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Accessibility
Semi-Automated Reconstruction of Neural Processes from Large Numbers of Fluorescence Images

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Abstract

We introduce a method for large scale reconstruction of complex bundles of neural processes from fluorescent image stacks. We imaged yellow fluorescent protein labeled axons that innervated a whole muscle, as well as dendrites in cerebral cortex, in transgenic mice, at the diffraction limit with a confocal microscope. Each image stack was digitally re-sampled along an orientation such that the majority of axons appeared in cross-section. A region growing algorithm was implemented in the open-source Reconstruct software and applied to the semi-automatic tracing of individual axons in three dimensions. The progression of region growing is constrained by user-specified criteria based on pixel values and object sizes, and the user has full control over the segmentation process. A full montage of reconstructed axons was assembled from the ~200 individually reconstructed stacks. Average reconstruction speed is ~0.5 mm per hour. We found an error rate in the automatic tracing mode of ~1 error per 250 um of axonal length. We demonstrated the capacity of the program by reconstructing the connectome of motor axons in a small mouse muscle.

Introduction

The nervous system is comprised of a large number of neurons with extensive and specific interconnections, but the wiring diagram is largely unknown. One approach to unravel neural circuits is to reconstruct the network by imaging its cellular components. A full wiring diagram (“connectome”) would require complete reconstruction of all the connections between all cells within the network, and has only been attempted rarely, the most notable example being the nervous system of the nematode C. elegans done by electron microscopy [1,2].

In recent years, with the adoption of confocal and two-photon microscopy as well as transgenic techniques to label neurons with fluorescent proteins [3,4], it becomes possible to do connectomic studies with fluorescent microscopy. However, a main technical challenge in connectomic reconstruction is to analyze the images and delineate neural processes. A number of programs have been developed to visualize and to trace neural processes in optical image stacks, allowing the user to interactively perform or monitor the tracing. Such programs include the NeuronJ plug-in to the open-source ImageJ platform [5], as well as commercial packages such as Imaris (Bitplane AG, Zurich, Switzerland), NeuroLucida (MicroBrightField, Inc., Williston, VT), Amira (Mercury Computer Systems, Inc., Chelmsford, MA), and Volocity (Improvision Inc., Lexington, MA).

These software packages do not perform satisfactorily when dealing with image stacks in which multiple neural processes branch and intertwine with each other. For instance, NeuronJ works on 2D image only, but the complexity of fasciculated nerve fibers makes it necessary to distinguish individual processes by exploring the full 3D data set. Moreover, when neural processes are closely apposed, the boundaries of such processes tend to smear into each other due to the diffraction-limited resolution of optical microscope and scattering. In this situation, the automatic or semi-automatic tracing functions provided by existing software do not guarantee correct tracing or segmentation. In addition, many of these programs do not allow segmentation tools to work on arbitrary slices. This limitation is serious because we find that reconstructing nerve fascicles is much easier from the cross-section orientation than a longitudinal one.

To facilitate the tracing of complex bundles of axons we enhanced the Reconstruct software [6], which was initially developed for manual segmentation of serial section electron microscopy. This platform permits the user to trace neural structures by delineating their profiles on each section of an image stack. In this way, the user can guarantee the correctness of the segmentation. The problem with this approach is that it cannot be done efficiently when large amounts of data need to be analyzed. We thus modified the software to allow faster, semi-automatic tracing of axons in image stacks. The modified program can be freely downloaded from the Yahoo group (http://tech.groups.yahoo.com/group/reconstruct_users/), which also provides a forum for user support and technical discussions. As a demonstration of the capacity of the program we reconstructed the full connectome of axons in a small mouse muscle, which required analysis of over 20,000 images.

Results

Image Acquisition

We imaged the axons innervating the omohyoid muscle of transgenic mice (the lby-1-YFP-16 line, [3]) that express cytoplasm-
mic YFP in all motor neurons. We also imaged dendrites of cortical pyramidal neurons of transgenic mice of the thy-1-YFP-H line [3]. Briefly, adult mice were fixed with paraformaldehyde; the muscles were removed, post-fixed, rinsed and mounted on slides. The mouse brain was removed, post-fixed, rinsed, sliced on a vibratome, and mounted on slides. A confocal microscope equipped with a motorized stage was used to automatically scan a montage of image stacks covering the entire area of muscle innervation. Technical details of sample preparation and image acquisition are discussed in the Methods section.

Pre-processing of Image Stacks
Image stacks were taken on a Zeiss Pascal confocal microscope with 12-bit dynamic range to ensure sufficient signal to noise ratio when the structures to be imaged were deep or dim. The Zeiss lsm file does not have native 12-bit format, so image stacks were saved in 16-bit format, with the highest four bits being zero. Hereafter these image stacks are referred to as "XY files" or "XY stacks."

We wrote Matlab scripts to preprocess these image stacks, but many of the operations are also available through other programs such as ImageJ plug-ins. As Zeiss lsm files are not among the standard file formats recognizable to the Matlab system, each stack (i.e., one lsm file) was converted into a series of individual 16-bit tiff files using ImageJ.

XY files were first converted to 8-bit, in which the dimmest pixel in the stack was mapped to value 0 and the brightest pixel in the stack mapped to value 255. In Matlab this was performed with the imadjust function. Each XY stack (Figure 1A) was then loaded in Matlab as a 3D array, and resampled along either the X-axis or the Y-axis with standard array manipulation functions in Matlab.

The axis for resampling (the preferred axis) was chosen so that the majority of axons in the stack would appear in their cross-sections orthogonal to their long axes (Figure 1B). Although images were taken at the Nyquist limit, we found that in many stacks the structures to be traced were not very densely compacted or highly complicated, and a lower resolution sufficed for reconstruction. In these cases we used a bicubic interpolation algorithm in Matlab (the imresize function with ‘bicubic’ option) to downsize the XY stacks before resampling to reduce the number of sections to be analyzed without losing the ability to track individual axons. This downsizing operation has two additional advantages: it in effect applies a mean filter to the original image and thus reduces the noise, and each resampled image will have square pixels as required by Reconstruct, since the original Z step size was twice that of the X-Y pixel size.

Semi-automatic Tracing of Axons
The original platform of the Reconstruct program allows a user to trace objects in serial sections by manually drawing the outline of each object on each section, which is time-consuming. We modified Reconstruct to enable semi-automatic tracing of axons using a region-growing algorithm called wildfire. The wildfire tool can be quickly guided by user input in an intuitive way, and generates a boundary enclosing the contiguous area of an axonal

Figure 1. Re-sampling of a XY stack along the Y axis. A. A fluorescent image stack rendered as volume data. The raw data set contained 159 z-direction optical sections. Each section is a 1024×1024 image. X-Y pixel size: 0.10×0.10 μm; z-step size: 0.20 μm. B. En face view of three virtual sections generated by resampling the stack in A along the Y-axis at positions schematically indicated by white lines in A.

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profile, while ignoring the outer halo of disjoint, scattered bright pixels common to confocal data.

The wildfire tool in Reconstruct allows the user to initiate region growing by selecting a “seed” pixel by a mouse-click. Region growing expands outward from the seed pixel to all 4-connected neighboring pixels (i.e., pixels with coordinates \((x+1,y), (x-1,y), (x,y+1),\) and \((x,y-1)\), given the seed pixel coordinate \((x,y)\)) that fail to satisfy user-specified stopping criteria based on hue, saturation and/or brightness. These pixels at which growth does not stop then serve as new “seeds” for the next iteration of growth. Region growth stops when all pixels at the frontier of growth satisfy the stopping criteria and thus provide no new seed. Once the growth process stops, a labeled boundary of the region is generated by tracing clockwise around the outermost frontier of pixels. The user can block region growing by using the mouse to define temporary boundaries.

When there are many fragments of the same structure appearing on the same section (e.g., at the highly branched neuromuscular junction), it is desirable to be able to trace all these fragments on a single section with a single command rather than requiring the user to click inside every profile. We thus implemented a feature to allow the user to specify a rectangular region by dragging the mouse across the image. The wildfire tool then traces all noncontiguous profiles in the rectangle using the region growth algorithm and the same stopping criteria. A user-specified size threshold is used to block the generation of outlines around isolated pixels.

Region growing is extended to serial sections by using the centroid of each trace to locate a seed pixel for wildfire on the next section. Successful region growing is thus repeated on successive sections automatically (Figure 2A). To control this propagation, constraints are imposed based on the knowledge that biological structures like axons typically do not make abrupt turns, or suddenly enlarge or shrink; therefore the cross-sections of the same object on successive sections should be similar to each other in location, shape and size. The area of each new region is compared with that on the previous section; if the two areas differ by a user-specified percentage (e.g. 50%), or the area is too small (e.g. less than 10 pixels), the propagation will stop. The user can re-initiate the wildfire tracing with a mouse click. The stopping criteria (such as the hue, saturation or brightness thresholds) can also be modified to improve performance after a stop. Another constraint is that different axons cannot overlap with each other. The user can set a minimal distance between axons (e.g., 3–5 pixels), and the region-growing procedure will stop when it reaches such “forbidden zones” defined by the boundaries that have been already traced.

The program also typically stops at branching points. Axons branch only at nodes of Ranvier, which show characteristically smaller diameters than the internode regions (Figure 2B and 3A) and subsequent emergence of two or more distinct profiles (Figure 3B). By recognizing this characteristic morphology, the user can easily re-initiate tracing on each of the branches with mouse clicks. Automatic tracing can continue with each of the branches, either one at a time or all together simultaneously.

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**Figure 2. Reconstruction of an un-branching nerve fascicle.** A. Axons in the nerve fascicle were traced out across multiple sections. Traces on the first 3 sections and the last 2 sections of the stack are shown. Scale bar: 10 μm. B. Traced axons were rendered in Reconstruct. Constrictions in the axons (arrows) represent nodes of Ranvier. Scale bars: 20 μm.

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Another difficulty lies where axons do not go parallel to the preferred axis of re-sampling. Sometimes axons fan out and go in all directions, and no matter which axis is chosen for re-sampling, there are always some axons (or parts of axons) that go almost perpendicular to it (Figure 4A). An axon in this category does not appear as a single ellipsoid on each cross-section, but often as a series of fragmented, elongated pieces with variable lengths (Figure 4B). Based on contiguity of the same axon across multiple sections, the user can trace all the sectioned pieces belonging to the axon by initiating *wildfire* on each piece.

The reconstruction procedure described above generates multiple 2D contours of each axon throughout the stack. These contours can be rendered as 3D objects in different ways for visualization and subsequent merging (for details of the rendering methods provided by the program, see [6] and the manual of the *Reconstruct* program provided at the download site).

We also tested this algorithm in tracing dendrites of cortical pyramidal neurons in a YFP-H transgenic mouse [3]. We traced the dendrites of two nearby neurons from their somata within a confocal stack. As shown in Figure 5, we could clearly distinguish the processes belonging to these two neurons from *en passant* processes of other cells.

**Concatenating Adjacent Stacks**

Although the *Reconstruct* software can montage multiple images in each section, the fact that different stacks were re-sampled in different directions made it necessary to use *Reconstruct* to trace one stack at a time. Within each stack, each distinct axon is recognized by the unique name the user assigns to it. However, axons go across multiple image stacks and it is important to make sure that the same axon is given the same name and color in all the stacks it traverses. If two adjacent stacks have the same preferred direction (Figure 6A and B), concatenation can be easily done through inspection of a single section in the overlapping region. For example, section 231 of the left stack (Figure 6C) is almost identical to section 001 of the right stack (Figure 6D). If axons in the right stack have been traced out, direct comparison of the two sections can unambiguously determine the correspondence between each axonal profile in the left stack with its counterpart in the right stack, and tracing the left stack can proceed with known axonal identity.

When the two adjacent stacks have different preferred directions (Figure 6E and F) it is no longer feasible to directly compare the sections in the overlapping region, as none of the re-sampled sections appear identical. The solution is to first reconstruct the two stacks independently, and then match the corresponding
axons in 3D rendered view, as Reconstruct allows arbitrary rotation of rendered objects. For instance, axons in the left stack (Figure 6G) are rendered, using a unique color for each distinct axon (axons that do not continue into the right stack are omitted from the rendering for clarity). The correspondence between identified axonal segments in the left stack and the reconstructed but unidentified segments in the right stack (Figure 6H, in gray) is easily established. Subsequently the names and colors of axonal segments in the right stack can be changed in Reconstruct to be consistent with that in the left stack.

**Assembly of Montage**

The reconstructed individual stacks need to be assembled into a full montage covering the entire sample. We first used Photoshop to manually montage the maximum intensity projection (MIP) images of all stacks (in our case monochromatic images) to provide a reference map. The overlap between adjacent stacks enables accurate alignment of the MIP images into a complete montage. This reference map facilitates obtaining the correct magnification for reconstructions from different stacks.

For each reconstructed stack, all axons were rendered in 3D in Reconstruct. The 3D rendering was rotated by a suitable angle to make it en face, i.e., viewed in the original XY orientation, and exported as a bmp or jpeg image. The rendering of all axons in the stack collectively was aligned onto the monochromatic montage with suitable resizing. Then each axon in the stack was rendered one by one and saved separately. These individual images were superimposed onto the montage subsequently, with one Photoshop layer per image. The collective rendering now serves as the reference for the alignment of individual axons. The reason to use a separate Photoshop layer per axon is to allow the user to turn on or off any axon from the view later. This procedure was repeated for each stack until the entire montage was aligned and colored. Then all layers belonging to the same axon were collapsed in Photoshop, allowing each axon to occupy a separate layer. In order to make the appearance of individual axons more distinguishable, we used the Photoshop magic wand tool to select one axon at a time on its layer, and used the paint bucket tool to fill its interior with a distinct color.

The procedure described above produces a 2D montage of the entire sample (Figure 7). However, as we already have the full 3D reconstruction of each stack, and as Reconstruct can export 3D rendering of objects in VRML formats, it should also be possible to do the alignment using VRML objects in a 3D modeling program.

**Evaluation of the reconstruction method**

We evaluated the effectiveness of the reconstruction method presented above in terms of the reconstruction speed and the error rate. The reconstruction speed depends on the complexity and layout of the axonal bundle, as well as image quality (e.g., signal to noise level). A stack that contains axons that are homogenously labeled, well separated, and imaged with high signal to noise ratio can be reconstructed without much user intervention, and the reconstruction speed approaches ~4 mm per hour. In this case most of the time is consumed by the delay (a fraction of a second) after generating a contour on each section, which is deliberately introduced to enable the user to see the result clearly. However, stacks that contain axons that “bleed” into each other, or are dimly labeled, or travel along non-preferred directions, take much more human intervention and manual reconstruction to complete, and the speed is consequently much slower. According to our experience, the average reconstruction speed for the whole muscle sample is ~0.5 mm per hour [7].

The error rate of segmentation algorithms is usually determined by comparing the results of the automatic segmentation and that of manual segmentation. For our semi-automated approach, however, it seems that the usual metric of “error rate” is not appropriate, because the program does not proceed all by itself.
Figure 6. Concatenation of adjacent stacks. A–B. Two adjacent stacks with the same preferred axis. Red lines: orientation of virtual sections. Scale bar: 20 μm. C–D. Cross-sections of the same axon in the two stacks were almost identical on corresponding virtual sections. C: section 231 of stack A. D: section 001 of stack B. Scale bar: 5 μm. E–F. Two stacks that overlapped but had different preferred orientations. Red lines: orientations of virtual sections. Cyan dotted lines: boundary of the overlapping region. Scale bar: 20 μm. G–H. Corresponding axons in E and F were identified using 3D rendered images based on their morphologies and relative positions in the overlapping region. G: Reconstruction of axons in E. Axons that did not go through both stacks were omitted for clarity. Arrows in H point to axons corresponding to the reconstructed ones in G. Arrow colors are matched to axon colors in G.

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and let the user correct the answers afterwards. In fact, the design of the semi-automated feature is to allow the user to discover any error in the wildfire segmentation as soon as it emerges, and correct it, so that the error does not propagate. Therefore, we believe that a better metric is the rate at which the semi-automated reconstruction process requires user intervention. This rate not only gives an estimation of the reliability of the automated processing, but affects the speed of reconstruction as well.

The rate of intervention depends critically on the complexity of the data. We thus used stacks of different complexity to estimate the rate of intervention. We reconstructed 9 axons from 2 “simple” stacks (Figure 2 and Figure 6B), and 6 axons from a “complex” stack (Figure 1A). Axons in the “simple” stacks have relatively homogeneous intensity and are well separated from each other. Axons in the “complex” stack are more variable in intensity and occasionally get very close to each other. Axons in the “complex” stack are more variable in intensity and occasionally get very close to each other. We further classified user interventions into “stops” and “errors”. “Stops” refer to the fact that the program automatically stops tracing and waits for user re-initiation. We identified 3 broad categories of events that can lead to stops: (1) the topology of the axonal profile changes (e.g., branching points), which makes the location and size of the axonal profiles on the subsequent section differ significantly from that on the previous section; (2) the intensity and/or size of the axonal profile changes sufficiently; (3) the shape of the axonal profile becomes concave (this may happen when a large mitochondrion is present, which is not labeled and thus shows up as a dark hole in the axon) and thus the “seed” pixel falls outside the contour of axonal profile and fails to initiate the new round of tracing. “Errors” refer to the case in which the program erroneously segments but does not stop by itself. A summary of rate of intervention is given in Table 1 (unit: number of occurrences per 100 µm of axons reconstructed). We excluded axons that were very dim and those that were very tightly intertwined with other axons from the analysis above. In these cases manual tracing would be preferred, given the large number of times the automated algorithm would require human intervention.

**Discussion**

In this paper we introduced a method for large scale reconstruction of neuronal processes from fluorescent image stacks. The processes are imaged at the diffraction limit with a confocal microscope. Images are pre-processed to remove noise and re-sampled so that tracing of axons can be performed along a

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**Figure 7. Full montage of reconstructed axons.** A. The entire connectome of an omohyoid muscle with 4 axons and 96 neuromuscular junctions. The white square indicates the size of one single image stack relative to the full montage of 168 stacks. Arrow: the entry point of the nerve into the muscle. Scale bar: 100 µm. B. Each axon in the connectome shown separately. Motor unit size: red (41), green (22), yellow (21), cyan (12). doi:10.1371/journal.pone.0005655.g007

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Table 1. Rate and Reasons of User Intervention in Reconstruction.

| Stack     | Topology Change | Size/Intensity Change | Initiation Failure | Errors |
|-----------|-----------------|-----------------------|--------------------|--------|
| Simple (n = 9) | 1.3            | 3.5                   | 0.8                | 0      |
| Complex (n = 6) | 2.2            | 7.9                   | 1.0                | 0.4    |

Unit: number of occurrences per 100 μm of axons reconstructed.
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convenient orientation (X, Y or Z axis) which shows the cross-
sections of the majority of axons. A semi-automatic program based
on the infrastructure of Reconstruct was developed and applied to
the tracing of individual image stacks. The program employs a
region-growing algorithm, and uses the centroid of an existing
axonal contour as the seed for region-growth on the next section in
order to proceed automatically. For a non-branching, well
segregated axonal process the program can automatically segment
it through the entire stack (e.g., 256 sections) without interruption
or human intervention in 2–3 min (~4 mm per hour). The
program stops when ambiguity arises, and the user has full control
over the segmentation process. A full montage can then be
assembled from the reconstructed stacks.

Several factors must be considered in the design of a program
for image reconstruction from large data sets. Obviously, it is
desirable to automate as many operations as possible. For
connectomics, automation is especially important, as the amount
of data to be processed is usually large, and manual segmentation
is one of the main bottlenecks. On the other hand, the variability
and complexity of the structure of the objects to be reconstructed
means that some user monitoring and intervention is necessary. A
user-friendly interface is thus required. If online user monitoring is
required, the algorithms used in the automatic segmentation
cannot be too time-consuming. This is the reason that we adopted
the fast and simple region-growing algorithm based on pixel values
for segmentation. If the strategy is to first go through the data
automatically and then let the user validate and correct the results,
the automatic processing can employ more sophisticated and
computationally expensive algorithms. Many image processing
algorithms, such as live wire [5], active contour or snake [8], level
sets [9], Kalman filter and optical flowlevel sets [10], wavelet-
based segmentation [11], and kernel-based tracking [12,13], have
been proposed for tracing 2D and 3D filamentous objects such as
axons and dendrites.

The Reconstruct program processes images in an essentially 2D
manner. Therefore one particular orientation must be selected and
maintained for each stack at the re-sampling step. When objects
within the stack assume very different main axes, this requirement
of a single orientation leads to some inconveniences for objects
along non-preferred directions. Manual segmentation is often
necessary for such objects as discussed above. An alternative
strategy would be to dynamically re-orient and re-sample the stack
along the local preferred direction as tracing proceeds. This will
ensure that at each step, the object is processed on its cross-section,
which is advantageous for segmentation. This approach, however,
is computationally more demanding, and remains to be fully
explored.

The reconstruction method presented in this paper is applicable to
the analysis of branching, tubular structures (e.g., neural
processes of both peripheral and central nervous system, blood
vessels, lung airways) imaged with fluorescent microscopy
techniques that can obtain volumetric data (e.g., confocal and
two photon microscopy). We also expect that the reconstruction
method is compatible with fluorescent image stacks taken by Array
Tomography [14], Selective Plane Illumination Microscopy [15],
as well as ultramicroscopy [16]. Images taken with electron
microscopes, however, may not be well segmented by the semi-
automated algorithm presented here, because in such images
neural structures are typically distinguished by their enclosing
membranes, which show up as closed contours, and there is no
universal intensity-based distinction between “signal” and “back-
ground.” Of course, these images may still be analyzed manually
with the Reconstruct program as reported previously [6]. In
summary, there is no intrinsic restriction on the type of tissue
preparation; as long as the structures of interest can be
distinguished from the background by their intensities (or hue/
saturation) in the image stack, the semi-automated segmentation
can be utilized.

Materials and Methods

Sample preparation

All animal experiments were conducted according to protocols
approved by Harvard University Institutional Animal Care and
Use Committee (IACUC). Transgenic mice of thy-1-YFP-16 line
(Feng et al. 2000, now available from the Jackson Lab, Bar
Harbor, ME) were used throughout these studies. Young adult
mice (~30 days old) received an intraperitoneal injection of 0.1 ml/20 g
ketamine-xylazine (Ketaset, Fort Dodge Animal
Health, U.S.A.), and were perfused transcardially with 4%
paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline
(PBS; pH 7.4). For the muscle preparation: the omohyoid muscle
from tissue surface to the coverslip was roughly constant. For the
brain slice preparation: the omoloyd muscle along with a short length of the innervating nerve was removed,
post-fixed in 4% PFA for 30 min, rinsed in PBS (room
temperature, 30 min×2), and then mounted on slides with the
Vectashield mounting medium (Vector Laboratories, Burlingame,
CA). Mounted slides were slightly squeezed between a pair of
small magnets over night to flatten the tissue so that the distance
from tissue surface to the coverslip was roughly constant. For the
brain slice preparation: the whole brain was removed from the
skull, post-fixed in 4% PFA over night, rinsed in PBS (room
temperature, 30 min×2), sliced at 50 or 100 μm thickness with a
vibratome (Leica VT1000S), and mounted on slides with the
Vectashield mounting medium.

Confocal Imaging

Samples were imaged using a confocal laser scanning
microscope (Zeiss Pascal, Carl Zeiss, Jena, Germany) equipped
with a motorized stage. We used a 63×1.4NA oil-immersion
objective and optically zoomed-in by a factor of 1.5. YFP
fluorescence was excited with a 488 nm Argon laser and detected
through a band-pass emission filter of 530–600 nm. Images were
sampled at the Nyquist frequency in the x-y direction (pixel
size = 0.1 μm) and over-sampled by a factor of ~2 in the z
direction (z-step size = 0.2 μm), with 12 bit dynamic range.
According to the well-known sampling theorem, a signal that
contains data at maximal frequency $f_{\text{max}}$ must be sampled at least at frequency $2f_{\text{max}}$ to ensure that the signal can be accurately recovered from the sampling [17]. This minimal sampling frequency is called the Nyquist frequency. In the imaging system, the maximal spatial frequency is determined by the resolution of the microscope, and for the particular imaging condition we used the resolution is $\sim 0.2 \, \mu m$ in the x-y plane, and $\sim 0.75 \, \mu m$ along the z axis [18]. Thus we used the optical zoom feature of the microscope to obtain pixel size that was at the corresponding Nyquist frequency. The motorized stage was controlled by the MultiTome\textsuperscript{2} macro (developed by Carl Zeiss) to set up the coordinates and imaging conditions for each stack in the montage. Adjacent stacks had 10% overlap to guarantee the precision of later alignment and tracing.

**Image Processing**

Image stacks were pre-processed with ImageJ (NIH, http://rsb.info.nih.gov/ij/) and custom-written programs in Matlab (The MathWorks, Inc.), and reconstructed with Reconstruct (http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm). Final assembly into a complete montage was done with Adobe Photoshop (Adobe Systems Inc.). See Results section for details.

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**Author Contributions**

Conceived and designed the experiments: JWL JL. Performed the experiments: JL JCF. Analyzed the data: JL. Contributed reagents/materials/analysis tools: JL JCF. Wrote the paper: JWL JL.

**References**

1. Hall DH, Russell RL (1991) The posterior nervous system of the nematode Caenorhabditis elegans: serial reconstruction of identified neurons and complete pattern of synaptic interactions. J Neurosci 11(1): 1–22.
2. White JG, Southgate E, Thomson JN, Brenner S (1986) The structure of the nervous system of the nematode Caenorhabditis elegans. Phil Trans R Soc Lond B 314: 1–340.
3. Feng G, McEwen RH, Bernstein M, Keller-Peck C, Nguyen QT, et al. (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28(1): 41–51.
4. Livet J, Weissman TA, Kang H, Drah RW, Lu J, et al. (2007) Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature 450(7166): 56–62.
5. Meijering E, Jacob M, Sarria JC, Steiner P, Hirling H, et al. (2004) Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A 58(2): 167–176.
6. Fiala JC (2005) Reconstruct: a free editor for serial section microscopy. J Microsc 218(Pt 1): 52–61.
7. Lu J, Tapia JC, White OL, Lichtman JW (2009) The intercerebralis muscle connectome. PLoS Biol 7(8): e22.
8. Cai H, Xu X, Lu J, Lichtman JW, Yung SP, et al. (2006) Repulsive force based snake model to segment and track neuronal axons in 3D microscopy image stacks. NeuroImage 32(4): 1608–1620.
9. Macke JH, Maaske N, Gupta R, Denk W, Scholkopf B, et al. (2006) Contour propagation algorithms for semi-automated reconstruction of neural processes. J Neurosci Methods 167(2): 349–357.
10. Jurrun E, Hardy M, Tasdizen T, Fletcher PT, Koshevoy P, et al. (2009) Axon tracking in serial block-face scanning electron microscopy. Med Image Anal 13(4): 180–188.
11. Dima A, Schölz M, Obermayer K (2002) Automatic segmentation and skeletonization of neurons from confocal microscopy images based on the 3-D wavelet transform. IEEE Trans Image Process 11(7): 790–801.
12. Al-Kofahi KA, Can A, Laske S, Szarowski DH, Dowell-Medlin N, et al. (2003) Median-based robust algorithms for tracing neurons from noisy confocal microscope images. IEEE Trans Inf Technol Biomed 7(4): 392–417.
13. Al-Kofahi KA, Laske S, Szarowski DH, Pace CJ, Nagy G, et al. (2002) Rapid automated three-dimensional tracing of neurons from confocal image stacks. IEEE Trans Inf Technol Biomed 6(2): 171–187.
14. Micheva KD, Smith SJ (2007) Array tomography: a new tool for imaging the molecular architecture and ultrastructure of neural circuits. Neuron 55(1): 25–36.
15. Verveer PJ, Swoger J, Pampaloni F, Greger K, Marcello M, et al. (2007) High-resolution three-dimensional imaging of large specimens with light sheet-based microscopy. Nat Methods 4(4): 311–313.
16. Doßt HU, Leischner U, Schierholz A, Jahrling N, Mauch CP, et al. (2007) Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain. Nat Methods 4(4): 331–336.
17. Castleman KR (1996) Digital Image Processing. Upper Saddle River, NJ: Prentice Hall.