

# Advances in automated 3D cortex image data acquisition

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## ABSTRACT

For many years neuroscientists have used electron microscopy to study the ultra-structure of the brain. This includes the use of transmission electron microscopy (TEM) to image sections of brain tissue and scanning electron microscopy (SEM) to provide the surface detail of freeze-fractured samples. Recently, this flexibility of the electron microscope to study cell structure has been extended with the use of a scanning technique that captures the electrons backscattered from within the surface of the tissue block face. Combined with a microscope that can section tissue samples - by using an ion beam (DualBeam™) - gives researchers the ability to section and analyze tissue *in situ* without the requirement of preparing thin sections. In this study we have explored the use of the DualBeam microscope to analyze the structure and connections of nerve cells in the adult brain, using tissue prepared for standard TEM analysis.

An important aspect in studying how the brain functions is understanding the details of the connectivity between its neurons. This occurs primarily through chemical synapses, of which there are a various types. Studying the detailed structure of these specialized sites of cell contact requires the use of high resolution imaging provided by the electron microscope. Recently this has been shown to be possible using the backscattered electrons from a block face that is sectioned *in situ* using a microtome and diamond knife [1]. In the study presented here, we have used a focused ion beam (FIB) on resin-embedded brain tissue and image synapses at high resolution using the backscattered electrons to evaluate the usefulness of the DualBeam approach for studying neuronal connectivity.

Focused Ion Beam milling, combined with scanning electron microscope in one device (DualBeam) started in the nineties as a tool for the semiconductor industry to enable analysis of semiconductor devices at specific sites in the circuit. The last five years has seen a large expansion of this technique into the material science and nanotechnologies for studying material properties on the nanoscale. And very recently, the DualBeam has been applied in the biological sciences (Drobne et al [2], Heymann et al [3]) to demonstrate the use of this technique for site-specific imaging as well as automated three dimensional reconstruction.

**Keywords:** focused ion beam, electron microscopy, neurons, synapses, serial section.

## 1 SPECIMEN PREPARATION

### 1.1 Tissue preparation

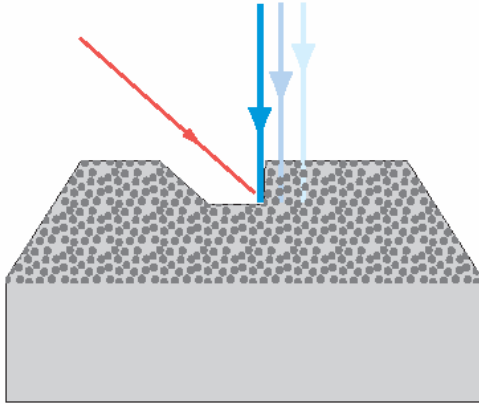
An adult mouse was deeply anesthetized with sodium pentobarbitone (30mg/100g body weight) and then fixed via cardiac perfusion using 0.2 % glutaraldehyde and 4% paraformaldehyde in PBS (0.1M). The brain was then removed and 60 micron thick vibratome sections cut coronally through the primary sensory cortex. The sections were then washed and postfixed in a combination of potassium ferrocyanide and osmium tetroxide followed by osmium tetroxide and uranyl acetate fixation. They were then dehydrated in alcohol and infiltrated with Durcupan resin.

### 1.2 Block Preparation

Once the resin had hardened, a block of the cortical tissue (1 mm x 1mm x 60 um) was cut from the embedded section, making sure that the neocortex was included. This piece was stuck to a blank resin block and trimmed down to a block face measuring 600 X 200 microns; typically the dimensions that are used to make serial sections for TEM. The final block was then mounted to a stub using silver paint and then coated with a platinum-palladium coating using a sputter coater.

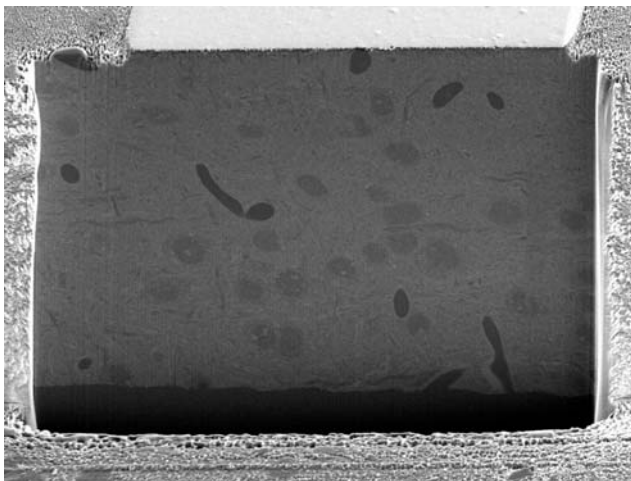
### Preparation for cross-sectional imaging

The Focused Ion Beam (blue arrows in figure below) was used to cut a trench into the upper surface of the block making an imaging face perpendicular to the upper surface (see diagram below). This face was then scanned with the electron beam to create images of the embedded cortical tissue. For making the initial trench an ion beam of high current was used. The geometry of the milling and imaging beams is shown below in the schematic diagram.



### 1.3 Imaging the FIB milled face

After a surface on the resin was milled to reveal an imaging face, its surface was “polished” with a low beam current to produce a flat surface, free of any relief that would introduce artifacts in to the images.

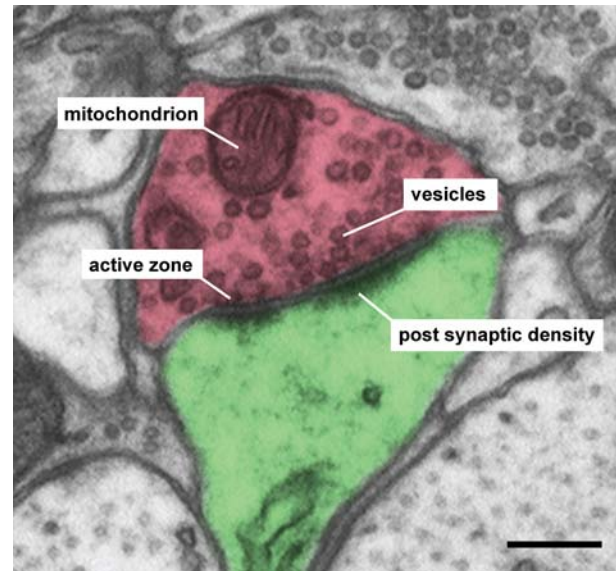


The above image shows a view of the trench and a full view of the block face that has been milled and is ready for scanning. The width of this face is approximately 100 microns.

### 1.4 Imaging resolution requirements

We investigated whether we could use the DualBeam approach of ion beam milling and imaging in backscatter mode to study the ultrastructure of the neuronal connections-the synapses. The following figure shows a TEM image of a synaptic contact and the features that can easily be seen to identify this cell specialization. This includes on the presynaptic side the axonal bouton (in red) containing the synaptic vesicles that are filled with the neurotransmitter. These vesicles will fuse with the presynaptic density that contains the active zone of proteins

involved in the fusion and release of the neurotransmitter from the vesicles and into the synaptic cleft between the active zone and the postsynaptic density. Facing this presynaptic part is the postsynaptic density of a dendritic spine (in green). This belongs to the next cell that will detect the released neurotransmitters via the receptors in this postsynaptic part. All these features are clearly visible in the transmission electron microscope.



To be a viable tool for studying the connectivity between neurons these features need to be seen with this DualBeam approach.

Since most synaptic vesicles of various different types of synapses have typical sizes of between 20 and 40 nm, we set our imaging resolution that would allow us to see these organelles at 5 nm per pixel. On one hand this would allow for the identification of the vesicles and therefore all synapses, but also, with a high resolution scan engine of the SEM, would also give a wide field of view. A 2kX2k pixel scan give a 10 um x 10 um field of view; 4kX4k pixel scan gives a 20um x 20um field of view.

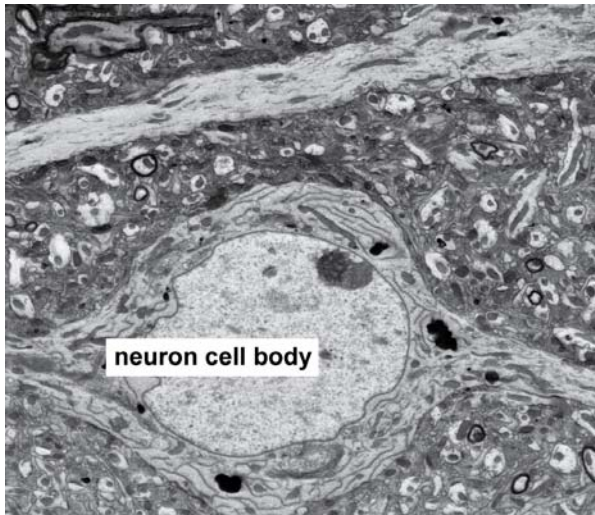
### 1.5 Imaging Detector Characteristics

Since the tissue is prepared with stains that typically have high molecular weights (uranyl acetate, osmium tetroxide) there is considerably more backscattering in regions with the greatest staining (ie membranes). The backscatter detector is optimized for collection of high energy electrons caused by the elastic scattering of the incoming electron beam within the sample. Because of larger amounts of backscattering where there are membranes these structures will appear brightest; opposite to the TEM where the heaviest staining causes the most elastic scattering and therefore the darkest region of the image. For this reason we have reversed the contrast of the images that we have recorded in the backscatter detector to make them more easily comparable with what we have obtained in the TEM.

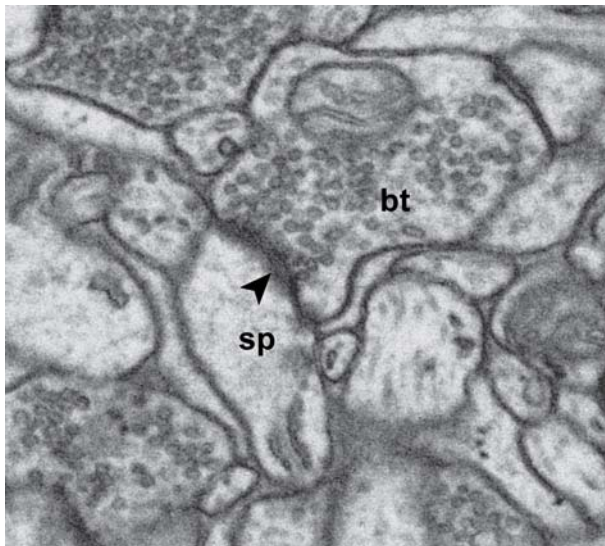
nm. These images have been used for a 3D visualization in video format that will be presented.

## REFERENCES

- [1] Denk W, Horstmann H (2004) Serial Block-Face Scanning Electron Microscopy to Reconstruct Three-Dimensional Tissue Nanostructure. *PLoS Biol* 2(11): e329 [doi:10.1371/journal.pbio.0020329](https://doi.org/10.1371/journal.pbio.0020329)
- [2] Drobne D, Milani M, Zrimec A, Zrimec M.B, Tatti F. and Drašlar K, *Scanning* 2005 Jan-Feb "Focused ion beam/scanning electron microscopy studies of *Porcellio scaber* (Isopoda, Crustacea) digestive gland epithelium cells"; 27(1):30-4.
- [3] Heymann J, Hayles M, Gestmann I, Giannuzzi L, Lich B. and Subramaniam S, "Site-specific 3D imaging of cells and tissues with a dualbeam microscope"; *Journal of structural Biology* July 2006; 155(1):63-73



The above image shows a 2k x 2k image of the imaged face taken at 5keV with a resolution of 10nm/pixel. This shows a neuron cell body (labeled) and the complexity of the neuropil that surrounds this cell containing axons dendrites and astrocytes. With these parameters it is not possible to distinguish the synapses.



Using a resolution of 4 nm/pixel and a voltage of 2keV the synapse are clearly distinguishable. The above image shows an axonal bouton (bt) containing vesicles that makes a synapse (arrowhead) with a dendritic spine (sp).

## 1.6 Automation

We have used automation software (Slice & View™) to run the DualBeam automatically to record more than 100 slides in a sequence with a slice thickness of approximately 40