Mapping mesoscale axonal projections in the mouse brain using a 3D convolutional network

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The projection targets of a neuronal population are a key feature of its anatomical characteristics. Historically, tissue sectioning, confocal microscopy, and manual scoring of specific regions of interest have been used to generate coarse summaries of mesoscale projectomes. We present here TrailMap, a three-dimensional (3D) convolutional network for extracting axonal projections from intact cleared mouse brains imaged by light-sheet microscopy. TrailMap allows region-based quantification of total axon content in large and complex 3D structures after registration to a standard reference atlas. The identification of axonal structures as thin as one voxel benefits from data augmentation but also requires a loss function that tolerates errors in annotation. A network trained with volumes of serotonergic axons in all major brain regions can be generalized to map and quantify axons from thalamocortical, deep cerebellar, and cortical projection neurons, validating transfer learning as a tool to adapt the model to novel categories of axonal morphology. Speed of training, ease of use, and accuracy improve over existing tools without a need for specialized computing hardware. Given the recent emphasis on genetically and functionally defining cell types in neural circuit analysis, TrailMap will facilitate automated extraction and quantification of axons from these specific cell types at the scale of the entire mouse brain, an essential component of deciphering their connectivity.

Volumetric imaging to visualize neurons in intact mouse brain tissue has become a widespread technique. Light-sheet microscopy has improved both the spatial and temporal resolution for live samples (1, 2), while advances in tissue clearing have lowered the barrier to imaging intact organs and entire organisms at cellular resolution (3, 4). Correspondingly, there is a growing need for computational tools to analyze the resultant large datasets in three dimensions. Tissue clearing methods such as CLARITY and iDISCO have been successfully applied to the study of neuronal populations in the mouse brain, and automated image analysis techniques have been developed for these volumetric datasets to localize and count simple objects, such as cell bodies of a given cell type or nuclei of recently active neurons (5–8). However, there has been less progress in software designed to segment and quantify axonal projections at the scale of the whole brains.

As single-cell sequencing techniques continue to dissect heterogeneity in neuronal populations (e.g., refs. 9–11) and as more genetic tools are generated to access these molecularly or functionally defined subpopulations, anatomical and circuit connectivity characterization is crucial to inform functional experiments (12, 13). Traditionally, and with some exceptions (e.g., refs. 14, 15), “projectome” analysis entails qualitatively scoring the density of axonal fibers in manually defined subregions selected from representative tissue sections and imaged by confocal microscopy. This introduces biases from the experimenter, including which thin tissue section best represents a large and complicated three-dimensional (3D) brain region; how to bin axonal densities into high, medium, and low groups; whether to consider thin and thick axons equally; and whether to average or ignore density variation within a target region. Additionally, it can be difficult to precisely align these images to a reference atlas (16). Volumetric imaging of stiff cleared samples and 3D registration to the Allen Brain Institute’s Common Coordinate Framework (CCF) has eliminated the need to select individual tissue sections (17). However, without a computational method for quantifying axon content, researchers must still select and score representative two-dimensional (2D) optical sections (18).

The automated identification and segmentation of axons from 3D images should circumvent these limitations. Recent application of deep convolutional neural networks (DCNNs) and Markov random fields to biomedical imaging have made excellent progress at segmenting grayscale computed tomography and MRI volumes for medical applications (19–23). Other fluorescence imaging strategies including light-sheet, FMOST, and serial two-photon tomography have been combined with software like TeraVR, Vaa3D, Ilastik, and NeuroGPS-Tree to trace and reconstruct individual neurons (24–28). However, accurate reconstruction often requires a technical strategy for sparse labeling. While reconstructions of sparsely labeled axons are informative for measuring projection variability among single cells, they are usually labor-intensive and underpowered when interrogating population-level statistics and rare collateralization patterns.

**Significance**

Simple, high-resolution methods for visualizing complex neural circuitry in 3D in the intact mammalian brain are revolutionizing the way researchers study brain connectivity and function. However, concomitant development of robust, open-source computational tools for the automated quantification and analysis of these volumetric data has not kept pace. We have developed a method to perform automated identifications of axonal projections in whole mouse brains. Our method takes advantage of recent advances in machine learning and outperforms existing methods in ease of use, speed, accuracy, and generalizability for axons from different types of neurons.


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Data deposition: Example image files, tutorials, and associated code for data handling, model training, and inference, as well as weights from the authors’ best model are available at https://github.com/AlbertPun/TrailMap.

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As one of the most successful current DCNNs, the U-Net architecture has been used for local tracing of neurites in small volumes and for whole-brain reconstruction of brightly labeled vasculature (29–33). To identify axons, a similarly regular structural element, we posited that a 3D U-Net would be well suited for the challenges of a much lower signal-to-noise ratio, dramatic class imbalance, uneven background in different brain regions, and difficult annotation strategy. In addition, whole-brain imaging of axons necessitates the inclusion of any artifacts that contaminate the sample. The paired clearing and analysis pipeline we present here mitigates the impact of autofluorescent myelin and nonspecific antibody labeling that interfere with the detection of thin axons. Our network, TrailMap (Tissue Registration and Automated Identification of Light-sheet Microscope Acquired Projectomes), provides a solution to all of these challenges. We demonstrate its generalization to multiple labeling strategies, cell types, and target brain regions. Alignment to the Allen Institute reference atlas allows for visualization and quantification of individual brain regions. We have also made available our best trained model (https://github.com/AlbertPun/TRAILMAP) such that any researcher with mesoscale projections in cleared brains can use TrailMap to process their image volumes or, with some additional training and transfer learning, adapt the model to their data.

**Results**

**TrailMap Procedures.** To generate training data, we imaged 18 separate intact brains containing fluorescently labeled serotonergic axons (SI Appendix, Fig. S1A). From these brains, we cropped 36 substacks with linear dimensions of 100 to 300 voxels (Fig. L4). Substacks were manually selected to represent the diversity of possible brain regions, background levels, and axon morphology. As with all neural networks, quality training data are crucial. Manual annotation of complete axonal structures in three dimensions is difficult, imprecise, and laborious for each volume to be annotated. One benefit of the 3D U-Net is the ability to input sparsely labeled 3D training data. We annotated 3 to 10 individual XY planes within each substack, at a spacing of 80 to 180 μm between labeled slices (Fig. L4). Drawings of axons even included single labeled voxels representing the cross-section of a thin axon passing through the labeled slice. In the same XY slice, a second label surrounding the axon annotation (“edges”) was automatically generated, and the remaining unlabeled voxels in the slice were given a label for “background.” Unannotated slices remained without a label as previously indicated.

Training data were derived from serotonergic axons labeled by multiple strategies to better generalize the network (SI Appendix, Fig. S1B). As serotonin neurons innervate nearly all forebrain structures, they provide excellent coverage for examples of axons in regions with variable cytoarchitecture and, therefore, variable background texture and brightness. Our focus on imaging intact

**Fig. 1.** Overview of the TrailMap workflow to extract axonal projections from volumetric data. (A) Annotation strategy for a single subvolume (120 × 120 × 101 voxels). Three planes are labeled with separate hand-drawn annotations for background, artifacts, and axons. The one-pixel-width “edges” label is automatically generated. (B) Basic architecture of the encoding and synthesis pathways of the 3D convolutional U-Net. Microscope volumetric data enters the network on the left and undergoes a series of convolutions and scaling steps, generating the feature maps shown in gray. Skip connections provide higher-resolution intermediate outputs to the decoder. Drawings of axons from the raw data entry are shown in white, with concatenated features in gray. More details are provided in ref. 30. (C) A network output thinning strategy produces skeletons faithful to the raw data but with grayscale intensity reflecting probability rather than signal intensity or axon thickness. XY and XZ projections of one subvolume are shown (122 × 609 × 122 μm). (D) A 2-mm-thick volumetric coronal slab, before and after the TrailMap procedure, which includes axon extraction, skeletonization, and alignment to the Allen Brain Atlas Common Coordinate Framework.
brains required the inclusion of contaminating artifacts from the staining and imaging protocol since these nonspecific and bright artifacts are common in cleared brains and interfere with methods for axon identification. We addressed this in two ways. First, we implemented a modified version of the AdipoClear tissue clearing protocol (34, 35) that reduces the auto-fluorescence of myelin. As fiber tracts composed of unlabeled axons share characteristics with the labeled axons we aim to extract, this reduces the rate of false positives in structures such as the striatum (SI Appendix, Fig. S1B). Second, we included 40 substacks containing representative examples of imaging artifacts and nonspecific background and generated a fourth annotation label, “artifacts,” for these structures.

From this set of 76 substacks, we cropped and augmented 10,000 separate cubes of 64 × 64 × 64 voxels to use as the training set. Our validation set comprised 1,700 cubes extracted from 17 separate substacks, each cropped from one of nine brains not used to generate training data. The U-Net architecture included two 3D convolutions with batch normalization at each layer, 2 × 2 × 2 max pooling between layers on the encoder path, and 2 × 2 × 2 upconvolution between layers on the decoder path (Fig. 1B). Skip connections provide information necessary for recovering a high-resolution segmentation from the final 1 × 1 × 1 convolution. The final network was trained for 188 epochs over 20 h, but typically reached a minimum in the validation loss 100 times faster. The way the network learns of the way the network learns of the weights of the network output. The most important was the inclusion of a weighted loss function to account for imperfect annotations of thin structures. The cross-entropy calculation for voxels defined as "edges" were valued 30× less in calculating loss than the voxels annotated as axons. Manually labeling structures with a width of a single pixel introduced a large amount of human variability (SI Appendix, Fig. S2C), and deemphasizing the boundaries of these annotations allowed the network’s axon prediction to “jitter” without producing unnatural thick predictions (SI Appendix, Fig. S2D). Relatedly, the total loss equation devalued background (7.5×) and artifact examples (1.875×) with respect to axon annotations—a set of weights that balances false positives and negatives and compensates for class imbalances in the training data (Fig. 2D and SI Appendix, Fig. S2D).

Details of Axonal Projections. To determine TrailMap’s ability to identify axons from intact cleared samples, we tested a whole brain containing axon collaterals of serotonin neurons projecting to the bed nucleus of stria terminalis. With the extracted axonal projection transformed into the Allen Institute reference space, the axon armature could be overlaid on a template to better highlight their presence, absence, and structure in local subregions (Fig. 3A and SI Appendix, Fig. S3). However, it was difficult to resolve steep changes in density or local hotspots of innervation without selectively viewing very thin sections. By averaging axons with a rolling sphere filter, a small hyperdense zone is revealed in amygdala that would have been missed in region-based quantifications (Fig. 3B, arrow). Total axon content in functionally or anatomically defined brain regions could be quantified (SI Appendix, Fig. S4) (36) or otherwise projected and visualized to retain local density information in three dimensions (Fig. 3 C and D and Movie 1).

An added benefit of the transformation into a reference coordinate system is that brain regions as defined by the Allen Institute could be used as masks for highlighting axons in individual regions of interest. As an example, the aforementioned density observed in amygdala is revealed to be contributed by two nearby dense zones in the basomedial and basolateral amygdala, respectively (Fig. 3E and Movie 2). These analyses of forebrain innervation by distantly located, genetically identified serotonergic neurons ensure certainty of axonal identity. However, in anatomical zones surrounding the labeled cell bodies in dorsal raphe, TrailMap does not distinguish among local axons, axons in passage, or intermingled dendritic arbors (SI Appendix, Fig. SS4). Without anatomical predictions, our imaging cannot morphologically distinguish these fiber types.

Generalization to Other Types of Neurons and Brain Regions. Given that TrailMap was trained exclusively on serotonergic neurons, it may not generalize to other cell types if their axons are of different sizes, tortuosity, or bouton density. The network may also fail if cells are labeled with a different genetic strategy or imaged at different magnification. However, our data augmentation provided enough variation in training data to produce excellent results in extracting axons from multiple additional cell types, fluorophores, viral transduction methods, and imaging setups. TrailMap successfully extracted brain-wide axons from pons-projecting cerebellar nuclei (CN) neurons retrogradely infected with AAVretro-Cre and locally injected in each CN with AAVDIO-tdTomato (Fig. 4A). Visualizing the whole brain (Fig. 4A)
or the thalamus (Fig. 4B and Movie 3) in 3D reveals the contours of the contralateral projections of these neurons without obscuring information at the injection site or ipsilateral targets.

We also tested TrailMap on functionally identified thalamocortical axons in barrel cortex labeled by conditional AAV-DIO-CHR2-mCherry and TRAP2 activity-dependent expression of Cre recombinase (37). The higher density of local innervation of these axons necessitates imaging at higher magnification, resulting in a change in axon scale and appearance in the imaging volume. Buoyed by the spatial scaling included in the data augmentation, TrailMap reliably revealed the dense thalamic innervation of somatosensory layer IV and weaker innervation of layer VI (Fig. 4C). In contrast, though the Ilastik classifier performs moderately well on serotonergic axons, it fails to generalize to thalamocortical axons, perhaps owing to the classifier's failure to identify layer-I axons; as a result, the trained network used the presence of low-intensity grayscale values outside the brain to influence the prediction for each test cube containing the edge of the sample. Using 17 new training substacks from brains with annotated superficial axons from PFC cortical projections and 5 new validation volumes, we performed transfer learning using our best model as the initial weights. After just five epochs, the model successfully identified these layer-I axons (Fig. 4D).

While TrailMap’s training and test data all originated from cleared mouse brains imaged by light-sheet microscopy, the network can also extract filamentous structures from 3D volumes imaged in other species and by other imaging methods. For example, variably bright neuronal processes in a Drosophila ventral nerve cord (38) were imaged by confocal microscopy were all extracted by our model and equalized in intensity by our thinning method (SI Appendix, Fig. S5C). This normalization reveals the full coverage of these fibers, independent of visual distractions from bright cell bodies or thick branches. This same thinning strategy, applied to a single Drosophila antennal lobe interneuron's dendritic arborization (SI Appendix, Fig. S5D), simplified the cell's structure to a wireframe which could aid in manual single-cell reconstructions. Lastly, dye-labeled vasculature (39) in cleared mouse brains also yielded high-quality segmentations of even the smallest and dimmest capillaries (SI Appendix, Fig. S5E), suggesting TrailMap’s utility extends beyond neuronal processes. Notably, all of these results were generated without any retraining of the network. With additional training data, we expect that TrailMap may apply to a wider range of cell types, species, and imaging modalities.

**Discussion**

Here we present an adaptation of a 3D U-Net tuned for identifying axonal structures within noisy whole-brain volumetric data. Our trained network, TrailMap, is specifically designed to extract mesoscale projectomes rather than reconstructions of axons from sparsely labeled individual neurons. For intact brains with hundreds of labeled neurons or zones of high-density axon terminals, we are not aware of a computational alternative that can reliably identify these axons.
Our clearing and image processing pipeline address a number of challenges that have prevented these mesoscale analyses until now. First, all clearing techniques have at least some issues with background or nonspecific labeling that can interfere with automated image analysis. Removing myelinated fiber tracts with a modified AdipoClear protocol greatly improved TrailMap’s precision in structures such as the striatum. Second, our weighted loss function considers manually annotated, nonspecific, bright signals separately.

Fig. 3. Volumetric visualizations highlight patterns of axonal innervation. (A) Coronal Z-projection of extracted serotonergic axons, color-coded by depth (0–500 μm) and overlaid on the CCF-aligned serial two-photon reference atlas. SI Appendix, Fig. S1 details viral-transgenic labeling strategy. (B) The same volumetric slab as in A presented as a density heatmap calculated by averaging a rolling sphere (radius = 225 μm). Green arrow highlights a density hotspot in the amygdala. (C) TrailMap-extracted serotonergic axons innervating forebrain are subdivided and color-coded based on their presence in ABA-defined target regions. (D) Same brain as in C as seen from a dorsal viewpoint, with major subdivisions spatially separated. Midline is represented by a dashed white line. Inset highlights the region indicated by the dashed box in A; Z-projection, 500 μm. (E, Left) Mesh shell armature of the combined structures of the lateral (LA), basolateral (BLA), and basomedial (BMA) amygdala in coronal and sagittal views. (Right) Density heatmap and extracted axons of the amygdala for the same views. LA, BLA, and BMA are color-coded in shades of green/blue by structure, and axonal entry/exit points to the amygdala are colored in red. White arrows highlight the same density hotspot indicated in B.
from other background areas—an essential step in reducing false positives. Relatedly, by devaluing the loss calculated for voxels adjacent to axon annotations, we reduced the rate of false negatives by allowing the network to err by deviations of a single voxel. Third, we present a strategy for thinning TrailMap’s output to construct an armature of predicted axons. One benefit of this thinning technique is a reduction in false breaks along dim axonal segments. Importantly, this benefit relies on the gradients in probability predicted by the cross-entropy-based loss calculation. The resultant armature improves visualizations and reduces biases in analysis and quantification by giving each axon equal thickness independent of staining intensity or imaging parameters.

Aligning armatures and density maps to the Allen Institute’s reference brain atlases which brain regions are preferentially innervated or avoided by a specific projection (36). Given that some brain regions are defined with less certainty, it will be possible to use the axon targeting of specific cell types to refine regional boundaries. TrailMap succeeds at separating thalamocortical projections to individual whisker barrels (Fig. 4C), a brain area with well-defined structure. Thus, it will be interesting to locate other areas with sharp axon density gradients that demarcate substructures within larger brain regions. These collateralization maps will also assist neuroanatomists investigating the efferent projection patterns of defined cell populations.

TrailMap has the added benefit of 3D, intact structures as the basis for quantification, but also the ability to process samples and images in parallel, reducing the active labor required to generate a complete dataset that spans the entire brain. TrailMap code is publicly available, along with the weights for our best model and example data. A consumer-grade GPU is sufficient for both processing samples and for performing transfer learning, while training a new model from scratch benefits from the speed and memory availability from cloud computing services. While 2D drawing tools are sufficient for generating quality training sets and reduce manual annotation time, we suspect that virtual reality-based visualization products would assist labeling strategies in three dimensions, potentially bolstering accuracy even further (24). We hope that TrailMap’s ease of use will lead to its implementation by users as they test brain clearing as a tool to visualize their neurons of interest. We expect that, as neuronal cell types are becoming increasingly defined by molecular markers, TrailMap can be used to map and quantify whole-brain projections of these neuronal types using viral-genetic and intersectional genetic strategies (e.g., ref. 36).

Materials and Methods

Animals. All animal procedures followed animal care guidelines approved by Stanford University’s Administrative Panel on Laboratory Animal Care (APLAC). Individual genetic lines include wildtype mice of the C57BL/6J and

Fig. 4. Generalization to other cell types with and without transfer learning. (A) Dorsal view of posterior brain with extracted axon collaterals from pons-projecting cerebellar nuclei neurons color-coded by their presence in major subregions. Injection sites in the right hemisphere lateral, interposed, and medial CN from the same brain are indicated. (B) Coronal (Top) and sagittal (Bottom) views of the extracted axons (orange) within the structure of thalamus (cyan) from the brain in A. (Scale bar, 500 μm.) Zoomed images (Right) of midline thalamus show exclusion of axons from specific subregions. VPPC, ventral posterior nucleus, parvcellular part; VPM, ventral postero medial nucleus; Po, posterior thalamic nuclear group. Midline shown as dashed vertical line. (Scale bar, 200 μm.) (C) Extracted thalamocortical axons in barrel cortex. XZ-projection of a 3D volume extracted from a flat-mount imaged cortex. Dashed lines indicate upper and lower bounds of cortex. Z-projection, 60 μm. (Scale bar, 100 μm.) (D, Left) Raw image of axons of prefrontal cortex neurons in posterior cortex. VIS, visual cortex; ECT, ectorhinal; ENTl, lateral entorhinal. (Right) Axons extracted by TrailMap model before (green) and after (red) transfer learning. Z-projection, 80 μm. (Scale bar, 100 μm.) Labeling procedure, virus, and transgenic mouse for all panels are described in SI Appendix, Fig. S1 and Methods.
CD1 strains, TRAP2 (Fox-CreER<sup>22</sup>; Jackson, stock no. 002175), and Sert-Cre (MMRRC, stock no. 017260-UCD). Mice were group-housed in plastic cages with disposable bedding on a 12-h light/dark cycle and water and food available ad libitum.

**Viruses.** Combinations of transgenic animals and viral constructs are outlined in <em>SI Appendix</em>, Fig. S1A. Viruses used to label serotoninergic axons include AAV-DJ+hsyn-DIO-HM3D<sub>2</sub>-mCherry (Stanford vector core), AAV-Ef1α-Z/EG-DIO-Eflu (Salk Institute GT3 core; ref. 40), AAV-retro-CAG-DIO-flp (Salk Institute GT3 core; ref. 40), AAV8-CAG-DIO-tsfTomato (UNC vector core, Boyden group), AAV<sub>vesc</sub>-eF1α-mCherry (Salk Institute GT3 core), AAV8-Ef1α-DIO-CHR2-mCherry (Stanford vector core), AAV8-hsyn-DIO-mGFP-2a-pansynaptoysin-mRuby (Stanford vector core, Addgene no. 71760), and Cav-Cre (Eric Kremers, Institut de Généétique Moléculaire, Montpellier, France; ref. 41).

**AdipoClear Labeling and Clearing Pipeline.** Mice were transiently perfused with 20 mL 1× PBS containing 10 μg/mL heparin followed by 20 mL ice-cold 4% PFA and postfixed overnight at 4°C. All steps in the labeling and clearing protocol are on a rocker at room temperature for a 1-h duration unless otherwise noted. Brains are washed 3× in 1× PBS and once in B11 before dehydrating stepwise into 100% methanol (20, 40, 60, 80% steps). Two additional washes in 100% methanol remove all of the water before an overnight incubation in 2:1 dichloromethane (DCM):methanol. The following day, two 100% DCM and methanol steps in methanol precede 4 h in a 5:1 methanol:30% hydrogen peroxide mixture. Stepwise brains are rehydrated into B11 (60, 40, 20% methanol), washed once in B11, and then permeabilized 2× in PTxwH containing 0.3 M glycine and 5% DMSO. Samples are washed 3× in PTxwH before adding primary antibody (chicken anti-GFP, 1:2,000; Aves Labs; rabbit anti-RFP, 1:1,000; Rockland). Incubation is for 7 to 11 d, rocking at 37°C, with subsequent washes also at this temperature. Brains are washed 3× in PTxwH over 12 h and then 1× each day for 2 additional days. Secondary antibody (donkey anti-rabbit, 1:1,000; Thermo; donkey anti-chicken, 1:2,000; Jackson) is incubated rocking at 37°C for 5 to 9 d. Washes were performed 5× in PTxwH over 12 h and then 1× each day for 2 additional days. Samples are dehydrated stepwise into methanol, as before, but with water as the counterpart, then washed 3× in 100% methanol, overnight in 2:1 DCM:methanol, and then 2× 100% DCM the next morning. The second wash in DCM is extended until the brain sinks, before transfer to dibenzyl ether (DBE) in a fresh tube and incubation with 4 h for before storing in another fresh tube of DCM at room temperature. Solutions were as follows: B11, 1:1,000 Triton X-100, 2% wt/vol glycine, 1:10,000 NaOH 10N, 0.02% sodium azide; and PTxwH in 1× PBS, 1:1,000 Triton X-100, 1,000 Tween-20, 2 μg/mL heparin, 0.02% sodium azide. This protocol diverges from the AdipoClear method subtly, by adding and extending the duration of exposure to DCM and by adjusting the duration of some steps. Most importantly, the overnight methanol/DCM steps and the hydrogen peroxide timing are crucial to image quality.

**Light-Sheet Imaging.** Image stacks were acquired with the LaVision Ultra-Light-Sheet Imaging. To improve image quality, the protocol diverges from the AdipoClear method subtly, by extending the duration of exposure to DCM and by adjusting the duration of some steps. Most importantly, the overnight methanol/DCM steps and the hydrogen peroxide timing are crucial to image quality.

**Data Availability.** Example image files, tutorials, and associated code for data handling, model training, and inference, as well as the weights from our best model are all available at https://github.com/AlbertPun/TRAILMAP.

**Model Evaluation.** To evaluate the network, we compared the validation set output by the model to ground truth human annotations. Each pixel was classified to be TP (true positive), TN (true negative), FP (false positive), or FN (false negative). It is important to note that we did not include pixels which were labeled as edges because these were predetermined to be ambiguous cases and would not be an accurate representation of the network’s performance. Using these four classes, we used the following formulas for evaluations.

- **True Positive Rate (TPR)**: \( TP/ (TP+FN) \)
- **False Positive Rate (FPR)**: \( FP/ (FP+TN) \)
- **Precision (P)**: \( TP/(TP+FP) \)
- **Recall (R)**: \( TP/(TP+FN) \)
- **F1 score (F1)**: \( 2 \times \frac{P \times R}{P+R} \)
- **Jaccard index (J)**: \( TP/(TP+FP+FN) \)

Human–human comparison was done by having 2 separate experts annotate the same 41 slices from each of 8 separate substacks before proceeding with the same set of evaluations.

**Skeletization and 3D Alignment to CCF.** Probabilistic volumes from the output of TrailMap were binarized at eight separate thresholds, from 0.2 to 0.9. Each of these eight volumes was skeletonized in three dimensions (skimage-morphology-skeletonize_3d). The logical binary skeleton outputs were each weighted by the initial probability threshold used to generate them and subsequently were summed together. The resulting armature thus retains information about TrailMap's prediction confidence without breaking connected structures by threshold segmentation. Small, truncated, and disconnected objects were removed as previously described (40). We downsamplled this armature from 4.0625 μm per pixel (XY) and 3 μm per pixel (Z) into a 5 × 5 × 5-μm space and also downsamplled the autofluorescence image into a 25 × 25 × 25-μm space. The autofluorescence channel was aligned to the Allen Institute's 25-μm reference brain acquired by serial two-photon tomography. These affine and b spline transformation parameters (modified from ref. 8) were used to warp the axons into the CCF at 5-μm scaling. Once in the CCF, ABA region masks can be implemented for pseudocoloring, cropping, and quantifying axon content on a region-by-region basis. Rolling sphere voxelization with a radius of 45 voxels (225 μm) operates by summing total axon content after binarization.
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