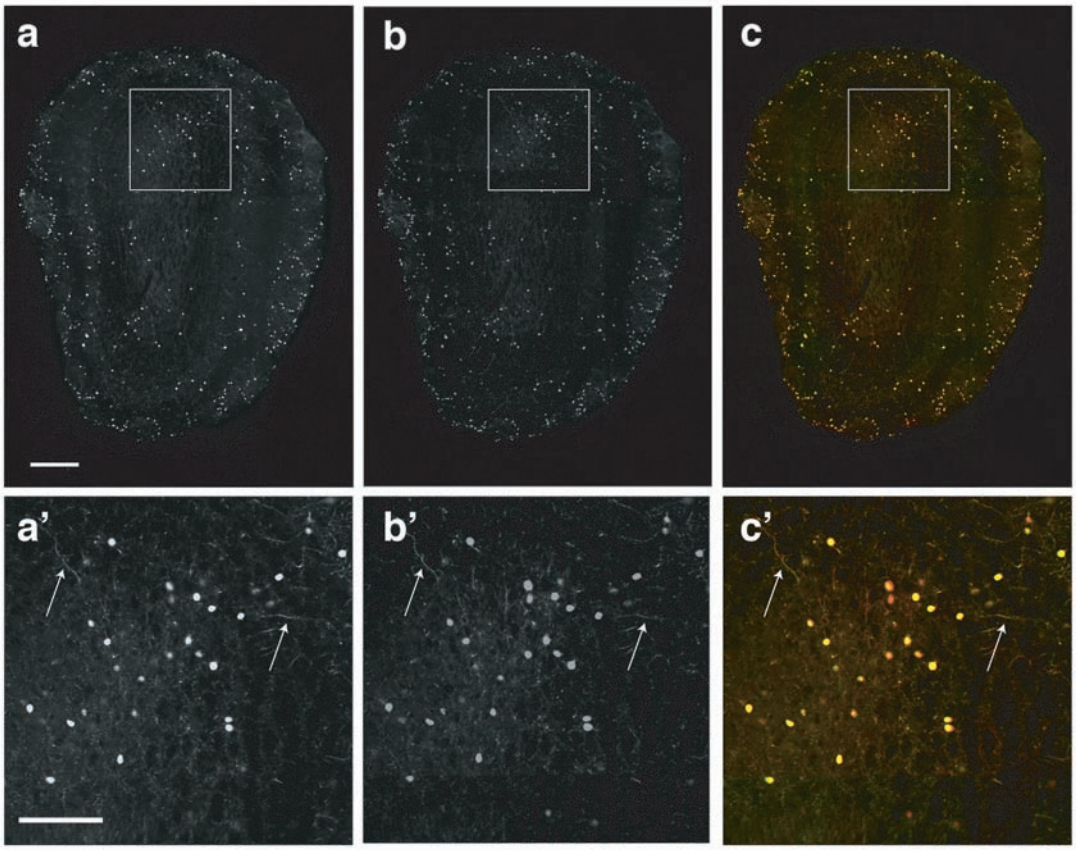
Supplementary Figure 4. Retrograde tracing by CTB-Alexa-488 (the same brain as shown in Fig. 2). (a) 3D views of selected coronal sections comprising the retrogradely labeled brain regions. (b) Brain areas marked up in (a) comprising: (i) ipsilateral and (ii) contralateral ventolateral orbital cortex (VLO) (Bregma = +2.2 mm); (iii) primary motor cortex (M1) (Bregma = +1.6 mm); (iv) claustrum (Cla) and (v) M1 (Bregma = +1.4); (vi) ectorhinal cortex (Ect), (vii) secondary somatosensory cortex (S2), (viii) barrel field primary somatosensory cortex (S1BF), (ix) ventral posteromedial thalamus (VPM) and posterior thalamus (PO) (Bregma = –1.8 mm). Note that to our knowledge, retrograde labeling of the contralateral VLO from S1BF has not been described before. The scale bar is 250 µm in panel (i) and 50 µm in the enlarged view of panel (ii). The Bregma estimates are based on comparison to the Mouse Brain Atlas by Paxinos and Franklin.

Supplementary Figure 5. Anterograde tracing by AAV-GFP.



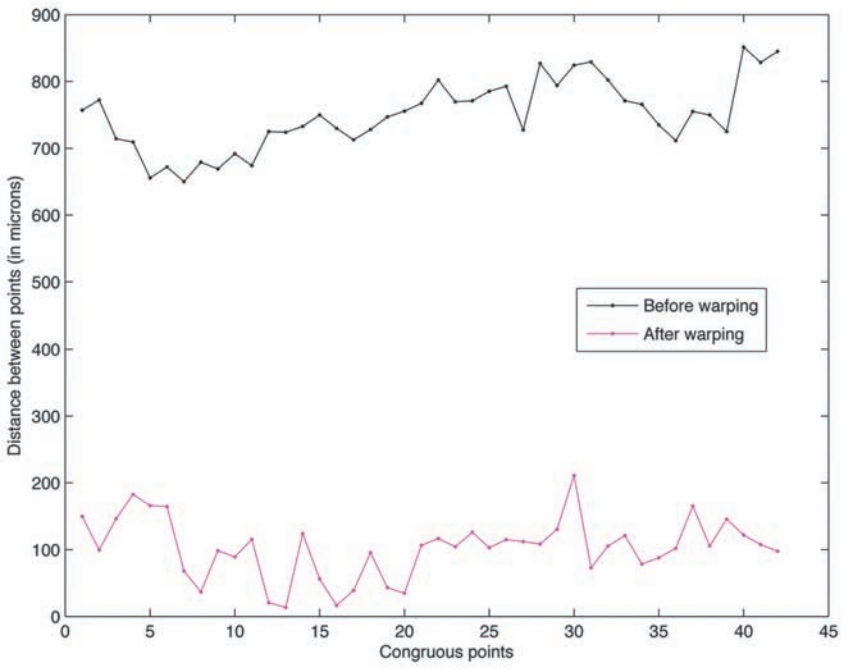
Supplementary Figure 5. Anterograde tracing by AAV-GFP (the same brain as shown in Fig. 3). (a) 3D views of selected coronal sections comprising anterogradely labeled brain regions. (b) Brain areas marked up in (a) comprising: (i) and (ii) ventolateral orbital cortex (VLO) (Bregma = +3.2, +2.1 mm, respectively); (iii) motor cortex (M1) and (iv) contralateral M1 (Bregma = 1.1 mm); (v) barrel cortex (S1BF), (vi) caudo-putamen (CP), and contralateral (vii) S1BF and (viii) CP (Bregma = –1.4 mm); (ix) perirhinal cortex (PRh), (x) ventral posteromedial thalamus (VPM) and posterior thalamus (PO), and (xi) zona incerta (ZI) (Bregma = –2.5 mm); (xii) anterior pretectal nucleus (APT) (Bregma = –3.1 mm); (xiii) superior colliculus (SC) and (xiv) pontine nucleus (PN) (Bregma = –4.1 mm); (xv) PN (Bregma = –4.4 mm); and (xvi) spinal trigeminal nucleus (SP5) (Bregma = –5.8 mm). Note that to our knowledge, anterograde labeling of contralateral motor cortex from S1BF has not been described before. The enlarged views show inverted grayscale images for better visualization of axon fibers and varicosities. The scale bar in both (i) and enlarged view of (i) is 250 µm. The Bregma estimates are based on the Mouse Brain Atlas by Paxinos and Franklin.

Supplementary Figure 6. Evaluation of Z-plane consistency before and after sectioning.



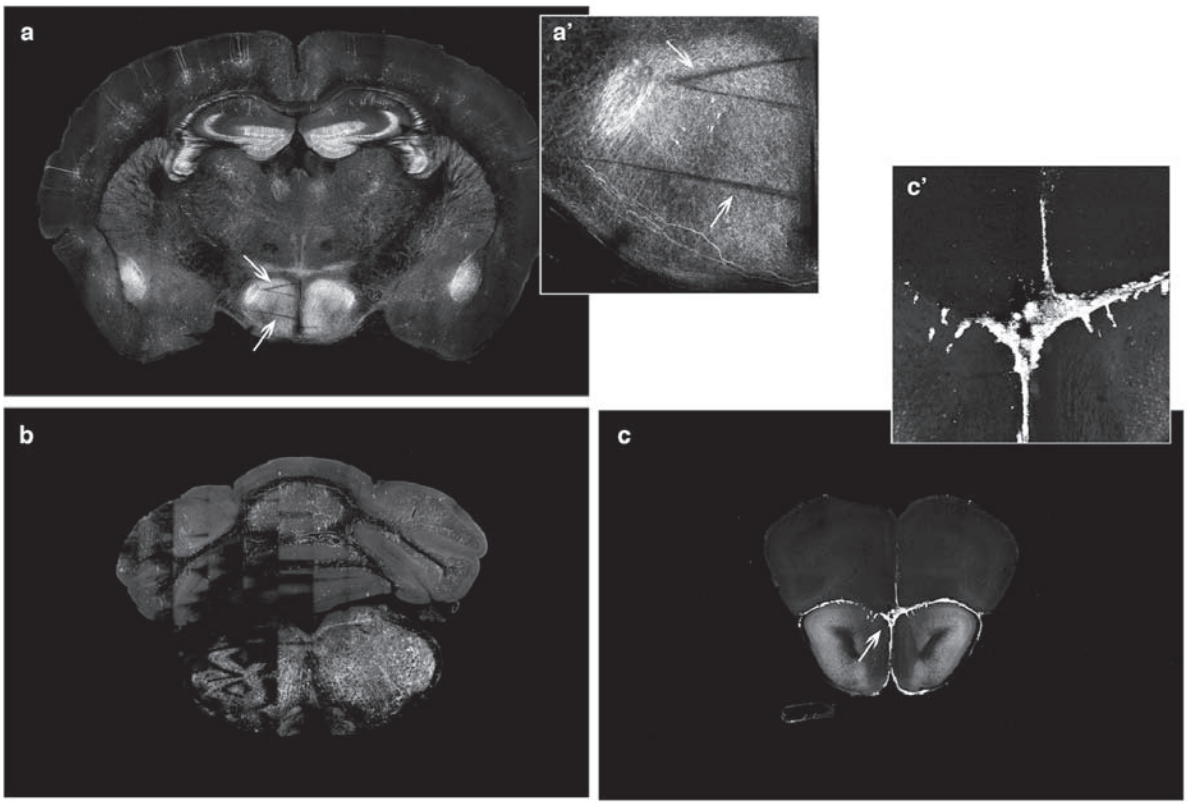
Supplementary Figure 6. Evaluation of Z-plane consistency before and after sectioning. (a, a') An optical plane imaged at Z-depth 90 µm below brain surface. (b, b') An optical plane imaged at Z-depth 40 µm below brain surface after cutting a single 50 µm thick section. (c, c') An overlay shows a close overlap of the two planes (pseudo-colored in green and red), demonstrating high consistency of the optical Z-plane before and after sectioning. Note the close overlap of labeled dendrites (long arrows). The scale are (a) 200 µm and (b) 100 µm. The image is taken from the SST-ires-Cre::Ai93 olfactory bulb (see Supplementary Movie 6).

Supplementary Figure 7. Quantification of warping accuracy.



Supplementary Figure 7. 42 landmark points of interest were manually selected in two different brains in the olfactory bulb, cortex, lateral ventricle, anterior commissure, lateral septum, fornix, hippocampus, optic track, amygadala, and cerebellum regions. The distance between each pair of corresponding points before and after warping is plotted. The mean (±SEM) of the displacement before and after warping was 749.5 ± 52.1 and 102.5 ± 45.0, respectively.

Supplementary Figure 8. Troubleshooting.



Supplementary Figure 8. Examples of artifacts that may occur during STP tomography (see Online Methods, section Troubleshooting). (a) Narrow shading (marked by arrows) is caused by a small strip of meninges that was not completely cut by the vibrating microtome. It appears rarely and typically does not persist for more than two to three sectioning cycles. (b) Large shading caused by a cut brain section floating between the objective and the tissue block. See troubleshooting on how to prevent this problem. (c) Bright autofluorescence from the meninges between the frontal cortex and accessory olfactory bulb. This only occurs in the rostral brain areas and since it is on the brain surface, it does not affect data interpretation.

Supplementary Table 1. Imaging conditions for STP tomography.

objective / NA	FOV (mm)	FOV (pixels)	Sampling rate *x*-*y* (μm)	Mosaic of FOVs	Pixel residence time (μs)	Time per 1 section (min:sec)	Time per 260 sections (hrs:min)
10x / 0.6	1.66 x 1.66	832x832	2.0	6 x 8	0.8	1:30	6:30
10x / 0.6	1.66 x 1.66	1664x1664	1.0	6 x 8	0.4	2:00	8:40
20x / 1.0	0.83 x 0.83	832x832	1.0	11 x 17	0.8	3:35	15:30
20x / 1.0	0.83 x 0.83	1664x1664	0.5	11 x 17	0.4	5:35	24:10

The time per 1 section and time per 260 sections correspond to imaging conditions with the 10x and 20 objectives, number of FOVs, sampling XY rate and pixel residence time as indicated. The time per 1 section comprises: 1) imaging time, 2) mosaicing movement of XY stages, and 3) sectioning time. Imaging time comprises most of the total time and varies based on sampling resolution and pixel residence time. The XY stage movement is about ~0.3 sec per move (~15 sec for 6x8 mosaic and ~1 min for 11x17 mosaic). The sectioning time, at stage movement of 1 mm per sec, is ~35 second per cycle.