



Key words:

PEEM, biological material, volume imaging, synapses, neural networks, connectomics

Volume-PEEM imaging of biological materials

Photoemission Electron Microscopy (PEEM) can obtain highly detailed spatial images with resolution below 10 nanometres. Although it is typically used to study surfaces in physics or chemistry, the direct imaging capabilities, non-destructive approach, significantly simpler sample preparation requirement and high spatial resolution make PEEM attractive for mapping of biological materials across large 3D volumes, which we term “volume-PEEM” (vPEEM). In this Note we present vPEEM results obtained on ultra-thin slices of a biological sample set in resin. The measurements reveal that PEEM can image finer structures down to the level of individual synapses. By imaging consecutive slices, we show that it is also possible to follow structures in 3D, thereby highlighting the potential of vPEEM to spatially map neural networks in biological systems, the connectivity of which is central to key functions in cognition.

Motivation

Imaging complex biological structures is a powerful way to understand their functions. A significant scientific challenge is to understand the workings of the human brain, and in particular the way in which the physical connections within network of neurons in the brain influence its function. State of the art high resolution methods of imaging such as Scanning Electron Microscopy (SEM) are limited by low data throughput due to the need to raster scan an electron beam across the sample and involves complicated sample preparation coupled with possible damage to the sample. On the other hand, optical imaging with high throughput is limited in the spatial resolution it can achieve. PEEM imaging is therefore an attractive alternative as it sits

between these two extremes, offering intermediate data rates with resolution down to a few nanometres. There is therefore immense potential for PEEM to drive significant developments within biological “connectomics” imaging-based research. The key goal of this work was to image the connections between neurons, which are called synapses, which requires resolution below 20 nm.

Method

A schematic of the volume imaging process is depicted in Fig. 1. In order to prepare biological materials for an ultra-high vacuum environment the sample is first set in a solid resin, which removes water from the sample and allows for simplified handling. The 3D sample is then cut into very thin slices of around 40 nm using a microtome. With this preparation, the sample is stable and a background pressure during measurements in the 10^{-10} mbar range is typical. Microscopy data of many individual slices are then obtained sequentially. Finally, a 3D representation of the sample can be reconstructed via an analysis of the 2D slices, by following the variation of features across different slices.

The key components and the electronic trajectories within an aberration corrected PEEM are highlighted schematically in Fig. 2. During a PEEM measurement,

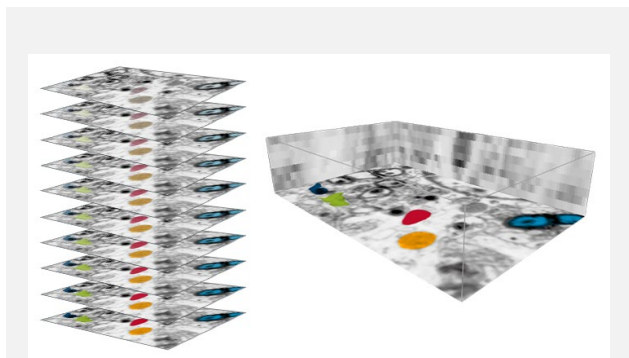
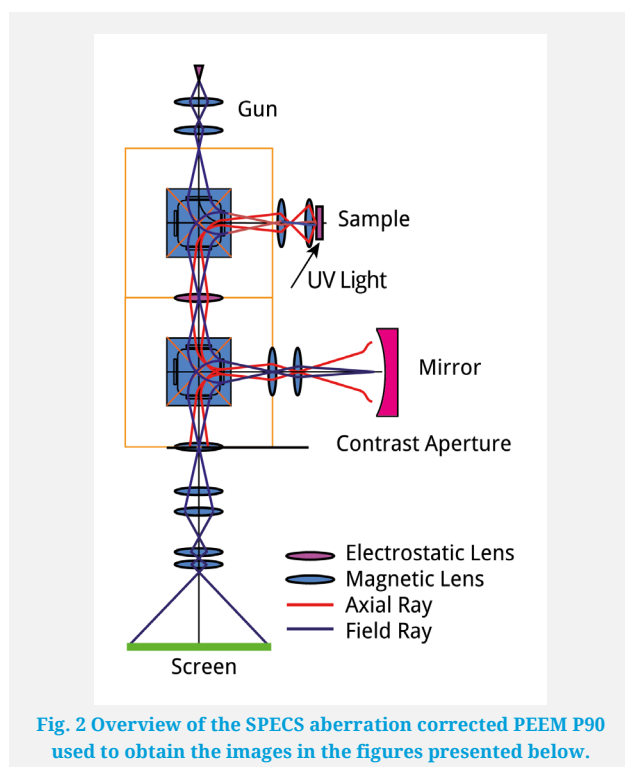


Fig. 1 A schematic of the volume imaging method. Individual slices are sequentially imaged to reconstruct a full 3D image. Images are regenerated using data from [1].

electrons are excited from the sample by a UV source. The optional electron gun is used only in LEEM measurements. Emitted electrons are then collected by the high voltage objective lens which is positioned a few millimetres in front of the sample surface. Following this, electrons pass through an electronic prism energy filter and an electrostatic lens before entering the aberration correction module which corrects for spherical and chromatic aberrations in the lens system. Finally, a contrast aperture selects a subset of the angular range of electrons contributing to the final image, and a focussed image is achieved using magnetic lenses.

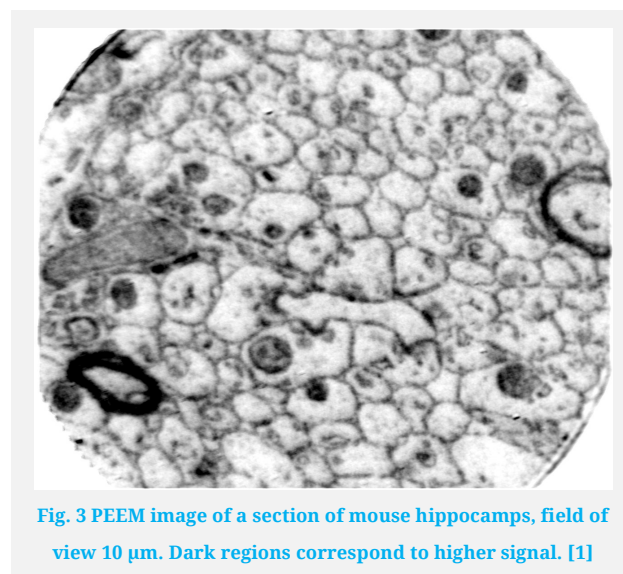


The samples imaged in this study were 40 nm thick slices of a mouse hippocampus on top of a Si substrate sputter coated with 50 nm of Au to improve adhesion of the sample. Si substrates without the Au layer were also measured and showed no significant differences. PEEM measurements were carried out using a SPECS P90 PEEM with aberration correction at the University of Leiden in the Netherlands. The UV source employed was a high flux Hg lamp equipped with a 250 nm filter to reduce sample degradation due to 300 nm light. For the samples investigated here, LEEM measurements using

the electron gun did not provide any contrast on the sample.

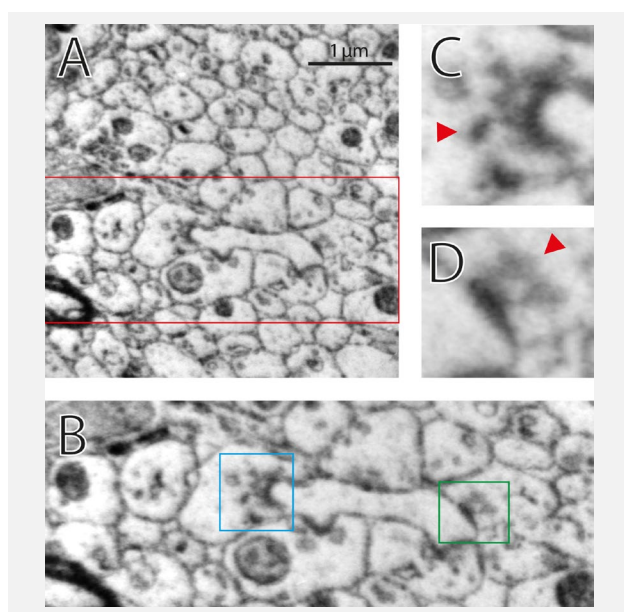
Results

A typical image with a field of view of 10 μm is presented in Fig. 3. The resolution in this image is 10 nm/pixel. A number of cell walls (dark boundaries) can be discerned across the image, including some where the nucleus is visible within the cell (filled circles). These cells are the neurons of the brain. A thicker, darker ring observed in the lower left corner of the image is a cross section of an insulating myelin sheath that is used to electrically isolate neurons that cover large distances, in order to preserve the electrical signal, they carry. The connections between neurons are called synapses, which form the basis for the brains function by making connections to other neurons thereby forming a neural network.



A key goal of the present study was to identify signatures of synapses in PEEM images. Synapses form the connections between neurons and therefore enable signals to be sent within the neural network. This connectivity is central to the brains function. In Fig. 4 we highlight regions within the data of Fig. 3 where signatures of synapses are observed. Two distinct features are observed, both of which are seen within the blue and green squares of Fig. 4B. The first feature is regions of the neuron cell wall that produce more signal compared with other parts i.e. they are darker. This signal is caused by

a thickening of the cell wall due to the connection of a synapse onto the neuron and is called the postsynaptic density. The second signature are clusters of small features within the neuron that are found close to the postsynaptic density, as highlighted by the red arrows in Fig. 4C, D. These are individual synaptic vesicles.



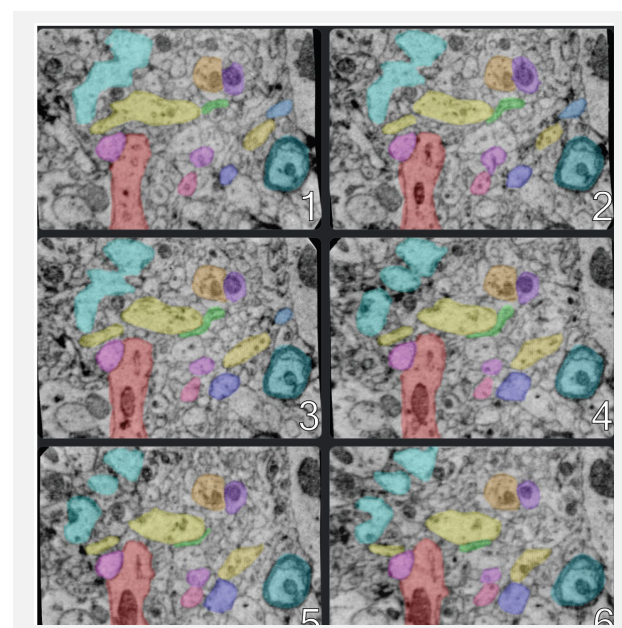
The vesicles store signalling molecules (neurotransmitters) that are released by the neuron into a synapse to send information to other cells. These data reveal that PEEM has sufficient resolution and contrast to resolve both synaptic density and individual synaptic vesicles, as well as many other features.

Having successfully identified synapses in the PEEM images, it remains to consider the “volume” aspect. To perform a proof of principle experiment, six neighbouring slices from a single sample were imaged sequentially. The resulting images from the six slices are shown in Fig. 5. In order to obtain a higher throughput rate, we imaged the samples at 30 nm/pixel resolution. Using openly available software algorithms typically used in the microscopy community, it was possible to identify and track the evolution of multiple neural strands (neurites) across the layers. As shown in the figure, the image is partially segmented into different regions as represented by different colours. Each region corresponds to an individual neurite and the changing form represents slices of the 3D evolution of the neurite as it expands, shrinks or bifurcates. This analysis would allow the

shape of the neuron, and their synaptic connections, to be reconstructed and visualised in 3D.

Conclusions

The present investigation of ultra-thin slices of mouse hippocampus demonstrates the unique capabilities of PEEM for imaging biological materials with a resolution



of 10 nm and without the need to raster scan the sample. Clear signatures of the fundamental connections present between neurons in the brain – synapses – were observed via postsynaptic density and the presence of individual synaptic vesicles. Furthermore, it was shown that volume-PEEM on biological samples can easily fit into existing analysis workflows for the 3D mapping of extended structures.

This proof-of-concept study highlights the significant potential for vPEEM in biological investigations involving connectomics, histology and cancer research.

1 G. Wildenberg et al, Photoemission electron microscopy for connectomics, Proc. Natl. Acad. Sci. U.S.A. 122 (48) e2521349122, <https://doi.org/10.1073/pnas.2521349122> (2025).

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