Supplementary Information for

Mapping Mesoscale Axonal Projections in the Mouse Brain Using A 3D Convolutional Network

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Other supplementary materials for this manuscript include the following:

Movies S1 to S3
**Fig. S1.** Viral-genetic strategies for axon labeling, clearing methods, and specs for network training. (A) Viral-genetic strategies used for labeling axons used in training and testing. Top row: three serotonergic neuron labeling strategies used for creating the network training data. Top right, strategy used to generate the whole-brain data in Figure 3. Bottom row: additional strategies used to label axons for transfer learning training data and as seen in Figure 4 A-B, C, and D respectively. (B) Left, iDISCO+ clearing protocol does not remove autofluorescent fiber tracts in striatum while AdipoClear (right) performs better. Z-projection 285 µm; scale bar, 100 µm. (C) Training set (blue) and validation set (magenta) loss across 188 epochs. Gray shading indicates a positive slope in the validation loss and overfitting to the training set. (D) Same as in C, except showing the F1 score; see Materials and Methods for equations for metrics. (E) Validation loss for each of five training runs with variable intensity scaling factors during data augmentation. Without Z-score normalization of training data, we used a random scaling factor between zero and the depicted values. Higher values tended to increase the validation loss minimum. The magenta trace is the same as in C and D.
**Fig. S2.** Comparisons of TrailMap to Ilastik classifiers and models trained without all annotation categories. (A) Additional examples comparing four separate Ilastik classifiers with TrailMap. From left to right: 2D classifier, trained on iDISCO+ cleared brains; 3D classifier trained with only axon and background annotations; 3D classifier trained with axons, background, artifact, and edges annotations; 2D classifier trained with every slice of raw data used in the TrailMap training set; segmentation of the 2D Ilastik classifier at $p > 0.5$; raw data; TrailMap armatures; and TrailMap probabilities. Colormaps as in Main Figure 2. (B) Top, performance of DeepNeuron (left) and TrailMap (right) on neurites imaged by fMOST. DeepNeuron-detected neurite signals shown in magenta and pruned connections in black. Red arrowhead marks a TrailMap false negative and blue arrowhead marks a dim axon missed by DeepNeuron. Bottom, performance of TrailMap and DeepNeuron on axons imaged by lightsheet. Left, DeepNeuron neurite connections before (magenta) and after (black) Smart Pruning. Arrowheads show false negatives as described above. (C) Example annotations from two separate users. The bright pixels are the manual annotation and dim pixels are automatically generated for the edge label. User 2 labeled more conservatively, with 94.53% of their annotation falling within the User 1 + edges zone. 74.75% of
User 1 annotations fall within the User 2 + edges zone. \( n = 8 \) substacks, 41 slices. (D) TrailMap output examples when excluding artifacts, edges, or both from the weighted loss equation. Arrowheads mark false negatives when excluding edges from the training data (top left) and false positives when excluding artifacts from training data (bottom center).
Fig. S3. Serotonergic axons across a whole brain as seen in Fig. 3A. Each panel represents 500 µm of Z-depth in the coronal axis, color-coded by depth as indicated in the top left.
Fig. S4. Example region-based quantification of axon density. (A) Whole-brain analysis of axon density in 2 example brains in each of 244 brain regions, clustered by major anatomical subdivisions. Density values represent axon content as a percentage of each region’s volume, normalized to the total axon content in each example brain. Brain A is the same as seen in Fig. 3 and Brain B is from a dataset used in (1). (B) Breakout comparison of innervation by layer in the summed total axon content in eight somatosensory cortical regions. Values normalized only to total brain axon content. (C) Representative slab (0.5 mm thickness) highlighting the overlaid projections of axons from the two brains described in (A); two of the eight regions corresponding to somatosensory cortex are indicated.
**Fig. S5.** (A) Fully connected, extracted serotonergic fibers in the region of dorsal raphe, sagittal view. The color code corresponds to distance along the fibers from a seed point in the axon fiber bundle exiting the image at the lower left. Cell soma positions are marked generally by “DR” and the fourth ventricle by “4V.” While false connections and false breaks prevent true tracing, the known anatomy in this region suggests that dendrites are accurately labeled with colors that place them further from the axon seed point than their cell somas. Color scale 0–1.7 mm, scale.
bar 300 µm. (B) Thalamocortical axons as seen in Fig. 4C. Top, raw data; middle, TrailMap extracted armature; bottom, Ilastik (2D classifier) probability map. All images are XZ-projections, scale bar 100 µm. (C) Center, neuronal processes in a Drosophila ventral nerve cord labeled by GFP (2), maximum intensity projection of 24 optical slices acquired by confocal. Left, TrailMap-extracted fibers. Right, merged view of TrailMap (red) and GFP (black) signals shows the complete coverage of thin GFP+ structures. The remaining black represents larger structures not extracted by TrailMap (e.g., cell bodies). (D) TrailMap thinning strategy applied to the segmentation (left) of a single Drosophila antennal lobe local interneuron (3) highlights the underlying structural armature (right). (E) WGA-stained vasculature imaged by lightsheet microscopy (4) and extracted by TrailMap. Red arrowhead indicates an unextracted structure of a larger size than presented to TrailMap during training. Blue arrowhead indicates dim capillaries extracted by TrailMap, but not included in the ground truth labeling. Right, ground truth labeling of vasculature as compared to TrailMap segmentation.
**Movie 1.** Rotation of the 3D whole-brain collateralization pattern of serotonergic axons as seen in Fig. 3D. Cortex and hippocampus are isolated and removed to better display the patterns of axons in the underlying structures. Axons of individual subregions are color coded as in Fig. 3.

**Movie 2.** Subregion-specific axons from right-hemisphere amygdala, as seen in Fig. 3E. Amygdala subregion color code matches Fig 3.

**Movie 3.** Extracted axons in dual-hemisphere thalamus, as seen in Fig. 4B. Thalamus color code: orange axons are present in sensory-motor cortex-related regions while pink axons are present in the polymodal association cortex-related regions.

**SI References**