doi: 10.1017/S1431927609099036
Microsc Microanal 15(Suppl 2), 2009

Serial Block-face Scanning Electron Microscopic Correlative Methods for Mapping Neuronal Interconnectivity

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Electron microscopy is the only technique capable of reconstructing wiring diagrams in their entirety [1], but until recently, complete electron microscopic circuit reconstruction has been a painstakingly slow process because of the sheer amount of data involved [1]. Serial block-face scanning electron microscopy (SBFSEM) automates the process of serial sectioning and imaging the tissue block face by incorporating a microtome into the vacuum chamber of a field emission scanning electron microscope [2-4]. In contrast to image generation in a transmission electron microscope (TEM), images in an SBFSEM are generated from electrons scattered off the surface of an embedded tissue sample (i.e. backscatter electrons), which allows the imaging of tissue block faces [2,-4]. Image stacks generated with this technology have a resolution sufficient to distinguish different cellular compartments, including synaptic structures, which makes it possible to obtain detailed neuroanatomical details of a neuronal circuit [1-4]. Our use of fluorescent and/or non fluorescent gap junction-permeant and gap junction-impermeant tracers to study axonal retrograde transport in living neurons permits the detection of mixed synapses on electron micrographs of serial sections and the tracing of axons and dendrites to their somas. As a result, we are able to identify synaptically coupled neurons. Adult Western Mosquitofish, *Gambusia affinis* (*Gambusia* hereafter) were anesthetized by immersion with benzocaine (1:2,000). Filter paper fibers saturated with fluorescent and/or non fluorescent gap junction-permeant and gap junction-impremeant tracers were implanted directly into crushed nerves innervating the deep muscles of the adult male gonopodium and of the adult female anal fin of *Gambusia*; fish were revived and the tracers was allowed to transport between 6.0-8.0 hrs. *Gambusia* were euthanized by immersion in benzocaine (1:4,000) and intracardially perfused with teleost buffer pH 7.4 followed with 4% formaldehyde made from freshly depolymerized paraformardyde (PFA) in teleost buffer pH 7.4. After fixation, the spinal cord associated with vertebral segments 7-17 was removed, dissected free and post-fixed overnight with 4% PFA in teleost buffer pH 7.4. Spinal cords were covered with mounting medium, cover-slipped and viewed using a Nikon C1 Laser Scanning Confocal Microscope. Three-dimensional (3D) volume reconstructions were rendered from light scanning confocal z stacks using the Imaris® 3D software. Volume reconstructions were obtained by thresholding intensity values and adjusting blending properties to create a solid and detailed volume model of the raw data acquired (see Figure 1A). Gap junction-permeant tracer coupling between motor neurons and interneurons, specifically commissural primary ascending interneurons (CoPA) occurred primarily through dendrodendritic and axosomatic glutamatergic mixed synapses[5]. We employed freeze-fracture replica immunogold labeling (FRIL) and three antibodies that identified the primary connexin isoform (connexin 35/36) within neuronal gap junction plaques (electrical component) and one antibody to glutamate receptors (the chemical component) in *Gambusia*[5]. For SBFSEM, the coverslip was removed and the spinal cord was washed three times in teleost buffer pH 7.4 to remove the mounting medium**.** The spinal cord was then post-fixed and stained with heavy-elements. The spinal cord was dehydrated, infiltrated with resin, embedded and polymerized at 60° C for 24.0 hr. After polymerization, the resin-embedded spinal cord sample was serially shaved (~50 nm), using a Gatan 3View® system.

Image stacks generated with SBFSEM have sufficient resolution to distinguish contour and segment structures such as synaptic contacts on the soma of motor MandM-00000850-00.pdf neurons (see Figure 1B-1D). Thus, we are able to develop detailed maps of mixed synapses between motor neurons and interneurons that, in turn, will assist in understanding how information moves between neural cells within regions of the spinal cord and elucidate various aspects of overt motor patterns.

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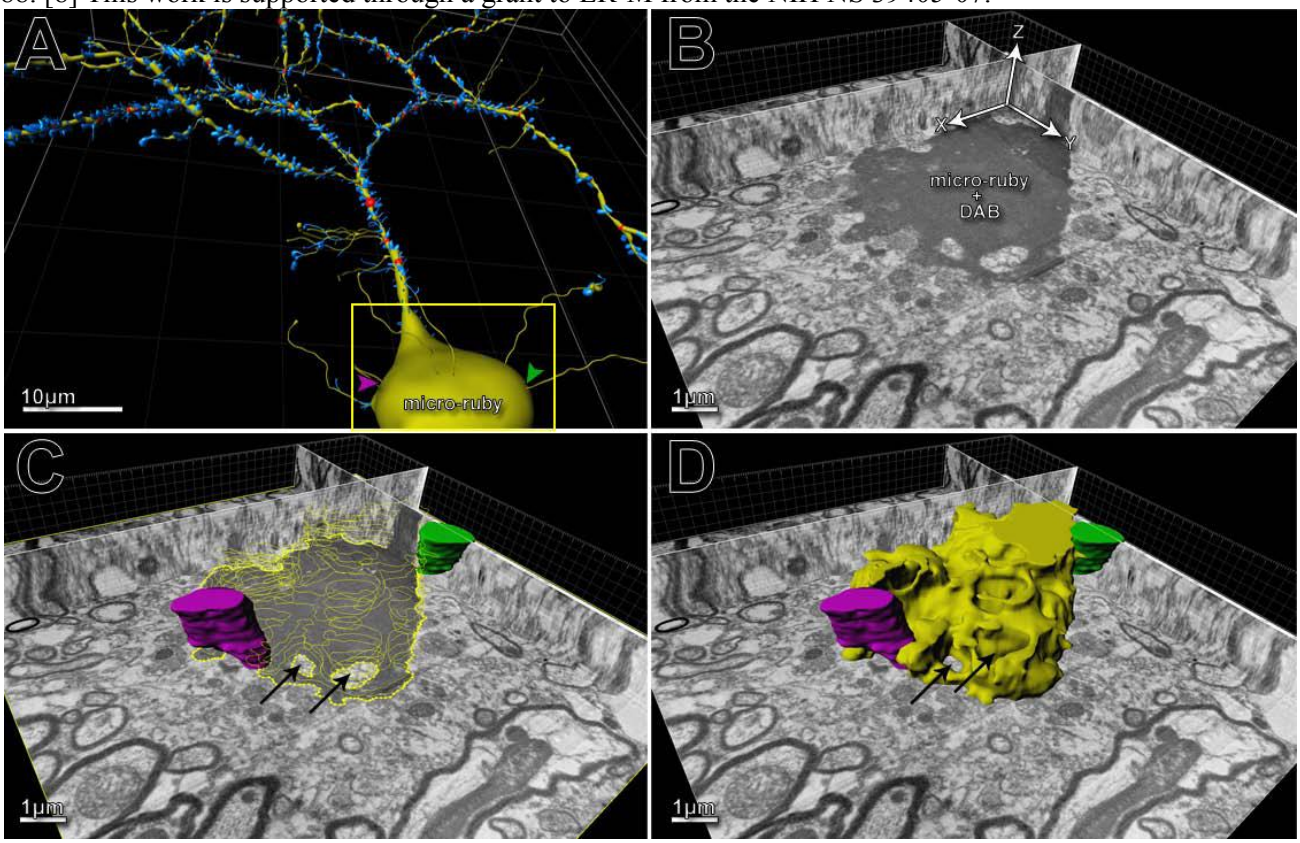

Figure 1. 3D volume rendering of type-1 motor neurons (A) retrogradely labeled with micro-ruby and visualized with diaminobenzadine (DAB; B) showing axosomatic contacts (purple and green arrow heads]. Image stacks generated with SBFSEM has the resolution sufficient to distinguish, contour and segment the axosomatic contacts purple and green arrow heads on the soma of a type-1 motor neurons (C; black arrows shows non-labeled axons). Note that the axosomatic contacts (purple and green axons appear embedded into the type1 motor neuron soma (D).

https://doi.org/10.1017/S1431927609099036 Published online by Cambridge University Press