Figures and figure supplements

Cell class-specific long-range axonal projections of neurons in mouse whisker-related somatosensory cortices

Yanqi Liu *et al*.

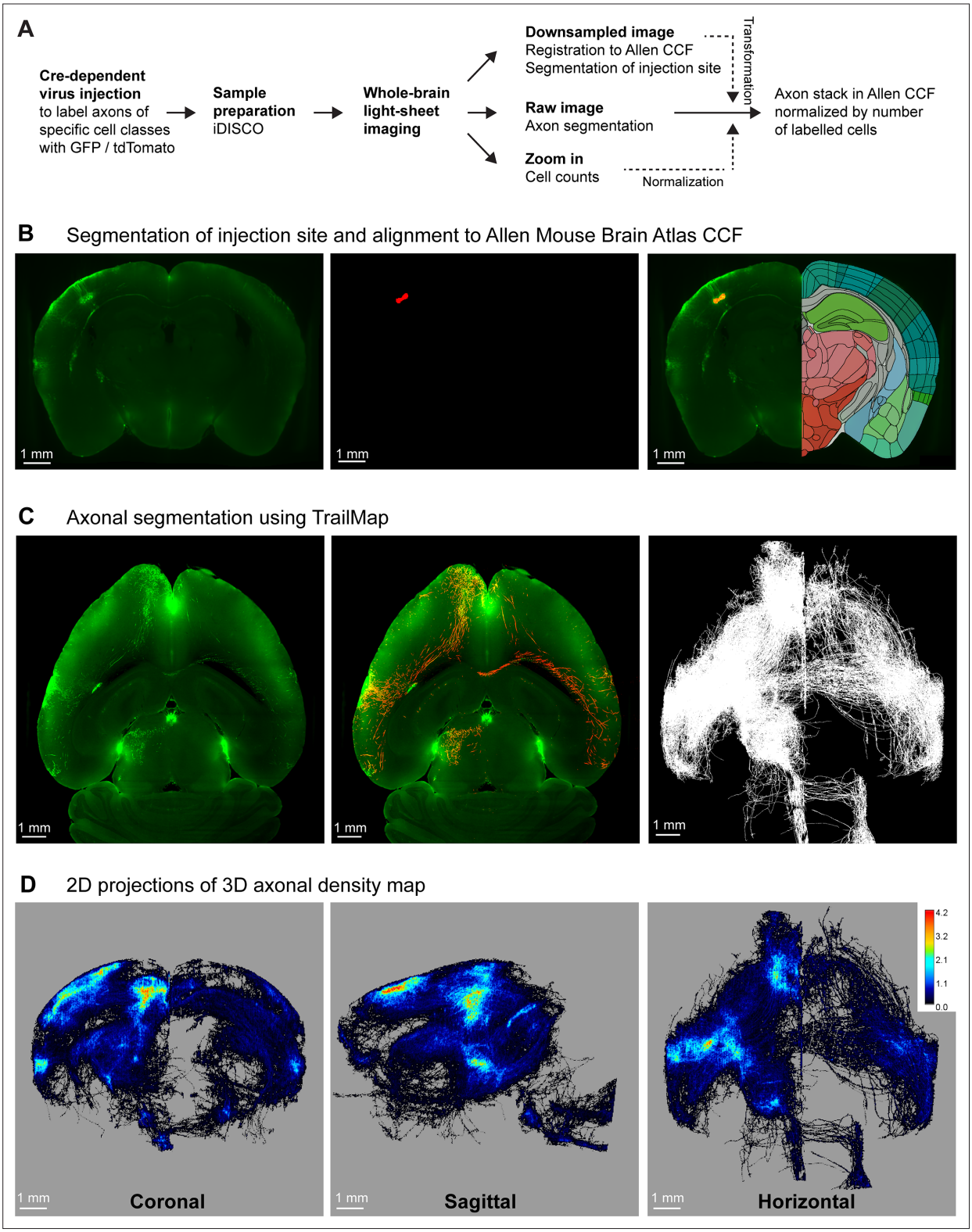

Figure 1. A pipeline for quantifying brain-wide axonal projections. (**A**) Cell class-specific labeling of axonal projections was achieved by injecting a Cre-dependent GFP or tdTomato reporter virus into posterior whisker-related SSp-bfd or SSs of transgenic mice expressing Cre in specific classes of cortical neurons. Samples then went through iDISCO treatment in order to achieve whole-brain immunolabeling and clearing. Volumetric brain imaging was performed using mesoscale selective plane illumination microscopy (MesoSPIM) to visualize axonal structures throughout the full brain. Axons were segmented on the original MesoSPIM images while atlas alignment and injection site segmentation were obtained with downsampled MesoSPIM

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images. To count the number of labeled cells, high-resolution images of a subregion around the injection site were obtained using a Zeiss Lightsheet 7 microscope. Finally, axons were aligned to the Allen CCF with the axonal density values normalized by the number of infected neurons. (**B**) An example downsampled plane (green color shows the fluorescence) aligned to the Allen CCF near the injection site in SSp-bfd of a Rbp4-Cre mouse (left). Segmentation of injection site (red color, center) and overlay in the Allen CCF space (right). This allowed the assignment of imaged voxels to voxels of the Allen CCF. (**C**) An example horizontal plane from the raw image stack obtained by MesoSPIM (left, green color raw fluorescence signal, same mouse as panel B), with the result of TrailMap axon segmentation (red) overlayed on the raw image (green) (center). Max horizontal projection (i.e. binary, axon or no axon) of the final axonal skeleton obtained from the axon segmentation (right). (**D**) Long-range axonal density maps in the Allen CCF in coronal (left), sagittal (middle), and horizontal (right) views, represented as summed projections of axonal voxel values normalized by the number of labeled neurons (same mouse as panels B and C).

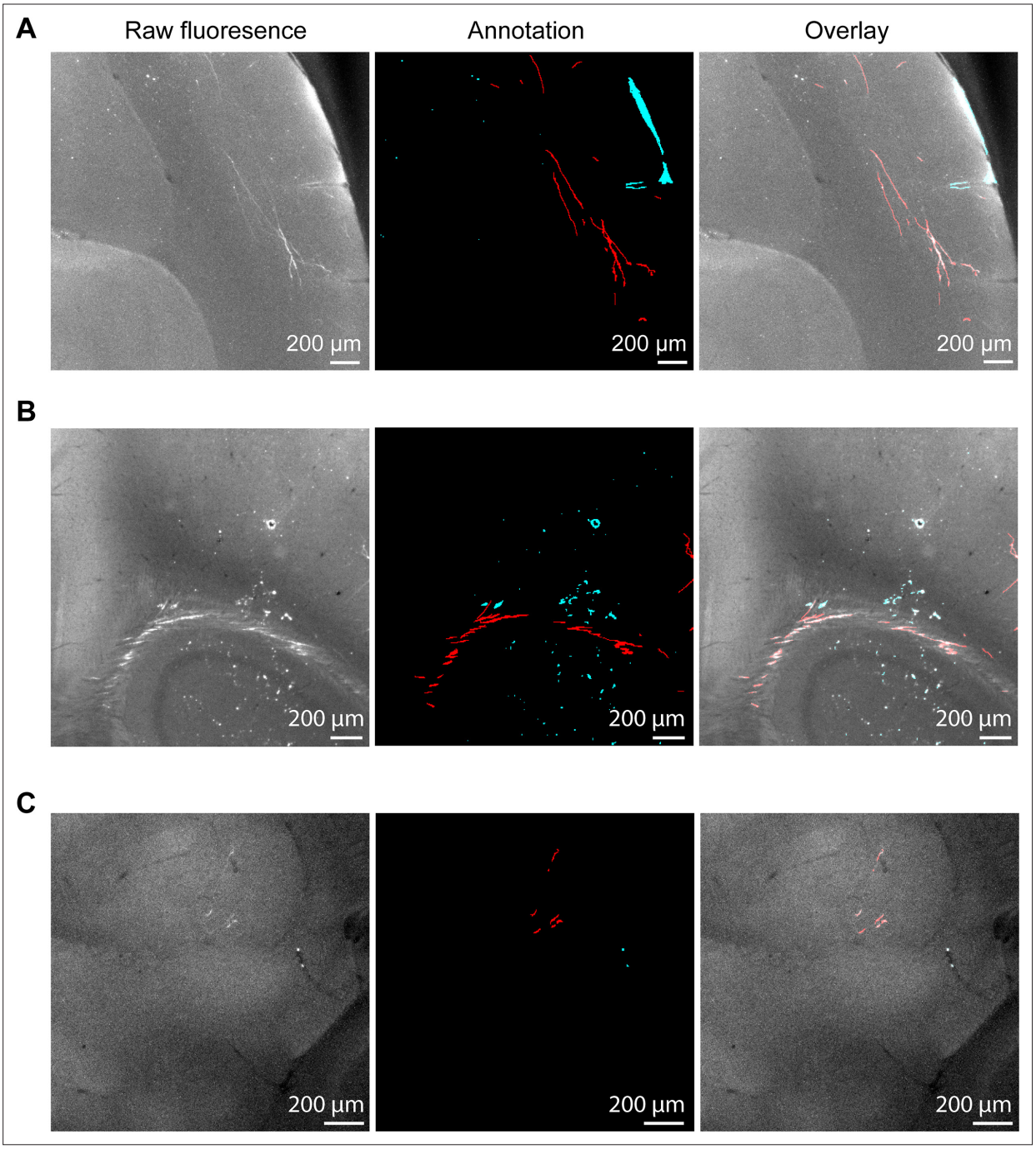

Figure 1—figure supplement 1. Training the TrailMap network. (**A**) An example image plane from an image sub-stack used to further train the Trailmap network (left). This image contains axons with delineated morphologies and blood vessel artifacts in the cortex. During training, pixels were manually labeled as axon (red) or artifact (cyan) (center). Overlay of the raw image with the labels (right). (**B**) Another example similar to A but demonstrating axon labeling in the corpus callosum. (**C**) Another example similar to A but demonstrating axon labeling in deeper brain regions.

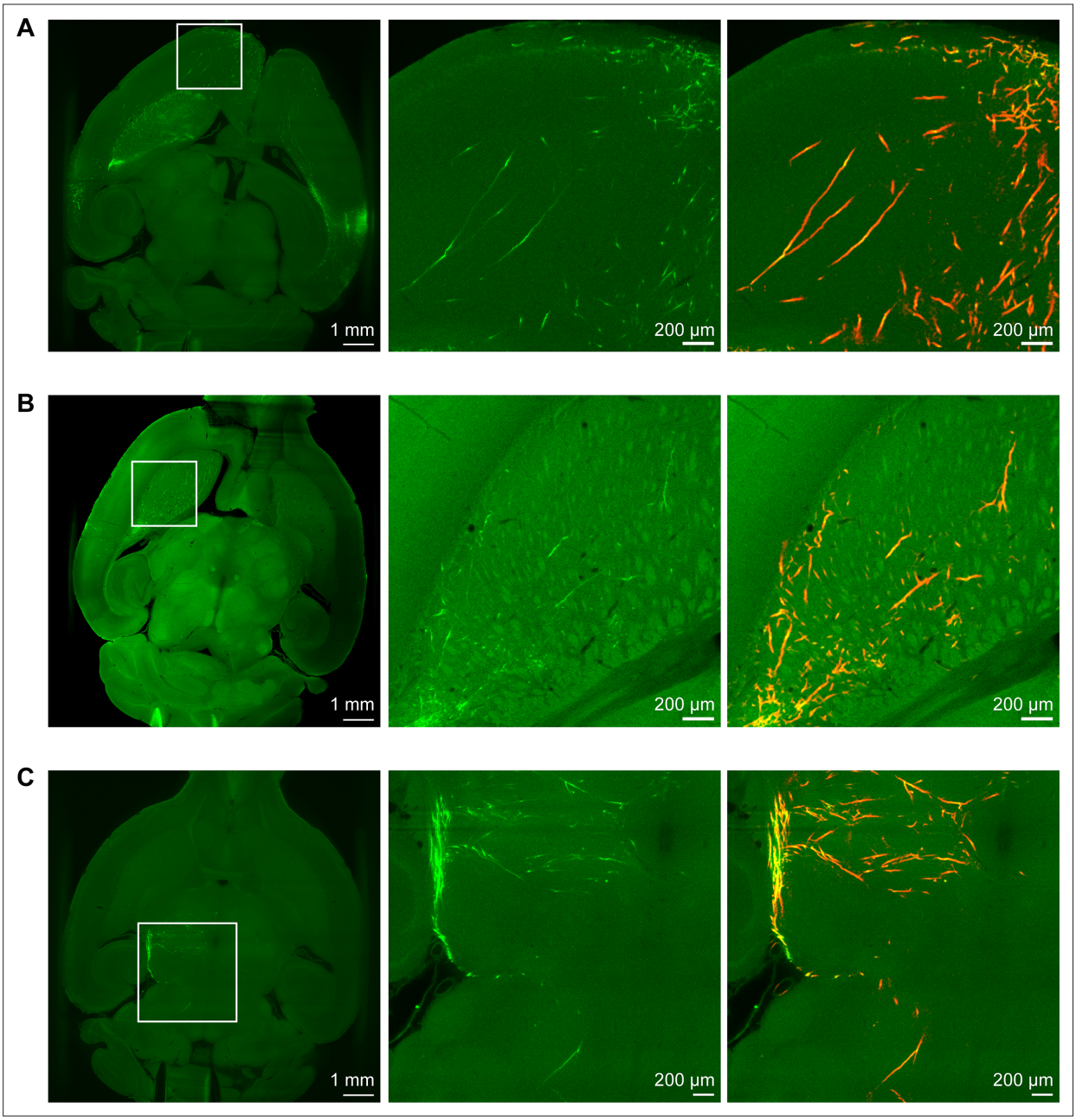

Figure 1—figure supplement 2. Segmentation of axons in highly branching regions. (**A**) *Left*, overview of one image plane in a Tlx3-Cre sample with region of interest in a frontal cortical area. *Middle*, zoomed in view of the region of interest displaying travelling and branching axons in the cortex. *Right*, zoomed in view of the region of interest overlayed with Trailmap segmentation (red). (**B**) Same as A but for a Scnn1a-Cre sample with the region of interest in the striatum. (**C**) Same as A but for a Sim1-Cre sample with the region of interest in the thalamus and brainstem region.

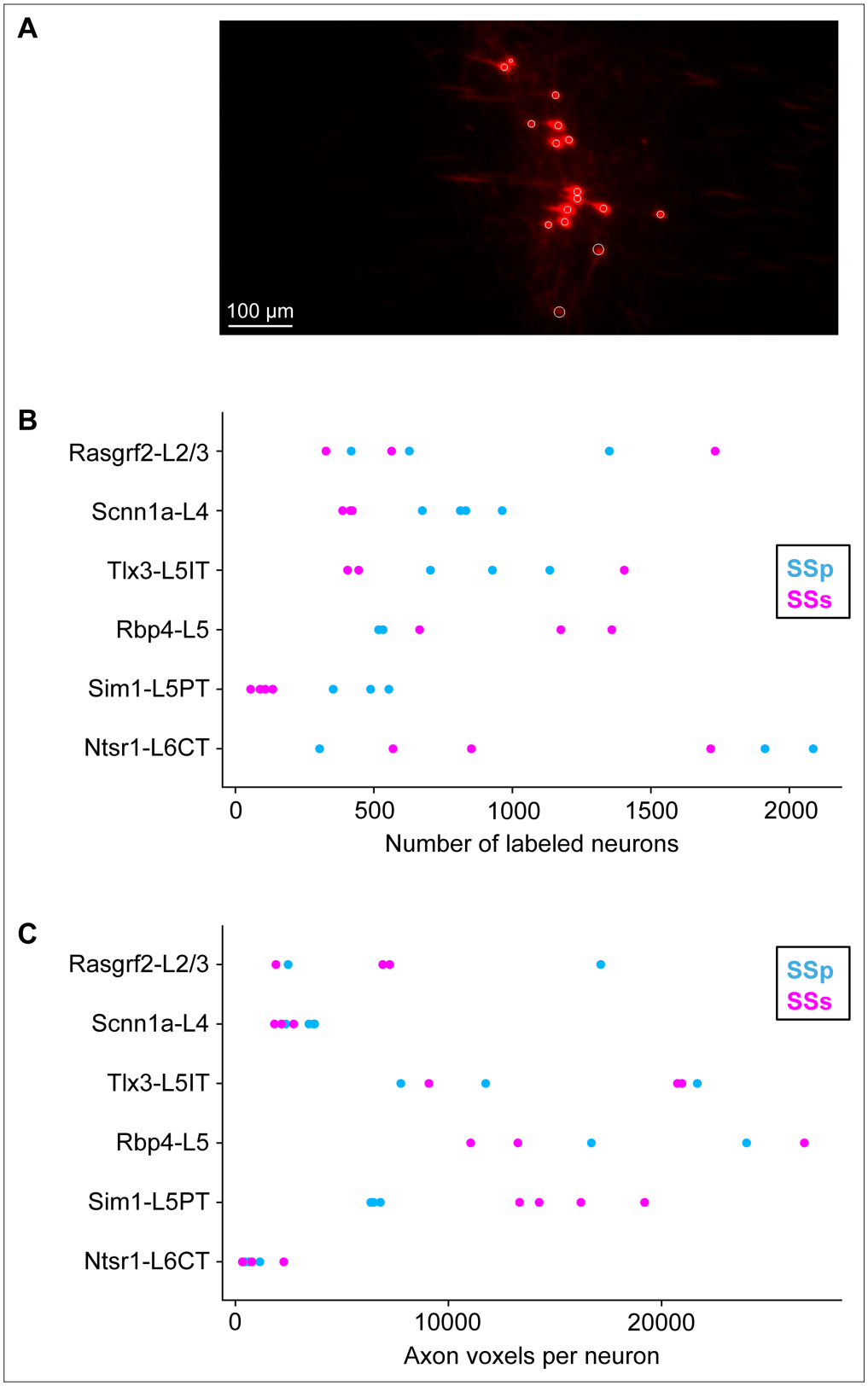

Figure 1—figure supplement 3. Quantification of the number of labeled cells in the injection site. (**A**) An example plane focused on the injection site with labeled cell bodies (white circles). Each sample was imaged at higher resolution near the injection to quantify the numbers of infected cells. Note that the sizes of white circles increase and decrease as the center of cell body comes in and out of plane. (**B**) Number of cells labeled in the injection site for each sample. (**C**) Number of axon voxels per labeled cell for each sample.

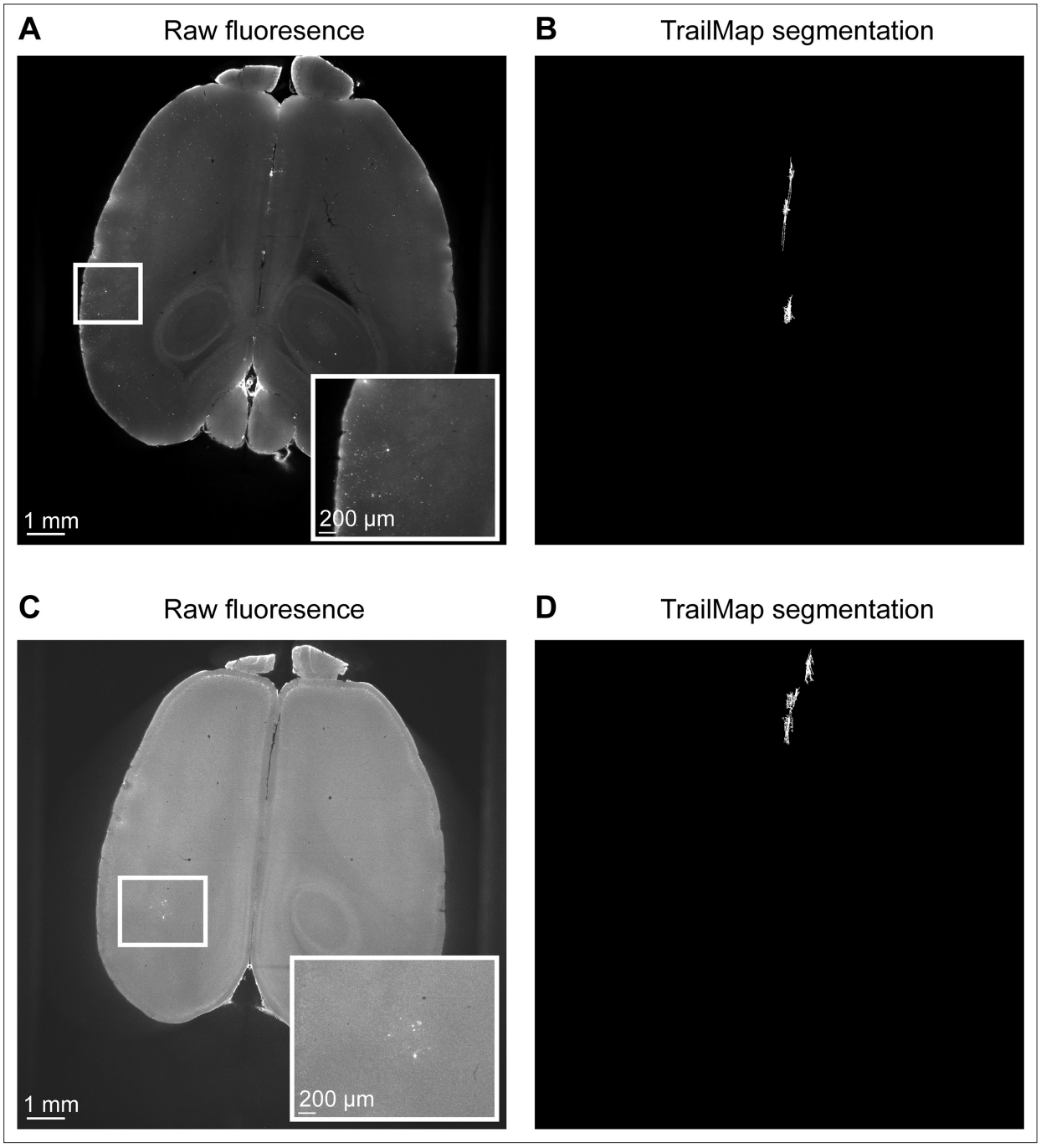

Figure 1—figure supplement 4. Absence of Cre-independent axonal labeling. (**A**) The sample preparation and analysis pipeline were repeated in wild-type mice to test for Cre-independent expression of the reporter viruses. A raw image plane from a control mouse injected with a Cre-dependent GFP-expressing virus in the SSp-bfd with region of interest indicating injection site (white box). Inset, zoomed in image of the region of interest showing few cell bodies with barely identifiable processes. (**B**) Max projection of connected components greater than 10,000 pixels after axon segmentation and processing of the same brain shown in panel A. These are mainly composed of midline artifacts which would then be eliminated in the visual inspection step. This suggests that our method specifically identifies axons from Cre-expressing neurons. (**C**) Same as panel A, but for a wild-type mouse injected with a Cre-dependent tdTomato -expressing virus in the SSs. (**D**) Same as panel B, but for the mouse investigated in panel C.

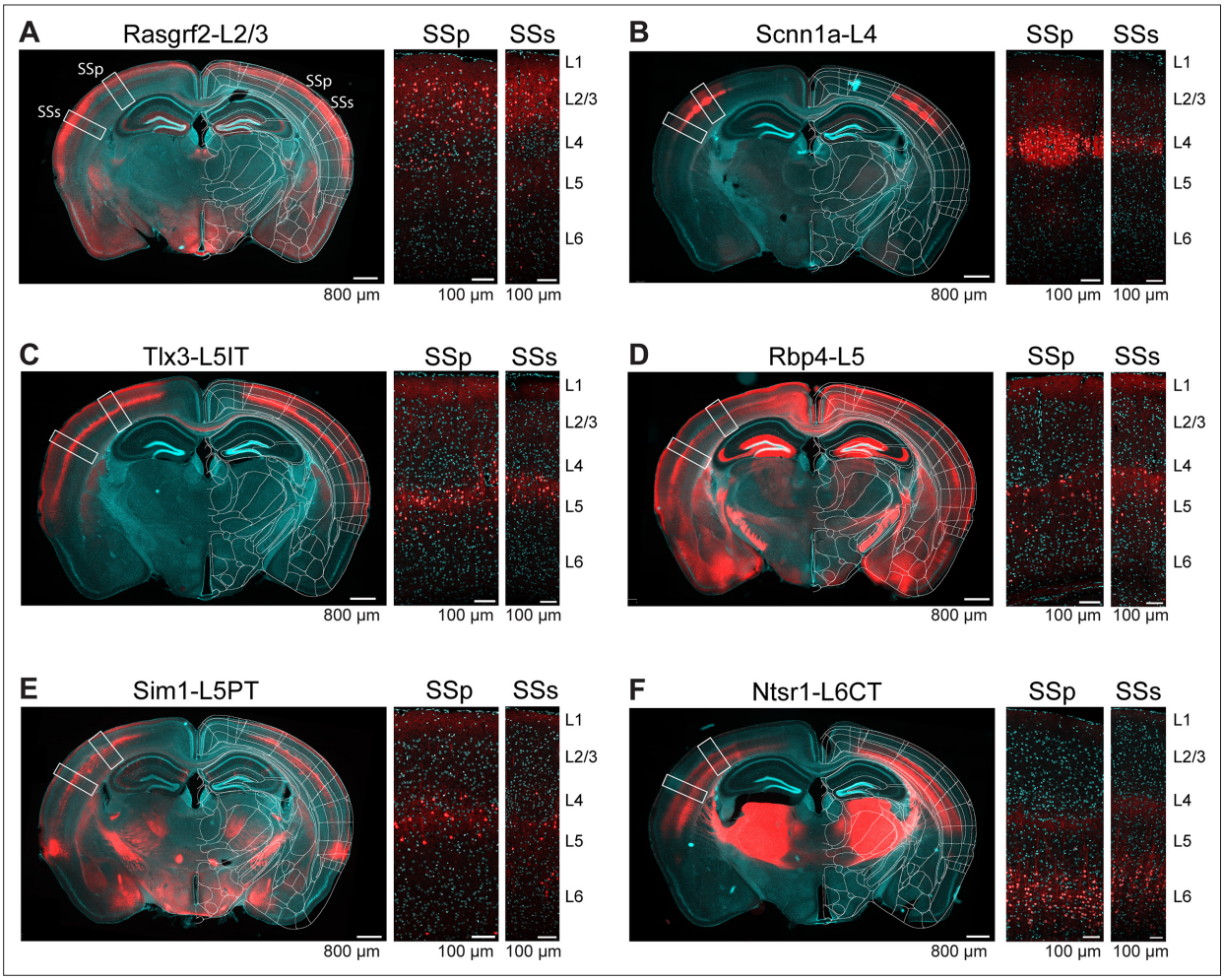

Figure 2. Layer-specific Cre-expression in SSp-bfd and SSs of six selected transgenic mouse lines. (**A**) Expression pattern of tdTomato in a coronal section including the posterior barrel field of a transgenic cross of a Rasgrf2-Cre mouse with a Cre-dependent tdTomato reporter mouse, presented as an overview image from an epifluorescent microscope overlayed with the corresponding annotation of the Allen Brain Atlas (left), and two confocal images from the locations of SSp-bfd (middle) and SSs (right) together with labels for the approximate cortical layer boundaries. Red, tdTomato. Cyan, DAPI. (**B**) As for panel A, but for Scnn1a-L4 neurons. (**C**) As for panel A, but for Tlx3-L5IT neurons. Labelling in layer 1 is likely of axonal or dendritic origin, and no cell bodies were labelled in this layer. (**D**) As for panel A, but for Rbp4-L5 neurons. Labelling in layer 1 is likely of axonal or dendritic origin, and no cell bodies were labelled in this layer. Note strong expression of Cre in neurons with cell bodies located in the hippocampus, which does not affect our analysis of axonal density based on virus injected locally into the neocortex. (**E**) As for panel A, but for Sim1-L5PT neurons. (**F**) As for panel A, but for Ntsr1-L6CT neurons.

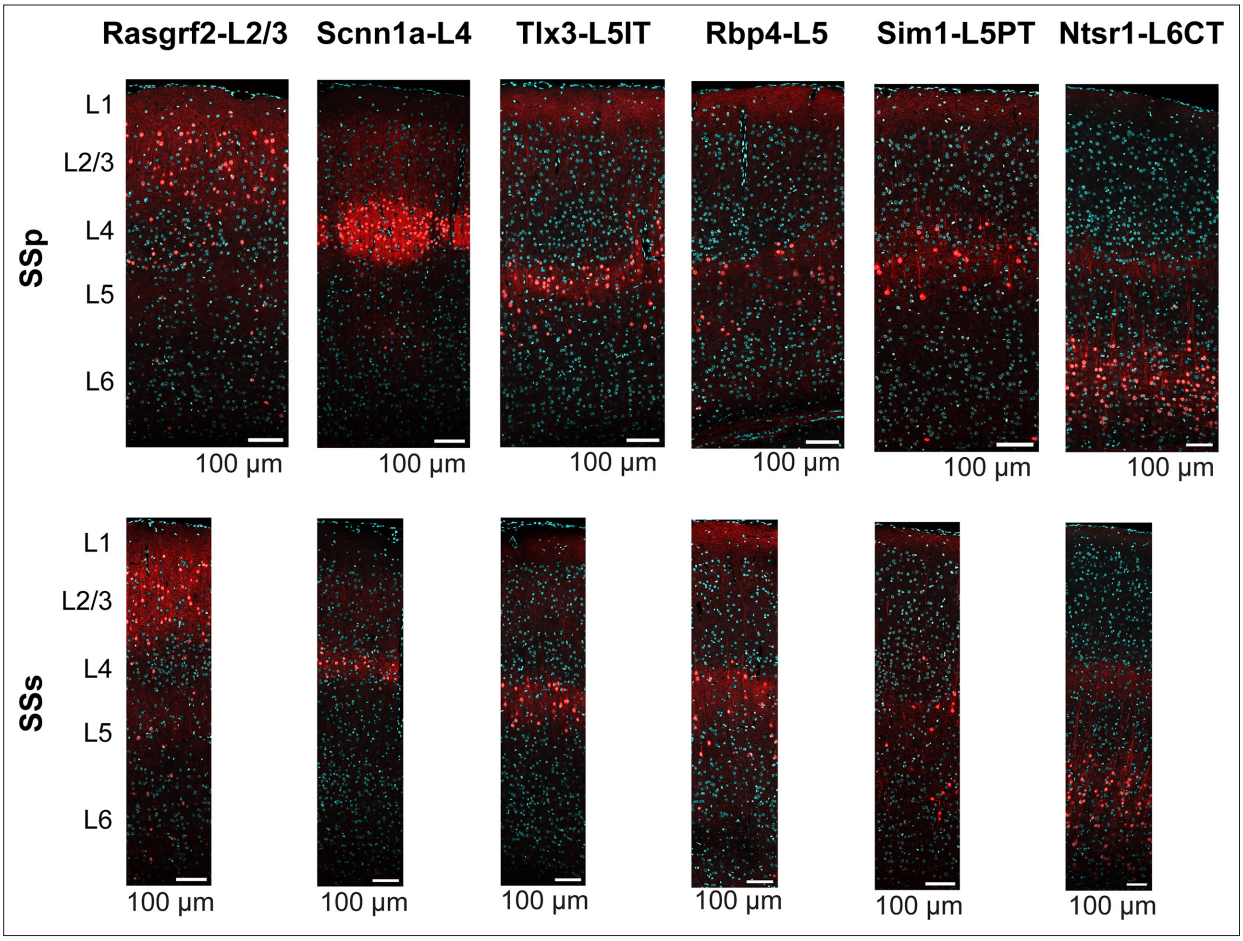

Figure 2—figure supplement 1. Higher resolution images of layer-specific Cre-expression in SSp-bfd and SSs. Same data as shown in ***Figure 2***, but showing the panels of SSp-bfd and SSs for each of the Cre-lines at higher resolution.

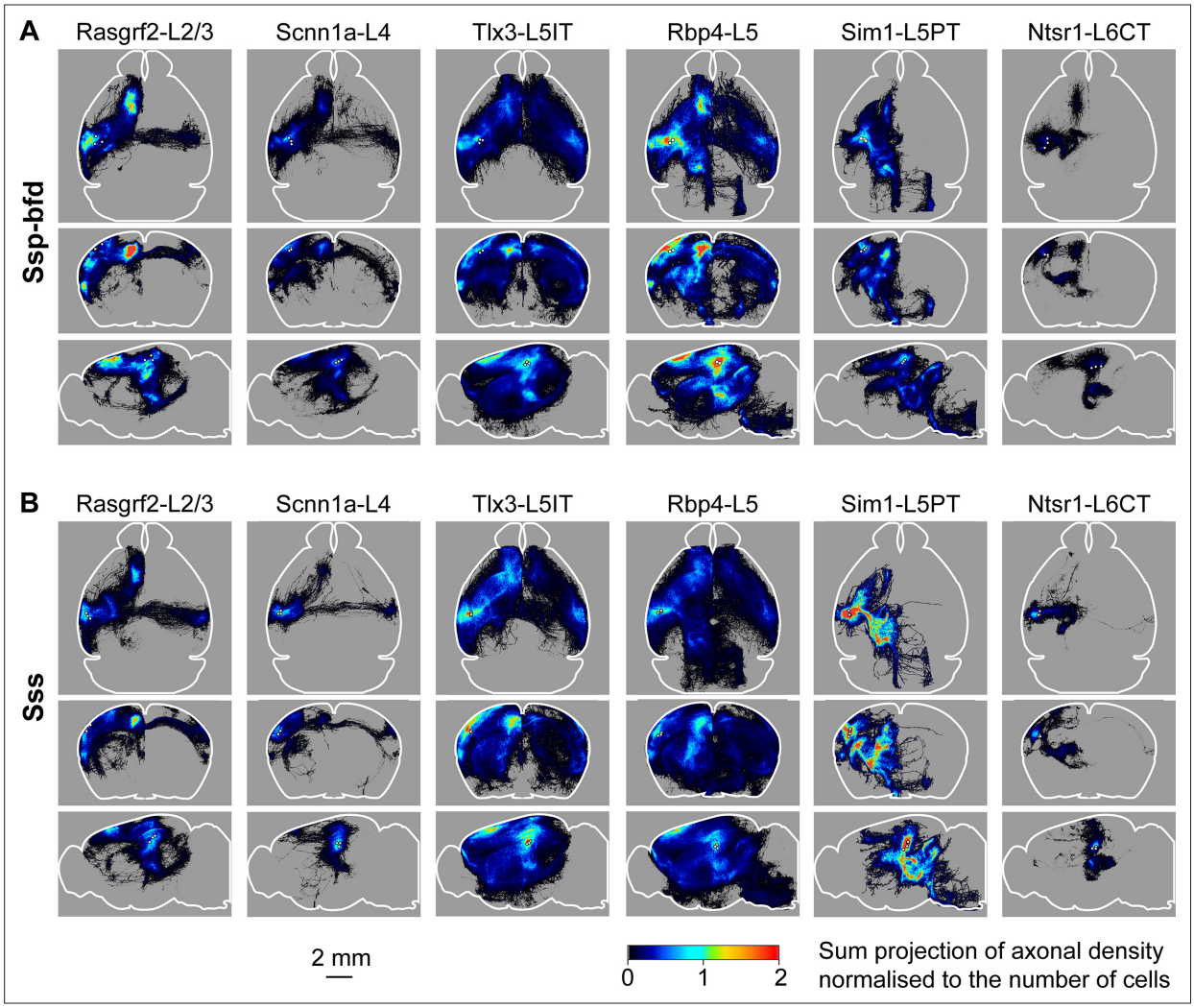

Figure 3. Overall axonal projection patterns are specific to cell classes but similar between SSp-bfd and SSs. (**A**) Averaged axonal density maps for each of the six transgenic mouse lines with axons originating from SSp-bfd presented in horizontal (top), coronal (middle) and sagittal (lower) sum-projection views. White dots represent the center of each injection site and color-coded pixel intensity represents the amount of axon voxels normalized with the number of labeled cells. For Rasgrf2-L2/3, n=3 injections; for Scnn1a-L4, n=4 injections; for Tlx3-L5IT, n=3 injections; for Rbp4-L5, n=2 injections, for Sim1-L5PT, n=3 injections; and for Ntsr1-L6CT, n=3 injections. (**B**) Same as panel A, but for SSs injections. For Rasgrf2-L2/3, n=3 injections; for Scnn1a-L4, n=3 injections; for Tlx3-L5IT, n=3 injections; for Rbp4-L5, n=3 injections, for Sim1-L5PT, n=4 injections; and for Ntsr1-L6CT, n=3 injections.

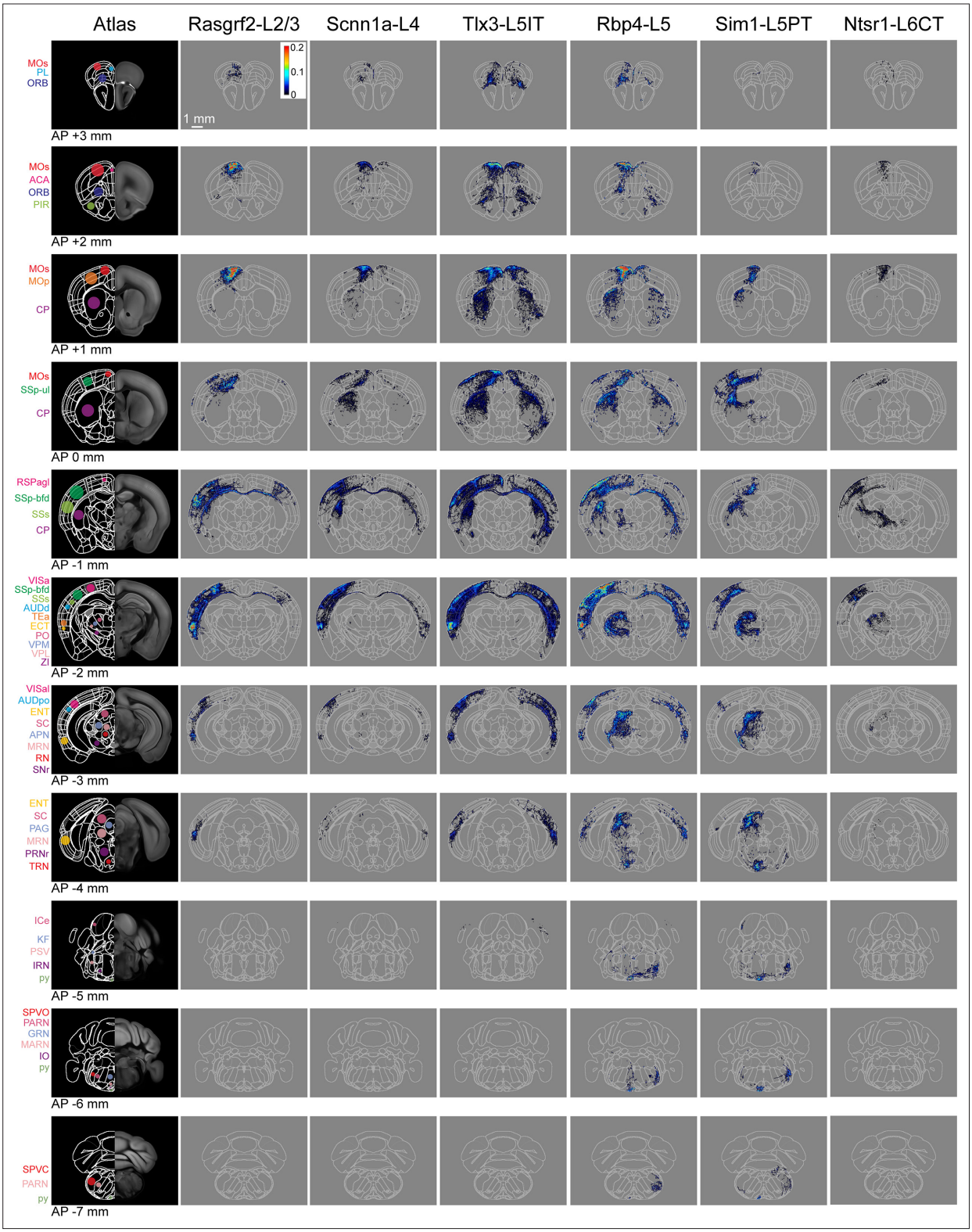

Figure 4. Serial coronal sections of group averages from each Cre-line with SSp-bfd injections. The leftmost column shows the Allen CCF atlas at different AP locations (from AP +3 mm in the top row to AP –7 mm in the bottom row). Some brain regions of interest are labeled with color-coded dots and acronyms. The next columns show the mean axonal density averaged across the injection sites in SSp-bfd for each of the six transgenic lines. Each axon image represents a 125 μm sum projection centered around the corresponding AP location with each 25x25 μm pixel indicating the color-coded number of 5 μm voxels containing axon per labeled neuron within the analysed 25 x 25 × 125 μm volume. Abbreviations: ACA, Anterior cingulate area;

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AUDd, Dorsal auditory area; AUDpo, Posterior auditory area; CP, Caudoputamen; ECT, Ectorhinal area; ENT, Entorhinal area; GRN, Gigantocellular reticular nucleus; ICe, Inferior colliculus external nucleus; IRN, Intermediate reticular nucleus; KF, Koelliker-Fuse subnucleus; MARN, Magnocellular reticular nucleus; MOp, Primary motor area; MOs, Secondary motor area; MRN, Midbrain reticular nucleus; ORB, Orbital area; PAG, Periaqueductal gray; PARN, Parvicellular reticular nucleus; PIR, Piriform area; PL, Prelimbic area; PO, Posterior complex of the thalamus; PRNr, Pontine reticular nucleus; PSV, Principal sensory nucleus of the trigeminal; py, pyramidal tract; RN, Red nucleus; RSPagl, Retrosplenial area, lateral agranular part; SC, Superior colliculus; SNr, Substantia nigra, reticular part; SPVC, Spinal nucleus of the trigeminal, caudal part; SPVO, Spinal nucleus of the trigeminal, oral part; SSp-bfd, Primary somatosensory area, barrel field; SSs, Supplemental somatosensory area; TEa, Temporal association area; TRN, Tegmental reticular nucleus; VISa, Anterior visual area; VISal, Anterolateral visual area; VPL, Ventral posterolateral nucleus of the thalamus; VPM, Ventral posteromedial nucleus of the thalamus; and ZI, Zona incerta.

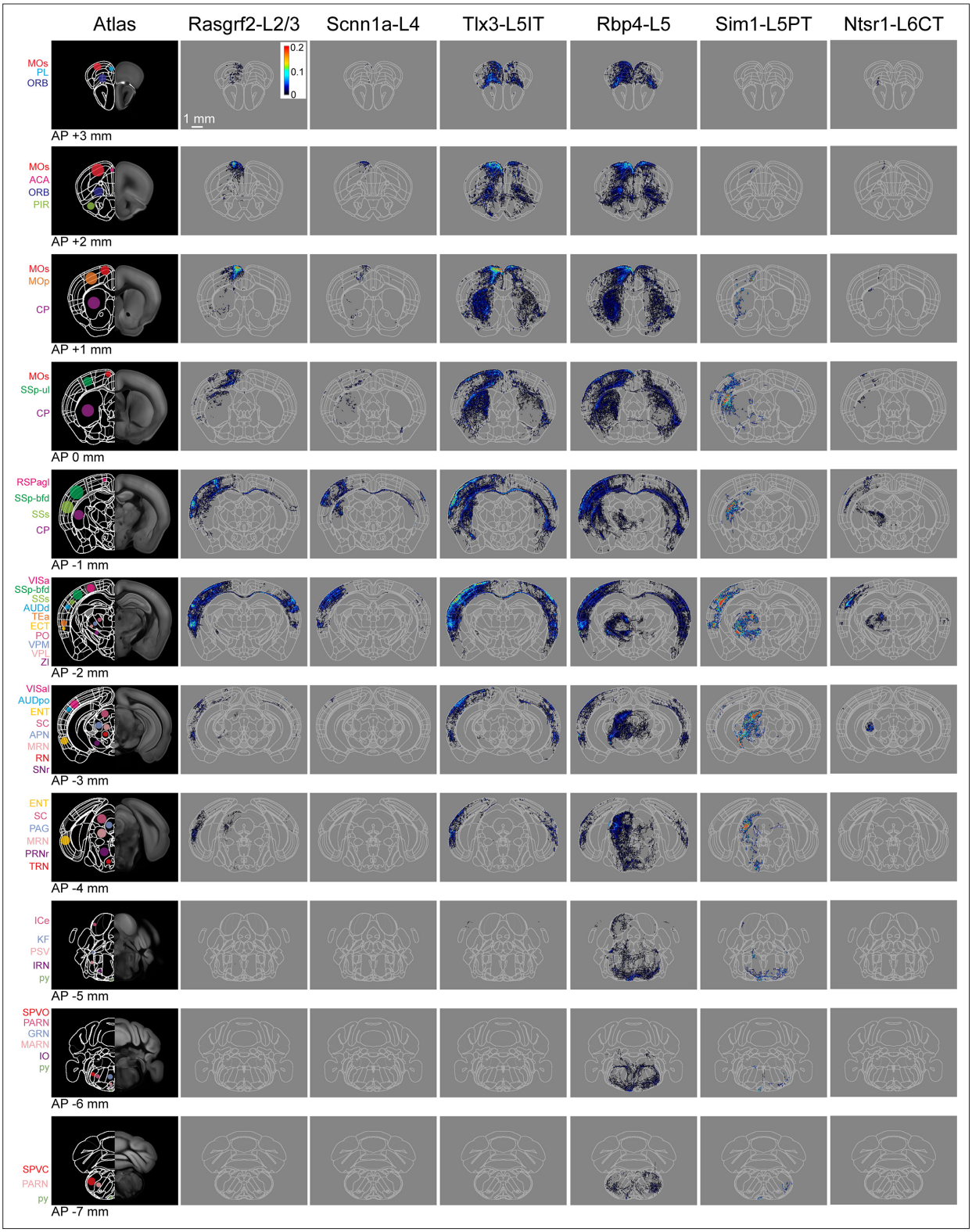

Figure 4—figure supplement 1. Serial coronal sections of group averages from each Cre-line with SSs injections. Same as ***Figure 4***, but for SSs injections. The leftmost column shows the Allen CCF atlas at different AP locations (from AP +3 mm in the top row to AP –7 mm in the bottom row). Some brain regions of interest are labeled with color-coded dots and acronyms. The next columns show the mean axonal density averaged across the injection sites in SSs for each of the six transgenic lines. Each axon image represents a 125 μm sum projection centered around the corresponding AP location. Abbreviations: ACA, Anterior cingulate area; AUDd, Dorsal auditory area; AUDpo, Posterior auditory area; CP, Caudoputamen; ECT, Ectorhinal

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area; ENT, Entorhinal area; GRN, Gigantocellular reticular nucleus; ICe, Inferior colliculus external nucleus; IRN, Intermediate reticular nucleus; KF, Koelliker-Fuse subnucleus; MARN, Magnocellular reticular nucleus; MOp, Primary motor area; MOs, Secondary motor area; MRN, Midbrain reticular nucleus; ORB, Orbital area; PAG, Periaqueductal gray; PARN, Parvicellular reticular nucleus; PIR, Piriform area; PL, Prelimbic area; PO, Posterior complex of the thalamus; PRNr, Pontine reticular nucleus; PSV, Principal sensory nucleus of the trigeminal; py, pyramidal tract; RN, Red nucleus; RSPagl, Retrosplenial area, lateral agranular part; SC, Superior colliculus; SNr, Substantia nigra, reticular part; SPVC, Spinal nucleus of the trigeminal, caudal part; SPVO, Spinal nucleus of the trigeminal, oral part; SSp-bfd, Primary somatosensory area, barrel field; SSs, Supplemental somatosensory area; TEa, Temporal association areas; TRN, Tegmental reticular nucleus; VISa, Anterior visual area; VISal, Anterolateral visual area; VPL, Ventral posterolateral nucleus of the thalamus; VPM, Ventral posteromedial nucleus of the thalamus; and ZI, Zona incerta.

Figure 5. Top 75 brain regions innervated by SSp-bfd and SSs neurons. The total number of axon voxels in various brain regions calculated across the group average for each Cre-line separately for SSp-bfd and SSs injection sites. Ipsilateral (left) and contralateral (right) innervation locations are indicated separately. The target areas were ranked in a descending order with respect to the average innervation density across all injections. The top 75 innervated regions are listed here with full anatomical names of each acronym presented in the leftmost column. Note that the values are represented in logarithmic scale, highlighting regions with little axon.

Figure 5—figure supplement 1. Top 75 brain regions innervated by SSp-bfd and SSs neurons shown on a linear color scale. Same as ***Figure 5***, but here the data are represented on a linear color scale, highlighting regions with dense axonal projections.

Figure 5—figure supplement 2. Axonal density in the isocortex. (**A**) Quantification of axons in the isocortex of the ipsilateral and contralateral hemispheres across all group averages. Regions are arranged such that functionally-related areas are placed together. Note the color-coded intensity scale is logarithmic. (**B**) Comparison of the number of isocortex regions in the contralateral hemisphere vs the ipsilateral hemisphere innervated by SSp-bfd (*left*) and SSs (*right*) samples. Regions with axon values below the 25th percentile across all data were considered as not being innervated.

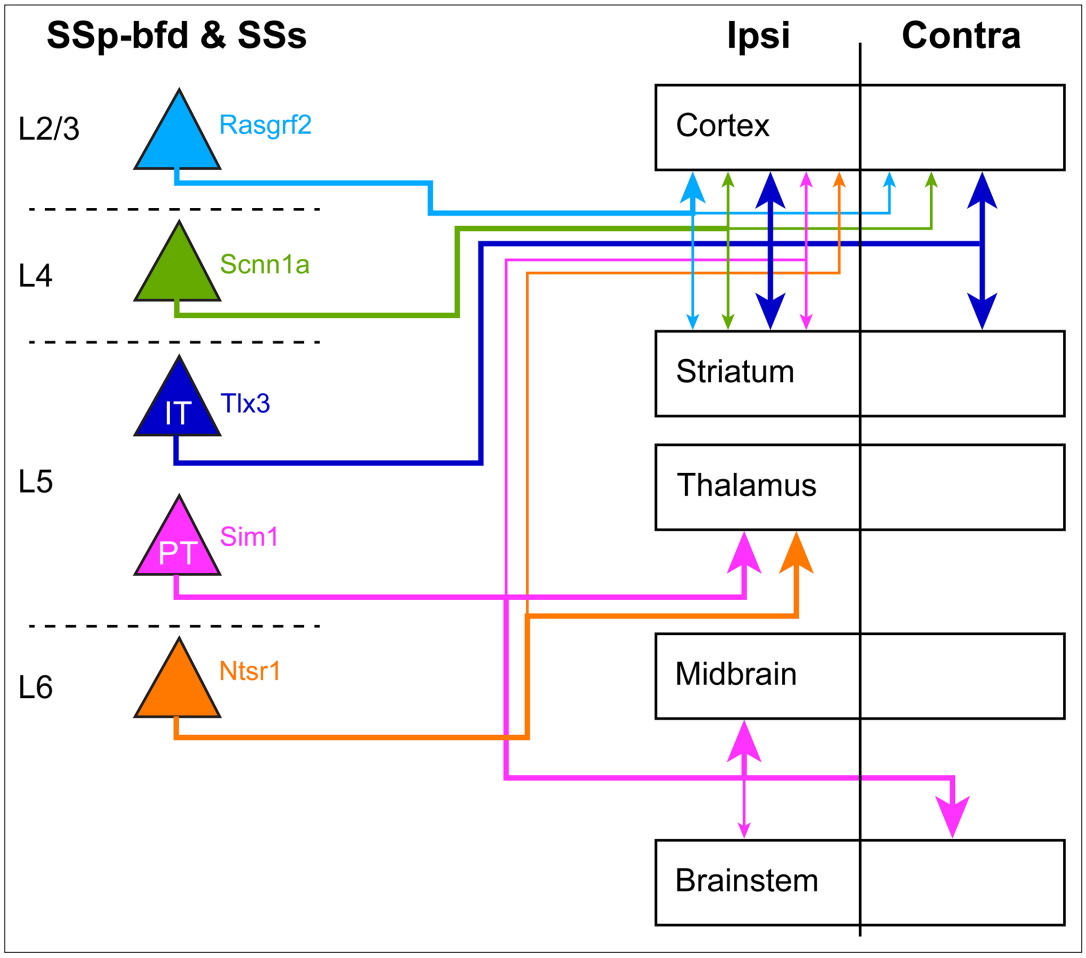

Figure 5—figure supplement 3. Schematic summary of long-range connectivity. Here we schematically represent the long-range projection patterns of the different genetically-defined classes of neurons in a highly simplified manner, which fails to capture the richness of the full data set, but might nonetheless serve as a useful high-level overview of some important aspects of the cell class-dependent connectivity.

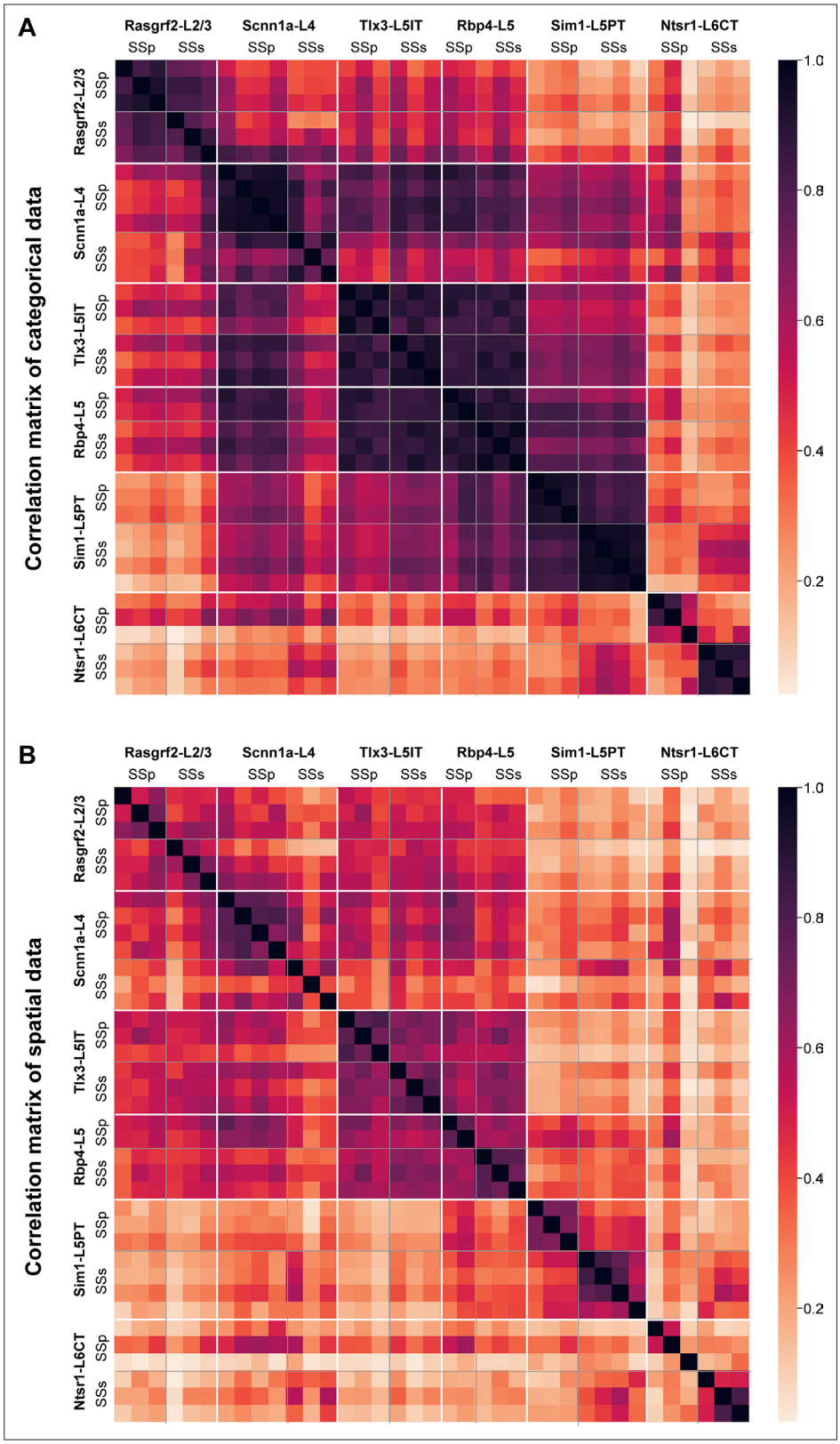

Figure 6. High correlations of axonal projection patterns between samples from the same mouse Cre-lines. (**A**) Pearson's correlation computed across injection sites for the total axonal innervation of each brain region parcellation of the Allen CCF at the most detailed level (such as various layers of the cerebral cortex). The samples were ordered first by Cre-lines, and then by mediolateral locations of the injection site center, with the most

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medial injection being first. (**B**) Similar to panel A, but computed using only spatial information obtained from the 25 μm resolution 3D stacks of axonal density in the Allen CCF (i.e. we did not use any parcellation annotations of the Allen CCF in this computation). The 3D stacks were first Gaussian filtered and then flattened to calculate correlations.

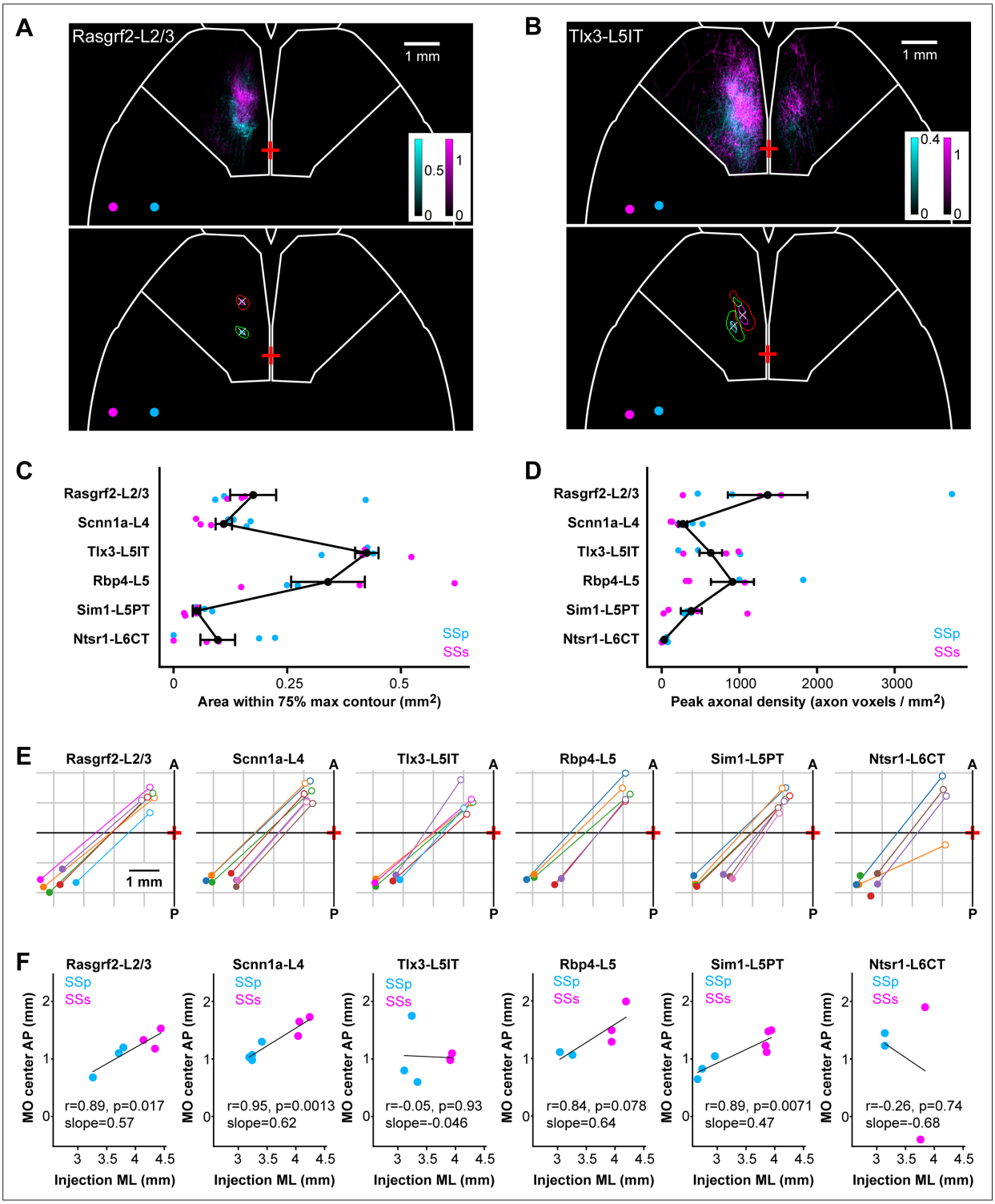

Figure 7. Mirror-reflected mapping of mediolateral neuronal location in somatosensory cortex with the anteroposterior location of the axonal innervation hotspot in motor cortex. (**A**) Example images of MO axons from two Rasgrf2-L2/3 injections represented with cyan (SSp-bfd injection site) and magenta (SSs injection site), and their centers of injection sites represented as circles with corresponding colors (above). Axons in layers 2/3 and 5 of motor cortex (MOp and MOs) were sum-projected in a horizontal view and axonal density per 25 x 25 µm pixel per labeled neuron represented on a color scale. Pixels with intensities ≥75% or≥95% of maximum intensity were segmented and a centroid of the 95% max segmentation was computed. Contours at 75% max (green and red) and 95% max (cyan and magenta) of the MO axons of the two injections as well as the centroid of the 95% contour (white crosses) were computed to help quantify axonal innervation patterns (below). Bregma is indicated with a red cross at the midline. White outline near the frontal region depicts the boundary for the MO region. (**B**) Same as panel A, but for two Tlx3-L5IT injections. (**C**) The cortical surface area

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within the 75% max contours of axonal innervation in MO for each Cre-line provides a measure of the horizontal spread of the MO innervation. Each dot represents an individual injection site with cyan indicating data from SSp-bfd injections and magenta from SSs injections. Black dots and error bars represent the group averages with standard errors. (**D**) Same as panel C, but indicating the sum-projected axonal density within a 225 x 225 µm ROI centered on the location of the peak axonal density. This is a measure of the peak innervation density in MO. (**E**) Center of injection sites and centroids of 95% contours of MO innervation mapped in the horizontal plane for each Cre-line. Each injection site and projection target is represented with the same color and a line drawn to connect the injection site center (filled circles) and its corresponding MO innervation center (open circles) in order to visualize a map reflected along the axis from anterolateral to posteromedial. Note that two injection sites from the Ntsr1-Cre transgenic line did not have any axons in MO. (**F**) Linear regression to measure the correlation between mediolateral positions (using the absolute values) of the injection site center and the anteroposterior positions of the MO innervation centers for each Cre-line (Rasgrf2-L2/3: r=0.89, p=0.017, slope = 0.57; Scnn1a-L4: r=0.95, p=0.0013, slope = 0.62; Tlx3-L5IT r=–0.05, p=0.93, slope = –0.046; Rbp4-L5: r=0.84, p=0.078, slope = 0.64; Sim1-L5PT: r=0.89, p=0.0071, slope = 0.47; and Ntsr1-L6CT: r=–0.26, p=0.74, slope = –0.68). Cyan data points show SSp-bfd injections whereas magenta data points are from SSs injections. Two Ntsr1-L6CT samples did not have any axon in MO, leaving only 4 data points for this mouse line.

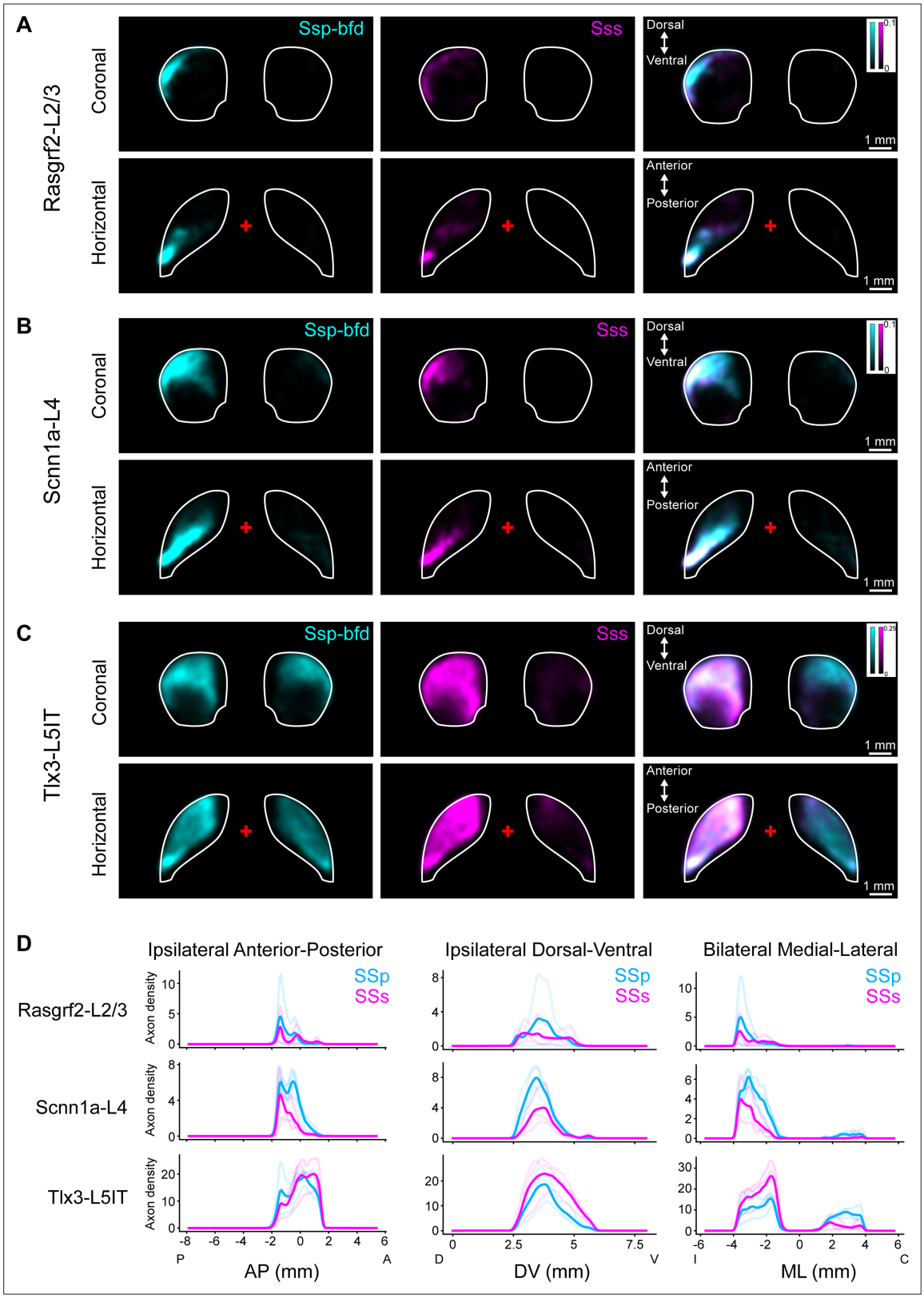

Figure 7—figure supplement 1. Cell class-specific axonal density in the striatum. (**A**) Axons in the caudoputamen from Rasgrf2-L2/3 neurons. *Left*, sum projections in coronal (*above*) and horizontal (*below*) views of Ssp-bfd (cyan) group average. *Middle*, sum projections in coronal and horizontal views of SSs (magenta) group average. *Right*, merge of Ssp-bfd and SSs group averages. Images are Gaussian filtered and red crosses in horizontal views indicates bregma. Axonal density from Rasgrf2-L2/3 neurons is concentrated in the most lateral, posterior, and dorsal part of the ipsilateral caudate

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putamen, displaying a 'crescent' shape in the coronal views. (**B**) Similar to panel A, but for Scnn1a-L4 neurons. The axonal projection patterns remain in the ipsilateral caudoputamen for Scnn1a-L4 neurons, but are somewhat broader than for Rasgrf2-L2/3 neurons. (**C**) Similar to panel A, but for Tlx3-L5IT neurons. The ipsilateral caudoputamen is broadly innervated by both SSp-bfd and SSs Tlx3-L5IT neurons. However, SSp-bfd Tlx3-L5IT neurons show greater innervation in the contralateral striatum compared to SSs Tlx3-L5IT neurons. (**D**) Sum axon intensity along the anterior-posterior axis and dorsal-ventral axis on the ipsilateral side, as well as bilateral medial-lateral axis were computed for each Rasgrf2-L2/3, Scnn1a-L4, and Tlx3-L5IT sample. Semi-transparent lines indicate individual samples, solid lines represent group averages for SSp-bfd (cyan) and SSs (magenta) injections.

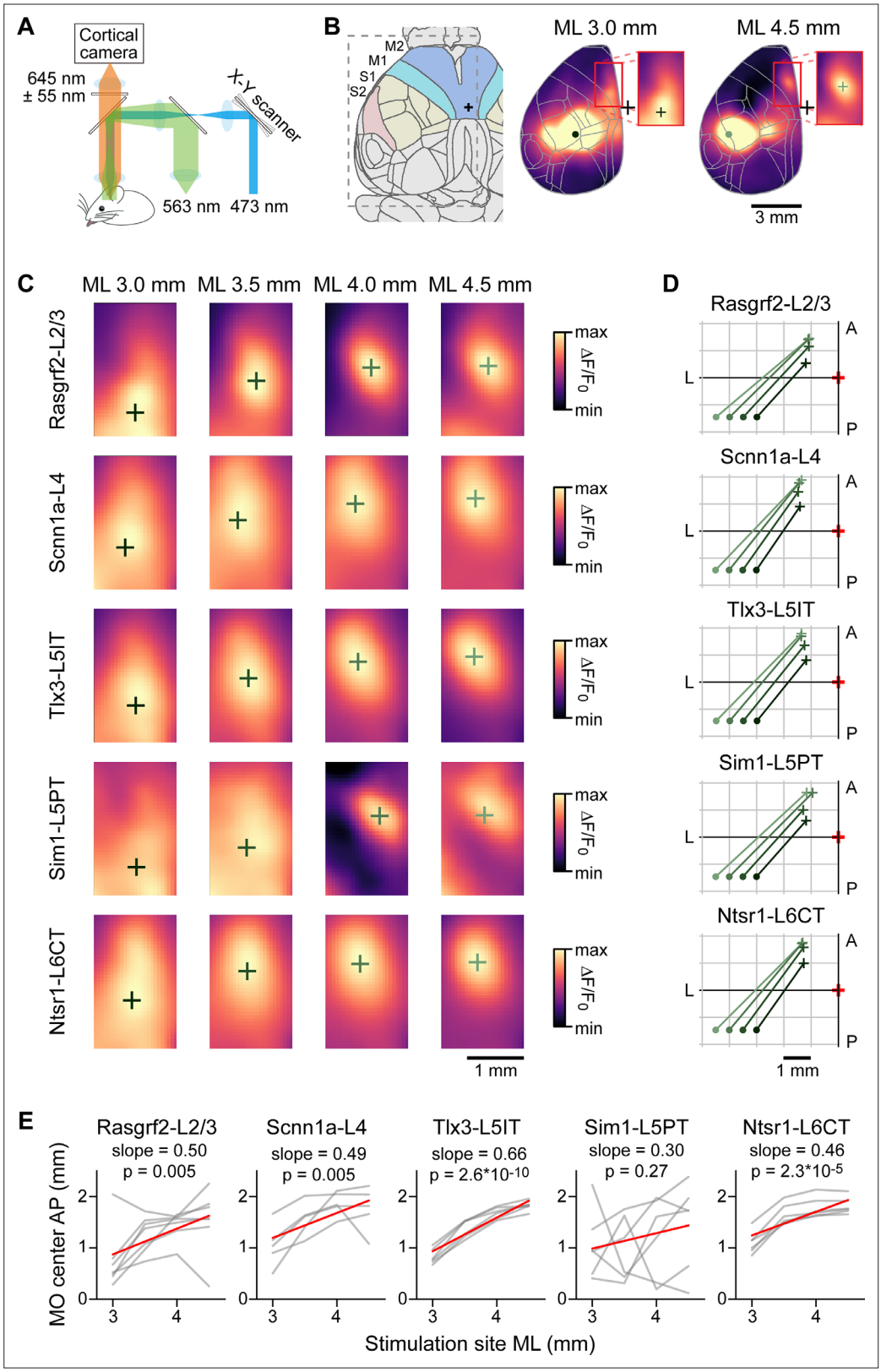

Figure 8. Optogenetic stimulation and wide-field calcium imaging support the hypothesis of reflected functional maps of sensory cortex in motor cortex. (**A**) Schematic of the experimental apparatus. Awake mice were trained to sit comfortably under a wide-field fluorescence macroscope with their head fixed to a metal pole. jRGECO1a excitation light (563 nm) traveled through a series of dichroic mirrors towards the transparent skull of the mice.

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Emission light was bandpass filtered (590–700 nm) and collected by an sCMOS camera. Interleaved with imaging frames, a 473 nm laser pulse train was directed by x-y galvoscan mirrors to generate an ~500 µm diameter cortical spot to photostimulate cell class-specific ChR2-expressing neurons. (**B**) Schematic of the Allen CCF parcellations of dorsal cortex with a 24 degree rotation around the anteroposterior axis (left). Example average functional images after stimulation for Rasgrf2-L2/3 neurons located 3 mm lateral to bregma (center) or 4.5 mm lateral to bregma (right). The red boxes (center and right panels) delineate the region used for the calculation of the center of mass. (**C**) Determination of centers of mass after stimulation of 4 cortical locations averaged for each mouse line. Stimulated points span 1.5 mm horizontally from SSp-bfd to SSs separated by 0.5 mm. The color scale is adjusted from min to max for each image. (**D**) Summary plot of the locations of the centers of mass in MO after stimulating each point in SSp-bfd and SSs, computed from the across mouse average images shown in panel C. (**E**) Correlation between mediolateral location of the stimulation points and the anteroposterior distribution of the centers of mass in MO. Individual gray lines correspond to individual mice and red lines represent the Pearson's correlation trendline calculated over the population. For Rasgrf2-L2/3 neurons: n=7 mice, r=0.52, p=0.005, slope = 0.50; Scnn1a-L4 neurons: n=5 mice, r=0.60, p=0.005, slope 0.49; Tlx3-L5IT neurons: n=6 mice, r=0.92, p=2.6 x 10^{-10}, slope 0.66; Sim1-L5 neurons: n=6 mice, r=0.25, p=0.27, slope 0.30; and Ntsr1-L6CT neurons: n=6 mice, r=0.75, p=2.3 x 10^{-5}, slope = 0.46.

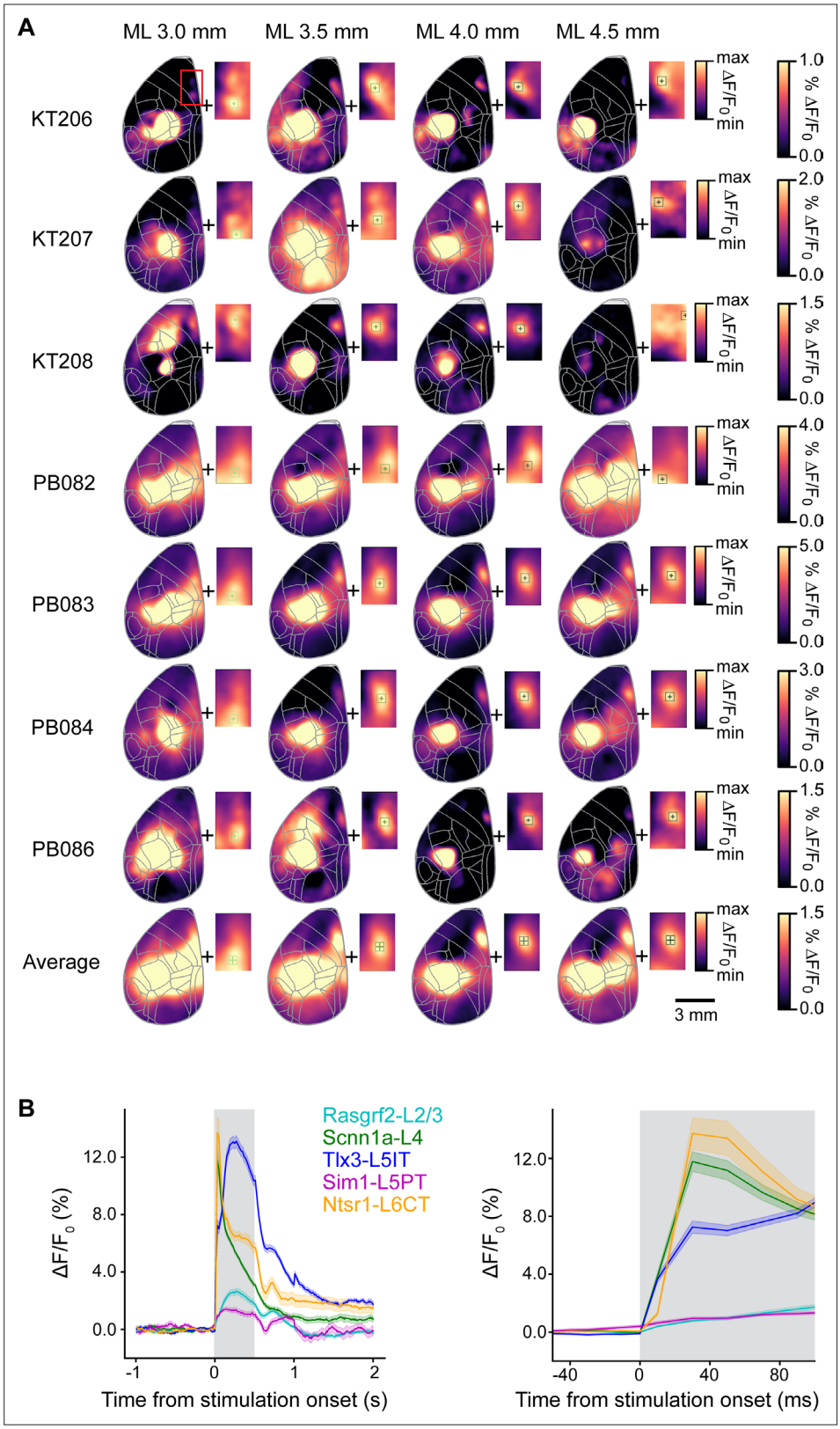

Figure 8—figure supplement 1. Individual example experiments and temporal dynamics of optogenetic stimulation combined with wide-field calcium imaging. (**A**) Data from the individual mice that comprise the average calcium image in Rasgrf2-L2/3 mice shown in ***Figure 8***. Color scales represent $\Delta F/F_0$ for each row of cortical images. Insets represent the region used to calculate the center of mass, with color scale adjusted from min to

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max. Crosses indicate center of mass and boxes the regions of interest used for quantification. (**B**) Grand average time course of $\Delta F/F_0$ for each mouse line computed around the center of mass of the frontal hotspot across the 3 s trial period (*left*) and at higher temporal resolution showing the first 100 ms following the onset of the 50 Hz optogenetic stimulus (*right*). The shaded gray area indicates the optogenetic stimulation period.