**Densely reconstructed volumes of hippocampal neuropil for testing computer-assisted segmentation and analysis of neural circuits.**

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**Abstract**  :

The resurgent interest in understanding the organization of neural circuits at the nanometer scale has prompted the development of computer-assisted tools
to facilitate reconstruction and analysis of the complex images generated . We have used serial section transmission electron microscopy ( ssTEM ) to image four volumes at <1 nm resolution across
100’s of serial sections each ~50 nm thin. We digitally downsized the images to ~2 nm resolution and used RECONSTRUCT
 TM  to trace manually all of the axons, dendrites, and
glial processes in three volumes of adult hippocampal neuropil located in the middle of s. radiatum . The images containing the reconstructed volumes comprise (\_\_\_\_\_ μ m  3  ; \_\_\_\_\_\_ μ m  3  ; \_\_\_\_\_\_ μ m  3  ; and \_\_\_\_\_ μ m  3  , respectively). The densely reconstructed sub volumes include: a cube surrounding a large dendritic spine (volume 1 = 8 μ m  3  ); a cylinder of 4 μ m diameter by \_\_\_\_\_ length surrounding ~3.35 μ m segment of a CA1 oblique dendritic branch for a total volume 2 = 44 μ m  3  ); and a box of 6 X 6 X\_\_\_\_\_ μ m surrounding ~3.55 μ
m segment length of a CA1 apical dendrite (volume 3 = 183.6
μ m  3  ).
The locations of synaptic contacts were also identified in these reconstructed volumes. All of the original unaligned
ssTEM
images, the manually aligned images, and the manual traces are available for download at \_\_\_\_\_\_\_.
The fourth volume of \_\_\_\_ images (\_\_\_\_\_ μ m  3  ), with traces encompassing \_\_\_\_\_\_\_ dendrites is also provided from a similar location in postnatal day 21 hippocampus
and contains sparse but complete reconstructions of individual dendrites and synapses
. The manually reconstructed volumes can serve as training volumes for developing and testing automatic segmentation tools. The image and reconstruction data in these volumes is dense and may also serve to investigate numerous
additional
properties such as: the relative volume occupancies of different objects, the degree of local connectivity, dimensions of a variety of spines and synapses, whether
glial
processes are present at specific locations, and dimensions and locations of intracellular organelles that comprise these structures. While manual reconstructions serve as ‘gold’ standards for identifying and following structures accurately through serial section, the specific placement of the traces is somewhat subjective, especially where membranes are obliquely sectioned. Furthermore, manual traces are subject to variations in hand movements that might accidently obscure objects, especially extracellular space. Hence,
these volumes provide excellent images for testing computer assisted tools and good traces for identifying objects
. Enhanced computer-assisted segmentation should aim to generate more objectively reliable reconstructions.

# *Methods*

**Tissue sources and photographic conditions:** 
All procedures followed NIH guidelines for the humane care and use of laboratory animals.
V1-3 were from hippocampal
area CA1 of a perfusion-fixed male rat of the Long-Evans strain weighing 310 gm (postnatal day 77,
(Harris and Stevens, 1989) ). Volume 4 was from a hippocampal
slice that was prepared from a postnatal day 21 male rat
(PND21)
of the Long-Evans strain and maintained in vitro for 3 hours prior to fixation as described (
Fiala et al., 2002). All volumes were from the middle of s.
radiatum about 150 to 200 microns from the hippocampal CA1 pyramidal cell soma. For volume 4
, the series was located at a depth between 100 and 200 µm from the cut air surfaces of the slice where excellent tissue preservation occurred.

The image volumes for V1 and V4 were obtained from digital scan s of
the original EM negatives that had been photographed to produce dendrite K24 (Harris and Stevens, 1989)
, and region PN21AA ( Fiala et al., 2002 ). V2 and V 3 were more recently sectioned from additional blocks from (Harris and Stevens, 1989). Series were cut according to our published protocols (Harris et al., 2007)
. Briefly, a diamond trimming tool (EMS, Electron Microscopy Sciences, Fort Washington, PA) was used to prepare small trapezoidal areas ~200 µm wide by 30-50µm high. Serial thin sections were cut at ~45-50 nm on an
ultramicrotome , mounted and counter stained with saturated ethanolic uranyl
acetate, followed by Reynolds lead citrate, each for 5 min. Individual grids were placed in grid cassettes and stored in numbered gelatin capsules. The cassettes were mounted in a rotating stage to obtain uniform orientation of the sections on adjacent grids and the series were photographed at
5,000x on a JEOL 1200EX (Volumes 1 - 3 ) or 10,000x on a JEOL 1230 ( V olume 4) transmission electron microscope (JEOL, Peabody, MA).

**Manual volume reconstructions** 
: Three-dimensional reconstructions and analyses were performed manually using the software entitled RECONSTRUCT
 ™  ( Fiala , 2005; Fiala and Harris, 2002) , which is freely available from <http://synapses.clm.utexas.edu>
). We digitally optimized images for brightness and contrast, and colorized reconstructions to visualize structures of interest. To align manually, we
placed five or more fiducial trace
s on adjacent pairs of serial sections that were in the same location (e.g. cross-sectioned mitochondria or microtubules).
We used the “Linear” alignment tool in RECONSTRUCT  TM  to align
most of these images. To test the quality of alignment, adjacent images were blended and viewed by paging through the series
.
In a well-aligned series, the blended images appear as a single well-focused image. If alignment is off, the blended images will appear blurred or as double-images in regions of sub-optimal alignment
; if so, additional feducial points were added in those regions . If a blended image could not be adequately aligned using the linear tool
, usually due to a flaw in the section , then the “ D eformal ” or “Q uadratic ” tool s were occasionally used ; however, these extreme alignments were not propagated to the rest of the series.

Pixel size was calibrated relative to a diffraction grating replica ( 0.463 um squares from Ernest F. Fullam , Latham, NY) that was photographed with each series , using the “Calibration” function in RECONSTRUCT  TM  . The calibrated digital images ha d spatial resolution s of ~2.2 nm/pixel. S
ection thickness was computed by dividing the diameters of longitudinally sectioned mitochondria by the number of sections they spanned
( Fiala and Harris, 2001a) and equaled ~50 nm.

We manually traced outlines of all objects in sub regions of the image volumes and identified them as axon s , dendrite s , glia , spine s, or synapse s . The traces generated for synapses in these reconstructed
volumes only provide a guide as to where a synapse is located
and a count per reconstructed volume .
Strategies to obtain realistic dimensions of the synaptic surface areas require
more detailed reconstructions as described elsewhere (e.g. \_\_\_\_\_\_\_ Harris references) , and have not yet been obtained for these volumes . RECONSTRUCT  ™  output provided calibrated volume s of dendrites, axons, and glia ; and 3D displays of reconstructed objects.

ADD HERE HOW THINGS ARE NAMED in the volumes
, and fix them to be uniform across the densely reconstructed
volumes – Assign this to John and Patrick.

Dendrites and Spine – Bright yellow outer line and pale yellow volume: values in 2x2 cube

# **RESULTS**

The f our image volume s were located in the middle of  *stratum radiatum*  in hippocampal area CA1 (Figure 1 A and Table 1 ) . Reconstruction through ssTEM provided una m biguous identif ication of axons, dendrites, glial processes and synapses using the foll owing characteristic features. Axons had boutons
containing synaptic vesicles interspersed along thin processes comprising a few microtubules, mitochondria or other organelles presumably in transit (Figure
1B ). Spiny dendrites had thickened post-synaptic densities ( PSDs
) characteristic of asymmetric excitatory synapses located on spine heads connected to their shafts through thin necks
(Figure 1C)
. Rarely, symmetric inhibitory synapses were located on
dendritic shaft s . These were unambiguously identified when associated axons were long enough to trace them to additional symmetric shaft synapses along their lengths (See V1 below) . Several
axons could not be uniquely distinguished in this fashion as too little of their length transected t
he image volumes . A few n onspiny dendrites were recognized by the presence of all or nearly all of their asymmetric and symmetric synapses located directly on the dendritic shafts. Small astroglial processes inter - digitated
irregularly among axons and dendrites, contained glycogen granules, and usually could be traced to larger processes with characteristic bundles of intermediate filaments
(Figure 1 D ). In volumes of the dimensions reported here,
g lial processes likely belong to one or at most a few astrocytes because they tile neuropil without substantial overlap between neighboring
astrocytes ( Bushong et al., 2002; Livet et al., 2007) ). Myelinated axons were recognized by the dense myelin sheaths as illustrated in Figure 2A below. Because the se characteristic features were often absent on a single ssTEM image of a particular object , the three-dimensional reconstruction s w ere essential for object identification.

The images comprising V1 were centered on a small dendrite
that traversed neuropil that was located between two longitudinally sectioned dendrites (Figure 2 A ). The image series contained \_\_\_\_\_ images and occupied ~ \_\_\_\_
KH check image volume – the automated segmentation volume was
35 µm  3  (Table 1). All objects located in a 2X2X2 μ 3  volume were reconstructed (Figure 2B). In 3D, a large dendritic
spine with a perforated synapse occupied most of the central region of this volume
(Figure 2C) although its edges were not completely contained by this particular cube
. In addition, \_\_\_\_\_ portions of other dendrit ic seg ments (or spines) enter ed th e
cube (Figure 2D, Table 2), along with \_\_\_\_ axons. Overall, there
we re \_\_\_\_ individual PSDs in the volume (Figure 2F). A ll of the astroglial segments have also been reconstructed (Figure 2G).

The images comprising V 2 were centered on a radial oblique dendrite as it coursed through the volume that occupied 338 µm  3  . V2 was photographed so as to be centered on an apical dendrite across \_\_\_\_\_\_ serial sections
that occupied 170 µm  3  (Figure 3A, B ). It contained \_\_\_ axons (Figure 3C, \_\_\_\_\_ dendritic segments, and \_\_\_\_\_ synapses.

The V4 from PND21 was randomly located in s. radiatum and occupied 131 µm  3  (Figure 1, Supp. Figure 1, and Table 1). Together the
image volumes occupied \_\_\_\_ µm  3 
while the densely reconstructed volumes occupied \_\_\_\_\_
µm  3

Extracellular space (ECS) was estimated in the manual segmentations by subtracting the total volumes of objects from the total manual volumes
(~Other)
. Estimates of ECS were \_\_\_\_ in the manually reconstructed sub-region of V1, \_\_\_\_\_ in V2, and \_\_\_\_\_ in V3. These are surely underestimates of true ECS
which have been further determined for V1 (see Kinney, 2009, Justin’s PhD Thesis)
. While
manual reconstructions can serve as ‘gold’ standards for identifying and following structures accurately through serial sections, the specific placement of the traces on membrane boundaries is somewhat subjective, especially where membranes are obliquely sectioned. Manual traces are also subject to variations in hand movements that might accidently obscure the extracellular space. In addition, when objects are obliquely sectioned at 45 nm, the ECS is buried within the depth of the section. From a technical standpoint,
ECS might be reduced by the dehydration steps needed for ultrastructural analyses (Thorne and Nicholson, 2006) .
Despite these caveats, the remaining amount of ECS corresponds well with the overall shrinkage of the tissue, suggesting that the objects themselves were not markedly shrunken or swollen, but rather might have moved closer to one another when part of the ECS
was lost (Refs, see KH comments).
It was impossible to determine whether the absolute volume of ECS changed locally; and hence we assumed it was uniform across these samples.

**DISCUSSION:**

Our expectation is that these densely reconstructed volumes of
hippocampal neuropil
will serve multiple purposes. They provide a basis from which to test the validity of computer generated segmentations of the same tissue volume and to accurately identify objects located in them. As such, they should prove to facilitate improvement of segmentation tools to generate larger densely reconstructed volumes of neuropil. They provide images that can be further mined to ascertain the relative frequencies of various objects and the likelihood that particular

**References:**

**1:**  [PLoS One.](javascript:AL_get(this,%20'jour',%20'PLoS%20One.');)  2009 May 21 ;4 (5):e5655.

[Links](javascript:PopUpMenu2_Set(Menu19479070);)

**Semi-automated reconstruction of neural processes from large numbers of fluorescence images.**

**[Lu J](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Lu%20J%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus)**  ,  **[Fiala JC](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Fiala%20JC%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus)**  ,  **[Lichtman JW](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&Cmd=Search&Term=%22Lichtman%20JW%22%5BAuthor%5D&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DiscoveryPanel.Pubmed_RVAbstractPlus)**  .

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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 appe
ared 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 approximately 200 individually reconstructed stacks. Average reconstruction speed is approximately 0.5 mm per hour. We found an error rate in the automatic tracing mode of approximately 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.

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Neurite tracing can be viewed as a process of grouping image
voxels
together into physically consistent objects. In previous work, this grouping has been accomplished using supervised learning methods based on
convolutional neural networks (Jain et al. 2007; Turaga et al. 2009).

Jain et al. 2007;

Turaga et al. 2009