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

**Kristen M. Harris, Josef Spacek (?John, or Bitao – if I include the database)**

**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 RECONSTRUCTTM 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 (\_\_\_\_\_μm3; \_\_\_\_\_\_μm3; \_\_\_\_\_\_μm3; and \_\_\_\_\_μm3, respectively). The densely reconstructed subvolumes include: a cube surrounding a large dendritic spine (volume 1 = 8 μm3); a cylinder of 4 μm diameter by \_\_\_\_\_ length surrounding ~3.35 μm segment of a CA1 oblique dendritic branch for a total volume 2 = 44 μm3); and a box of 6 X 6 X\_\_\_\_\_μm surrounding ~3.55 μm segment length of a CA1 apical dendrite (volume 3 = 183.6 μm3). 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 (\_\_\_\_\_μm3), 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 scans 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 V3 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 JEOL1230 (Volume 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 traces 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 RECONSTRUCTTM 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 “Deformal” or “Quadratic” tools 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 RECONSTRUCTTM. The calibrated digital images had spatial resolutions of ~2.2 nm/pixel. Section 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 axons, dendrites, glia, spines, or synapses. 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 volumes 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 four image volumes were located in the middle of *stratum radiatum* in hippocampal area CA1 (Figure 1A and Table 1). Reconstruction through ssTEM provided unambiguous identification of axons, dendrites, glial processes and synapses using the following 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 shafts. 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 the image volumes. A few nonspiny 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 1D). In volumes of the dimensions reported here, glial 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 these characteristic features were often absent on a single ssTEM image of a particular object, the three-dimensional reconstructions were 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 2A). The image series contained \_\_\_\_\_ images and occupied ~\_\_\_\_ KH check image volume – the automated segmentation volume was 35 µm3 (Table 1). All objects located in a 2X2X2μ3 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 dendritic segments (or spines) entered the cube (Figure 2D, Table 2), along with \_\_\_\_ axons. Overall, there were \_\_\_\_ individual PSDs in the volume (Figure 2F). All of the astroglial segments have also been reconstructed (Figure 2G).

The images comprising V2 were centered on a radial oblique dendrite as it coursed through the volume that occupied 338 µm3. V2 was photographed so as to be centered on an apical dendrite across \_\_\_\_\_\_ serial sections that occupied 170 µm3 (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 µm3 (Figure 1, Supp. Figure 1, and Table 1). Together the image volumes occupied \_\_\_\_ µm3 while the densely reconstructed volumes occupied \_\_\_\_\_ µm3

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)**.

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, United States of America.

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 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