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SNT: a unifying toolbox for quantification of neuronal anatomy

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SNT is an end-to-end framework for neuronal morphometry and whole-brain connectomics that supports tracing, proof-editing, visualization, quantification and modeling of neuroanatomy. With an open architecture, a large user base, community-based documentation, support for complex imagery and several model organisms, SNT is a flexible resource for the broad neuroscience community. SNT is both a desktop application and multi-language scripting library, and it is available through the Fiji distribution of ImageJ.

Quantification of neuronal anatomy is essential for mapping information flow in the brain and classification of cell types in the central nervous system. Although digital reconstruction ('tracing') of the tree-like structures of neurons—axons and dendrites—remains a laborious task, recent improvements in labeling and imaging techniques allow faster and more efficient reconstructions, with neuroscientists sharing more than 145,000 (ref. [1]) reconstructed cells across several databases. Powerful toolboxes have been developed for neuronal morphometry (Supplementary Note). However, such tools can be tethered to specific imaging modalities or remain specialized on specific aspects of neuroanatomy workflows. To address these limitations, we established a unifying framework for neuronal morphometry and analysis of single-cell connectomics for the widely used Fiji and ImageJ platforms[2,3].

We built on the popular Simple Neurite Tracer program[4] to create an open-source, end-to-end solution for semiautomated tracing, visualization, quantitative analyses and modeling of neuronal morphology. All aspects of our software, named SNT—Simple Neurite Tracer's moniker—can be controlled from a user-friendly graphical interface or programmatically, using a wide variety of scripting languages (Fig. 1a).

For semiautomated tracing we implemented a host of features (Supplementary Note), including support for multi-channel and time-lapse images, optimized search algorithms and image processing routines that detect neuronal processes and make it possible to reconstruct simple morphologies directly from thresholded images. With time-lapse sequences, traced paths can be automatically matched across frames so that growth dynamics of individual neurites can be monitored across time (Fig. 1b). To expedite the proof-editing of traced structures, SNT allows users to edit, tag, sort, filter and rank the traced segments, using either ad hoc labels or morphometric traits. Altogether, these features improve reconstruction accuracy and tracing efficiency.

For visualization, SNT features an interactive three-dimensional (3D) viewer dedicated to neuron morphology—Reconstruction Viewer—that is hardware accelerated, supports rendering of meshes and detailed annotation of morphometry data. In addition, SNT also integrates with sciview[5], a visualization tool for mesh-based data and arbitrarily large image volumes, supporting virtual and augmented reality (Supplementary Note).

For data retrieval, SNT provides seamless integration with the ImageJ platform, and thus tracing and reconstruction analyses can be intermingled with image processing workflows. To exemplify this, we used SNT to quantify challenging datasets: expression of synaptic markers along dendrites (Fig. 1c) and fluorescence in situ hybridization imaging of messenger RNA in the same volume in which dendrites of pyramidal cells were reconstructed (Fig. 1d).

Another strength of SNT is that it can connect directly to the major neuroanatomy databases, including FlyCircuit[6], InsectBrain[7], MouseLight[8], NeuroMorpho[1] and Virtual Fly Brain[9], supporting several multi-species brain atlases (*Drosophila*, mouse, zebrafish; Fig. 1a, Supplementary Note and Supplementary Video 1). While such online databases are highly queryable, they remain constrained by website design limitations. Scripting frameworks that can programmatically parse their data bypass those restrictions, facilitate data sharing, scientific reproducibility, bridge isolated data repositories and promote the development of additional tools and features. Since SNT adopts the SciJava framework[10] (Supplementary Video 2) it can be scripted using several computer languages such as Python (and Jupyter notebooks) through pyimagej, Clojure, Groovy, JavaScript, Jython, MATLAB, R, Ruby or Scala.

To demonstrate the analytical power of SNT, we parsed the MouseLight database, currently containing the most complex reconstructions described in the literature[8]. In particular, we focused on quantifying the repertoire of strategies adopted by individual cells to broadcast information across the brain. Since no synaptic strengths are currently known for MouseLight neurons, projection strength to target areas must be inferred from morphometric surrogates. In a programmatic, unbiased approach, we used two morphological criteria (normalized cable length and number of axonal endings) to retrieve the number of anatomical brain areas innervated by individual axons (Fig. 2a). In doing so, we identified two extremes of connectivity: cells that connect exclusively to a single projection brain area, and cells that project broadly over a multitude of brain areas (Fig. 2b). A key feature of SNT is the ability to generate streamlined connectivity diagrams, holding quantitative information determined from the intersection or union of multiple morphometric criteria that can be customized using SNT's interactive tool Graph Viewer. This type of diagram can be generated at the

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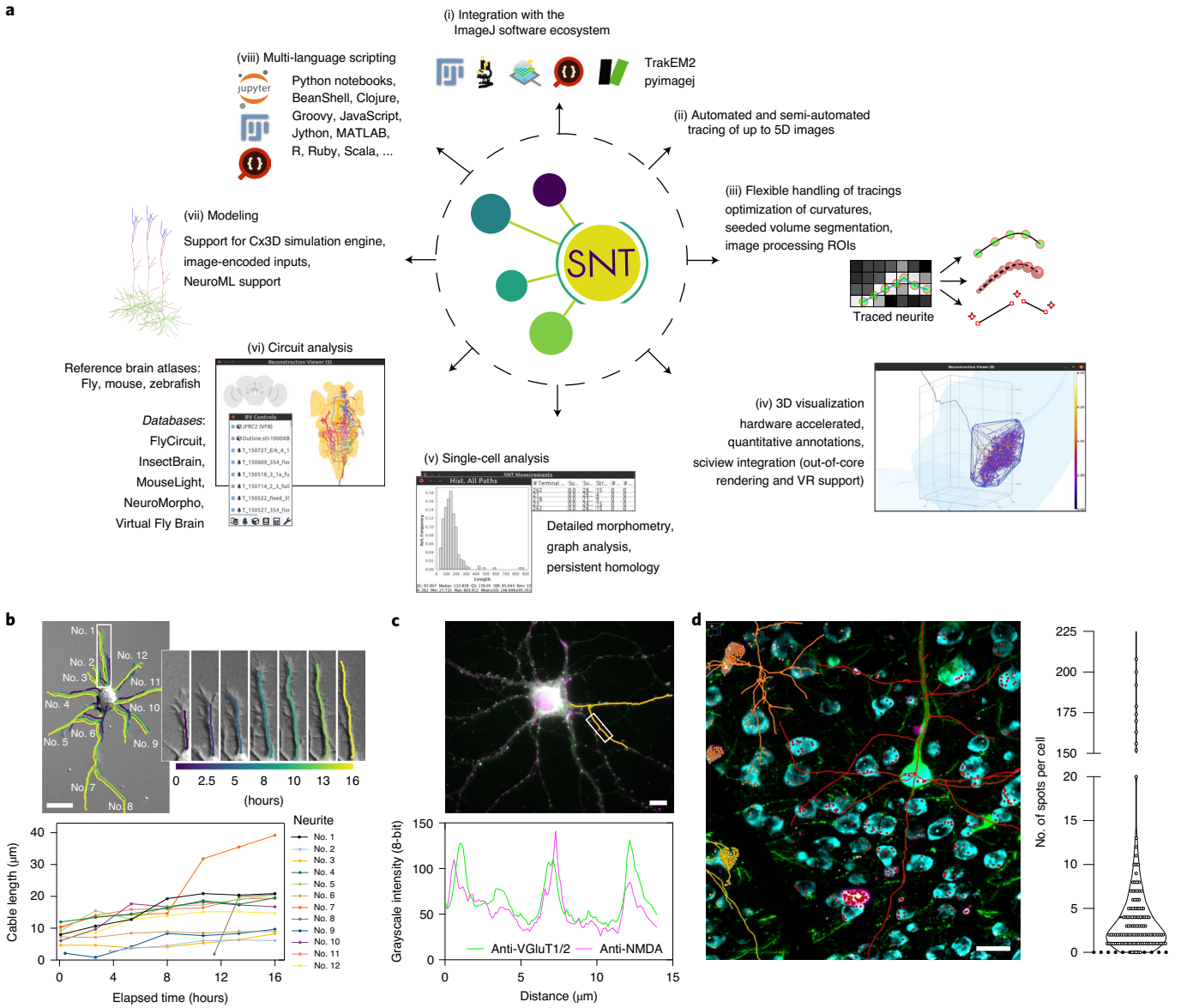

Fig. 1 | SNT as an end-to-end platform for data retrieval, visualization, quantification and modeling of neuroanatomical data. a, Schematic diagram of functionality: (i) SNT is powered by the stack of ImageJ-based software, including: Fiji, ImageJ2, sciview, SciJava, ImgLib2, TrakEM2 and pyimagej. (ii) Reconstructions can be obtained directly from thresholded images or using semiautomated procedures that support time-lapse and multi-channel light-microscopy imagery. (iii) Once center-line reconstructions (tracings) are obtained, they can be conveniently processed in subsequent image processing routines. (iv) Dedicated neuroanatomy viewers allow for effective quantitative visualization of complex data. VR, virtual reality. (v) In addition to single-cell morphometry, (vi) circuit analyses are facilitated through support of several online databases and reference brain atlases (*Drosophila*, mouse and zebrafish). (vii) Biophysical modeling of neuronal growth is achieved through Cx3D integration (Supplementary Note). (viii) Users may use SNT as a standalone interactive program and as a multi-language scripting library. **b**, Static frame from a nonfluorescent time-lapse video monitoring the development of neuron polarity in a rat hippocampal neuron growing in vitro[19]. The 12 highlighted neurites were traced throughout the video sequence and color coded across time as per color ramp. Inset details the growth of neurite no. 1 at selected time points. The plot depicts growth dynamics of individual neurites across time. **c**, Multi-channel image of a rat hippocampal neuron stained in vitro for the presynatic markers VGluT1-2 (green) and the postsynaptic *N*-methyl-D-aspartate receptor (magenta)[20]. Dendrites were traced (orange) and intensity profiles obtained directly from the tracings. Profiled maxima from the marked region depict synaptic locations. **d**, Maximum intensity projection of a three-channel 3D image of a mouse brain section processed for ExFISH. Dendrites of green fluorescent protein-labeled neurons (green) were traced in SNT (center lines for three cells are displayed in orange, red and yellow). Foci reporting on SST mRNA (magenta) were detected on neighboring somata, segmented from a counterstain for total RNA (cyan). Point region of interest (ROI) reporting foci (circles) were labeled with the same hue of the closest traced cell. All procedures were performed within ImageJ. Right, a violin plot of SST expression for segmented cells in the subvolume ($N = 147$ cells). Scale bars **b**,**c**, 10 and **d**, 20 µm.

single-cell level (Fig. 2c) or from cell populations (Supplementary Fig. 1), and is a valuable visualization tool for connectomics[11].

SNT provides support for generative models of artificial neurons by using the neurodevelopmental simulation framework, Cx3D[12]. This not only provides capabilities for the algorithmic generation of neuronal morphologies, but also enables image-based modeling for cellular neuroscience. On the latter, we provide a proof-of-concept example, where artificial neurons are seeded in an image derived

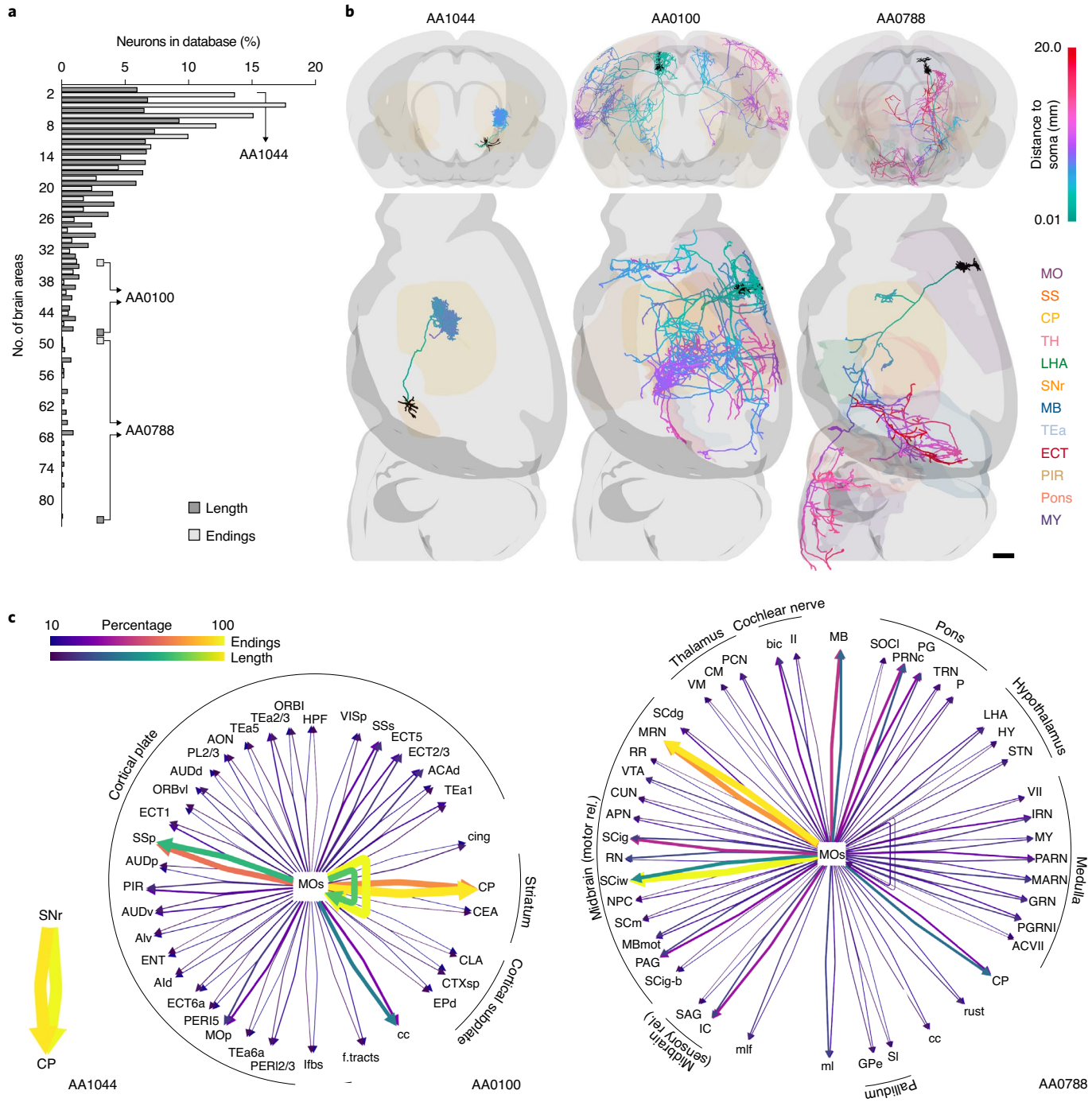

Fig. 2 | Comprehensive analytical tools enable discovery. a, Frequency histogram of number of brain areas innervated by single cells from the MouseLight database. The mouse brain was divided into 141 brain areas, and the presence of axons from each cell in the database (N=1,094) quantified at each area under to criteria: cable length (normalized to size of profiled area) and number of axonal endings at each area. **b**, Examples of axonal connectivity rendered in SNT's Reconstruction Viewer. Left, the SNr neuron projecting exclusively to the caudoputamen (MouseLight ID AA1044). Its single axonal tuft can cover as much as 4% of the ipsilateral lobe of the target area. Center, the SS neuron projecting to many areas in the isocortex (MouseLight ID AA0100). Right, the pyramidal-tract neuron projecting to many areas in the isocortex, midbrain and hindbrain (MouseLight ID AA0788). Dendrites are depicted in black and axonal arbors color coded by 'path distance to soma', as per the color ramp legend. Selected brain regions are depicted according to color-coded abbreviations (MO, somatomotor areas; SS, somatosensory areas; CP, caudoputamen; TH, thalamus; LHA, lateral hypothalamic area; SNr, substantia nigra, reticular part; MB, midbrain; TEa, temporal association areas; ECT, ectorhinal area; PIR, piriform area and MY, medulla). Scale bar, 1 mm. **c**, Connectivity diagrams for the three examples in **b** were programmatically generated in SNT's Graph Viewer. In this 'Ferris wheel' diagram, the neuron's target areas are displayed around the brain area associated with the cell soma (SNr and MOs (secondary motor area)), with connecting edges indicating projection strength and self-connecting edges depicting local innervation. Edges were scaled and color coded according to the two criteria used in **a**, as per color ramp legend. This representation can also be extended to cell populations (Supplementary Fig. 1). When generating such diagrams, SNT automatically sorts target areas by projection strength and groups them by parental ontology (labeled in external arcs). As a reference, the total axonal lengths of each cell are: 28.598 cm (AA1044), 44.649 cm (AA0100) and 18.454 cm (AA0788). Abbreviations reflect the Allen Mouse Brain Common Coordinate Framework[21] nomenclature.

from an in vitro chemotaxis assay (Supplementary Video 3). On the former, we challenged SNT's ability to morphometrically distinguish closely related reconstructions. First, we generated different mathematical gene-regulatory networks (GRNs)[13] capable of controlling neuronal growth by regulating extension, branching and directionality of neurites to define in silico morphologies. Second, we generated thousands of artificial neurons constrained by these patterns. Third, we used built-in metrics[14,15] to statistically differentiate between these computer-generated 'neuronal types'. We found we could distinguish with high confidence all of the morphological classes, including those closely related (Supplementary Note). Altogether, these experiments demonstrate how SNT can bridge experimental and modeled data to support model evaluation for both inference and predictive modeling.

In summary, SNT is a powerful tool for tracing, proof-editing, visualization, quantification and modeling of neuroanatomy. It is based on recent technologies, supports modern microscopy data, integrates well with the ImageJ platform, interacts with major online repositories and synergizes with postreconstruction analysis software and recent data-mining frameworks[16,17]. With a large user base and thorough community-based documentation (https://imagej.net/SNT), SNT is an accessible, scalable and standardized framework for efficient quantification of neuronal morphology. SNT can be installed by subscription to Fiji's 'Neuroanatomy' update site. The source code is available at https://github.com/morphonets/SNT (ref. [18]).

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-021-01105-7.

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Methods

The figures and analyses from this manuscript can be generated programmatically (see Data availability for details). Technical details and benchmarks are included in Supplementary Note.

Programming. SNT was programmed with Eclipse Java IDE v.4.4–4.16 (Eclipse Foundation), IntelliJ IDEA 2020 (JetBrains) and Fiji's built-in Script Editor on an Intel i7 laptop running Ubuntu v.18.10–20.04.

Cell image library imagery. Analyses were performed manually from SNT's graphical user interface with the following modifications to the original images: CIL701 (ref. [19]) (an unannotated *Z*-series) was converted to a time-series stack and CIL810 (ref. [20]) (an RGB image) was converted to a multi-channel composite. Please refer to the original publications for details on the datasets.

Expansion fluorescence in situ hybridization (ExFISH). For histology, ExFISH was performed using an optimized protocol[22]. In short, 150 µm-thick cortical slices of an heterozygous Thy-1-GFP-M[23] adult mouse were embedded in a hydrogel using standard embedding procedures for thick tissue[24] mRNA was detected using HCR v.3.0 (ref. [25]). somatostatin (SST) probes and fluorescent hairpins were obtained from Molecular Instruments (molecularinstruments.com). Gelled sections (roughly 2× expanded) were imaged in PBS on a commercial Zeiss Z1 lightsheet microscope. For spot quantification, the signal from total RNA labeling was segmented using Labkit (https://imagej.net/Labkit). Individual cells were then masked using watershed filtering and labeling of connected components using MorphoLibJ[26]. Ill-segmented somata were manually eliminated with the aid of BAR tools[27]. Spot density (no. of spots per cell) of SST signal was determined by iteratively running 3DMaximaFinder (https://imagej.net/3D_ImageJ_Suite)[28] at locations of each connected component. It should be noted that this approach is rather elementary: it was designed as a proof-of-principle image processing routine that can be performed mid-way through a tracing session using accessible ImageJ tools.

MouseLight single-cell connectivity. The MouseLight database was programmatically parsed to obtain the number of brain areas (Allen Mouse Common Coordinate Framework[21] compartments) associated with individual axonal arbors using two criteria: (1) axonal length within an anatomical compartment and (2) number of axonal endings at target area. Only CCF compartments of ontology depth 7 (mid-level) with public meshes available were considered ($N = 141$). For criterion 1, all nodes within a compartment were taken and the distances to their parent nodes summed. To exclude en passant axons, a cell was considered to be associated with the compartment if such length would be at least 5% of the compartment's bounding-box diagonal. For criterion 2, only neurons with at least two axonal end points were considered. Selected examples in Fig. 2 were chosen by sorting cells by number of associated areas and selecting those with the largest axonal cable length (AA1044, 28.598 cm; AA0100, 44.649 cm and AA0788, 18.454 cm). CCFv2.5 ('ML legacy') annotations were used since CCFv3 annotations were not available on the MouseLight database at the time of writing. In total, 1,094 cells were parsed.

Tracing and path fitting benchmarks. Tracing benchmarks and fitting procedures were performed programmatically and can be reproduced using the scripts available at https://github.com/morphonets/SNTmanuscript (ref. [29]). DIADEM scores[30] were computed with default thresholds and retrieved in 'post-DIADEM competition' mode. For degradation of traces (Supplementary Note), each node in the reconstruction was displaced to a random position within a 1 µm neighborhood around each axis.

Synthetic morphologies. For the chemoatraction assay (Supplementary Video 3), the code is accessible from github.com/morphonets/SNTManuscript (ref. [29]). For generating GRNs (Supplementary Note), the code is available at github.com/morphonets/cx3d/ (ref. [31]) and the five GRNs used in this study are made available at https://github.com/morphonets/SNTManuscript (together with remaining analysis scripts). Tools for inspecting GRNs are available at github.com/morphonets/grneat (ref. [32]). For morphometric analysis, the default metrics provided by SNT were retrieved for all artificial neurons. Data were normalized and analyzed using principal components analysis, t-SNE[33] (t-stochastic neighbor embedding) and uniform manifold approximation and projection (UMAP)[34]. Group comparisons on principal components, t-SNE features and UMAP components were performed using two-sample Kolmogorov–Smirnov tests adapted for multivariate data. *P* values were combined using Fisher's combined probability test. For density maps and exemplars, soma-aligned cells were skeletonized and their skeletons projected into the *xy* plane using SNT's core functionality. Binary masks of skeletons were then summed up and resulting image normalized to the number of cells. Exemplars were chosen from a random pool of ten cells. The metrics used were as follows: average branch length, average contraction, average fractal dimension, average fragmentation, average partition asymmetry, average remote bif. angle, cable length, depth, height, highest path order, Horton–Strahler bifurcation ratio, Horton–Strahler number, length of inner branches (sum), length of primary branches (sum), length of terminal branches (sum), mean radius, no. of branch points, no. of branches, no. of inner branches, no. of nodes, no. of primary branches, no. of terminal branches, no. of tips, width (Sholl-based metrics), centroid, centroid radius, decay, degree of polynomial fit, enclosing radius, intercept, kurtosis, maximum, maximum (fitted), maximum (fitted) radius, mean, median, no. of maxima, no. of secondary maxima, skewness, sum and variance.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data required to generate the figures and analyses described in this manuscript are available at https://github.com/morphonets/SNTManuscript and https://doi.org/10.5281/zenodo.4568540.

Code availability

SNT source code is available at github.com/morphonets/SNT (ref. [18]) under the GNU General Public License v3.0. Technical aspects of the software are described in Supplementary Note. The SNT application is available in Fiji by subscription to the 'Neuroanatomy' update site (https://imagej.net/SNT#Installation). User documentation, manuals and video-tutorials are available at https://imagej.net/SNT. https://github.com/morphonets/SNT (ref. [18]).

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Author contributions

T.A.F. conceived and supervised the project. T.A.F. and C.A. wrote the core SNT. K.I.S.H. and U.G. implemented Cx3D/sciview integration. K.I.S.H. designed and ran simulations. M.E. performed the ExFISH experiment. T.A.F. analyzed the data. T.A.F. and K.I.S.H. wrote the paper.

Competing interests
The authors declare no competing interests.

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Software and code

Policy information about availability of computer code

Data collection	Imaging of smFISH-probed tissue was performed on a Zeiss lightsheet microscope using Zen Microscope Software v9.2.0.0.
Data analysis	Core analyses: - SNT v3.1.112 and Fiji v2020-08-02 (binaries at https://github.com/morphonets/SNTmanuscript/releases/tag/v2.0). Third-party libraries used by SNT are listed at https://github.com/morphonets/SNT/blob/master/NOTES.md Analyses using Python notebooks (https://github.com/morphonets/SNT/tree/master/notebooks): - pyimagej 1.0.0 (https://pypi.org/project/pyimagej/); openjdk8 (https://adoptopenjdk.net/); python3.9 (https://www.python.org/downloads/) Source code: - SNT: https://github.com/morphonets/SNTmanuscript; Manuscript figures: https://github.com/morphonets/SNTmanuscript

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https://ml-neuronbrowser.janelia.org/; http://neuromorpho.org/; https://virtualflybrain.org; https://insectbraindb.org

Used datasets (imagery):
http://cellimagelibrary.org/images/701 (doi:10.7295/W9CIL701); http://cellimagelibrary.org/images/810 (doi:10.7295/W9CIL810); http://www.diademchallenge.org/ (no DOI)

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https://github.com/morphonets/SNTmanuscript

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample-size computation was performed because this is a computational study
Data exclusions	No data was excluded from the study
Replication	Replication not applicable because this is a computational study
Randomization	Randomization not required. Benchmarks were performed programmatically
Blinding	Blinding was not required because this is a computational study

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging