ESRF	**Experiment title:** **Structure of myelinated nerves by tomographic x-ray propagation imaging**	**Experiment number**: SC 3574
Beamline: ID22NI	**Date of experiment**: from: January, 22th 2013 to: January, 29th 2013	**Date of report**: August 27th,2013
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Report:

We have studied myelinated mouse nerves of the peripheral and central nervous system with hard x-ray phase contrast tomography in the Fresnel near field (holotomography [1]). The myelin sheath is a concentric multilamellar membrane stack which is wrapped around axons of neurons. The segmental structure of the insulating myelin sheath along the axon, with segments separated by so-called nodes of Ranvier (where myelin is lacking), enables the saltatory conduction of nerve impulses, needed for fast signal transduction. While multiple scattering, fluorescence staining and resolution issues pose a limit to visible light microscopy studies, preparation difficulties such as cutting and staining as well as limited penetration depth restrict the applications of electron microscopy. Therefore, the aim of the experiment was to investigate whether x-ray holography is capable of visualizing thousands of axons simultaneously within unsliced nerve tissue. To this end we have followed different sample prepration strategies and used varying geometrical settings and phase retrieval methods.

Fig. 1 Preparation of nerve samples (both stained with OsO_4). Left: Immersion fixated nerve embedded in agarose. Right: High-pressure frozen nerve embedded in EPON.

Sciatic, optical and saphenus nerves of wildtype mouse were prepared either by high pressure freezing followed by EPON embedding or immersion fixation followed by agarose embedding (sample preparation performed by Wiebke Möbius, Max-Planck Institut für Experimentelle Medizin, Göttingen) [2]. EPON blocks were milled into cylindrical shape while nerves embedded in agarose where kept in capton tubes (Fig.1). For (optional) specific myelin labeling samples were stained with osmium tetroxide OsO_4. The samples were imaged in cone beam geometry at beamline ID22NI. The energy of the pink photon beam was set to 17 keV for the current experiment and the focus size was about 80nm x 80nm (FWHM). Tomographic datasets were recorded at different geometric magnifications and effective pixel sizes using the FReLoN detector system

with a pixel size of 756 nm in the detection plane at a distance of 526 mm from the focus. Low resolution datasets with effective pixel sizes of 430 nm were recorded at a single source-sample distance, higher resolution datasets with 100 nm effective pixel size were recorded using four different planes with relative distances 1 mm, 5 mm and 15 mm, in order to circumvent artifacts due to zeros in the contrast transfer functions of Fresnel diffraction [1]. For tomography 1499 projections distributed among 360° were recorded. The illumination time was altered between 0.1, 0.5 and 1 seconds for a single projection, depending on the focal distance and sample preparation method.
Phase and tomographic reconstructions were succsessful for unstained imersion fixated saphenus nerves (peripheral nervous system) and reveal sub-structure within the nerve. (Fig. 2a). While nuclei of Schwann cells (which form the myelin) are visible, axons are at most vaguely identifyable. Therefore, we have investigated the effect of osmium tetroxide staining on image contrast and axon visibilty (Fig 2.b). Staining of lipids with electron dense osmium reverses the relative electron density contrast of lipids against water as can obsiously be seen for fat. Moreover, staining allows to visualize axons unambiguously since surrounding myelin is resolved (inset of b). The observeed structures correlate well with optical micrographs obatined of a semithin section of an EPON-embedded saphenus nerves (Fig 2.c).

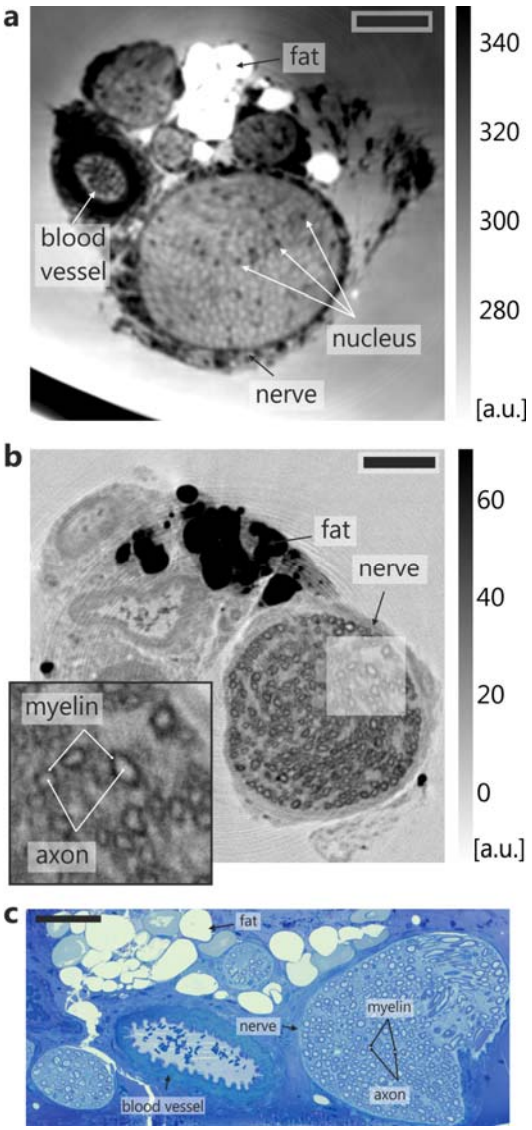

Fig 2. Slice through reconstructed 3D electron density of immersion fixated saphenus nerves (a) without and (b) with osmium tetroxide stain. (c) Optical micrograph of a semithin section of an EPON-embedded saphenus nerve. Scale bars, (a,b) 200 µm, (c) 50 µm.

Next, nerves of the central nervous system with thinner and less strongly myelinated axons were investigated. By using immersion fixated, osmium stained and agarose embedded optical nerves tomograms with 100 nm voxel size could be obtained (Fig. 3a). Axons with surrounding myelin are resolved down to axon diameters of 1 µm (inset) and structures can readiliy correletated to optical micrographs of corresponding semithin sections of optical nerves (Fig 3.b). However, sample shrinkage and movement during recording induced reconstruction artifacts and limited the available resolution.

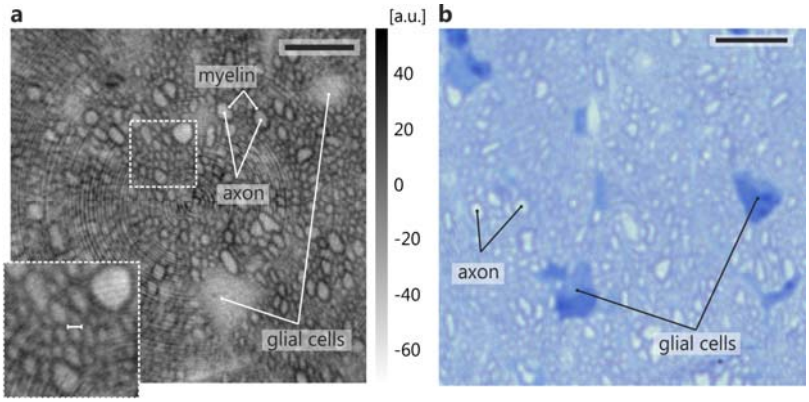

Fig 3. (a) Slice through reconstructed 3D electron density of immersion fixated optical nerve. (b) Optical micrograph of a semithin section of an EPON-embedded optical nerve. Scale bars, 10 µm, 1 µm (inset).

In order to achieve highest resolution and circumvent artifacts high pressure frozen and EPON-embedded sciatic nerves were investigated. Tomograms with 100 nm voxel size were sucsessfully reconstructed. The small focus size of ID22NI resulted in high-resolution data wich allows to cleary identify myelin (Fig.4, here stained with OsO_4). In contrast to electron microscopy studies, a huge volume of $(200\mu m)^3$ was reconstructed at once, containing more than 1000 axons running in parallel within the nerve. Surprisingly, contrast and resolution suffices to resolve myelin substructure, i.e. nodes of Ranvier and Schmidt-Lantermann incisures. To illustrate the unique information content of such a dataset, 20 axons were visualized. Data analysis is still ongoing but preliminary data suggest that Schmidt-Lantermann incisures may predominately be found in the vicinity of nodes of Ranvier of neighbouring axons.

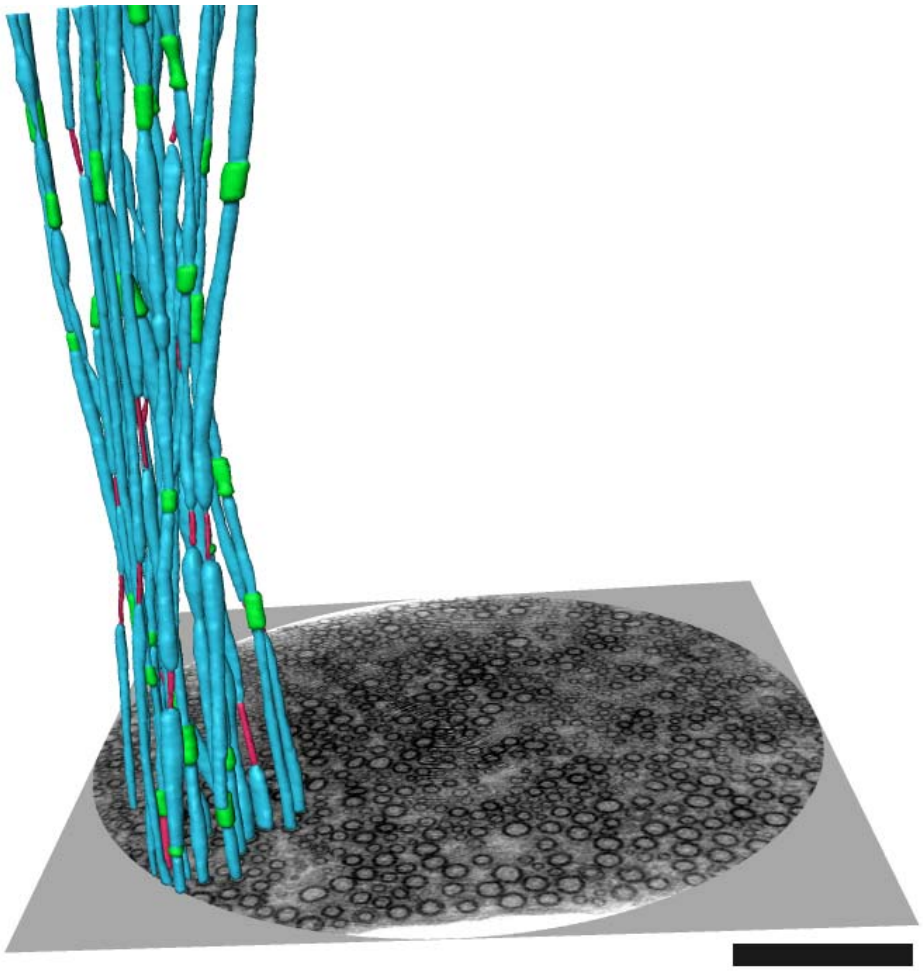

Fig 4. Slice through the reconstructed electron density of a high pressure frozen sciatic nerve with 100 nm voxel size, aling with the three-dimensional visualisation of 20 axons (turquoise). Nodes of Ranvier are rendered red, Schmidt-Lantermann incisures green. Scale bar, 100 µm.

The present experiment demonstrates that hard x-ray phase contrast tomography is capable of reconstructing the three-dimensional structure of entire nerve tissue with high contrast and resolution. Nerves of the peripheral and central nervous system (optical, saphenus and sciatic nerves) were investigated. The technique may help to evaluate distribution, orientation and relative location of biological relevant features of entire nerves in pysiological and pathological states. Future experiments should focus on higher spatial resolution which should be feasible with smaller x-ray focus sizes as delivered for example by the coming NINA beamline of ESRF.

References

[1] P. Cloetens, W. Ludwig, J. Baruchel, D. V. Dyck, J. V. Landuyt, J. P. Guigay, and M. Schlenker. Holotomography: Quantitative phase tomography with micrometer resolution using hard synchrotron radiation x rays. Applied Physics Letters, 75:2912, 1999.
[2] W. Möbius, B. Cooper, W. Kaufmann, C. Imig, T. Ruhwedel, N. Snaidero, A. Saab, and F. Varoqueaux. Electron microscopy of the mouse central nervous system. Methods in Cell Biology, 96:475, 2010.