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# Extending two-dimensional histology into the third dimension through conventional micro computed tomography

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ABSTRACT

Histological examination achieves sub-micrometer resolution laterally. In the third dimension, however, resolution is limited to section thickness. In addition, histological sectioning and mounting sections on glass slides introduce tissue-dependent stress and strain. In contrast, state-of-the-art hard X-ray micro computed tomography (μCT) systems provide isotropic sub-micrometer resolution and avoid sectioning artefacts. The drawback of μCT in the absorption contrast mode for visualising physically soft tissue is a low attenuation difference between anatomical features. In this communication, we demonstrate that formalin-fixed paraffin-embedded human cerebellum yields appropriate absorption contrast in laboratory-based μCT data, comparable to conventional histological sections. Purkinje cells, for example, are readily visible. In order to investigate the pros and cons of complementary approaches, two- and three-dimensional data were manually and automatically registered. The joint histogram of histology and the related μCT slice allows for a detailed discussion on how to integrate two-dimensional information from histology into a three-dimensional tomography dataset. This methodology is not only rewarding for the analysis of the human cerebellum, but it also has relevance for investigations of tissue biopsies and post-mortem applications. Our data indicate that laboratory-based μCT as a modality can fill the gap between synchrotron radiation-based μCT and histology for a variety of tissues. As the information from haematoxylin and eosin (H&E) stained sections and μCT data is related, one can colourise local X-ray absorption values according to the H&E stain. Hence, μCT data can correlate and virtually extend two-dimensional (2D) histology data into the third dimension.



## Introduction

The microanatomy, i.e. microstructures and morphology, of tissue components is generally characterised by means of histological sectioning, as this sort of examination can provide a true (sub-)micrometer resolution in two lateral dimensions, when neglecting processing-derived shrinkage artefacts, and the option of functional staining for the contrast (Müller et al., 2006; Irshad et al., 2014). By means of optical microscopy individual intra- and extracellular components are visualised (Irshad et al., 2014; Kandel et al., 2012; Fuchs and Buhmann, 2011). Currently established protocols, however, are often time-consuming, and individual steps involved in the preparation procedure induce stress- and strain-related artefacts in the tissue (Schulz et al., 2010a; Müller et al., 2012; Germann et al., 2008). Moreover, for histological examinations, the tissue has to be irreversibly cut into slices (Lang et al., 2014). As the sections are a few micrometers thick, isotropic lateral resolution is lost in the third dimension. Therefore, non-destructive three-dimensional imaging is a promising complement to provide volumetric morphological information (Schulz et al., 2010a).

Confocal microscopy yields images of cells in layers well below the surface. However, the limited transmission of visible light does not allow for the comprehensive visualisation of human tissue in its three-dimensional (3D) state (Müller et al., 2006). Multiphoton fluorescence in general and one- or two-photon microscopy (Wolf et al., 2015) in particular can provide information from tissue layers up to hundreds of micrometers, while decreasing the spatial resolution (So, 2002), for example in zebrafish larval brain (Vladimirov et al., 2014). Tissue-clearing methods significantly increase the accessible depth (Richardson and Lichtman, 2015). For example, tissue-transformation method CLARITY can increase the achievable imaging depth up to 5 or even 6 mm (Chung et al., 2013). The SWITCH method improves the light penetration as well, demonstrating clearing of a whole adult

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mouse brain, lung, kidney, heart, liver and spinal cord with a required tissue-clearing time between five and ten days (Murray et al., 2015). Combining tissue clearing with confocal light sheet and light sheet fluorescence microscopy allows for the visualisation of entire mouse brains (Dodt et al., 2007; Silvestri et al., 2012; Costantini et al., 2015). Nevertheless, these procedures are often technically demanding, expensive, time-consuming, induce significant tissue deformation, and restricted to a particular tissue type with sizes not exceeding a thickness of some millimeters.

Another means of obtaining 3D morphology is serial sectioning, which is time-consuming and is mainly applied to small tissue volumes (Chung et al., 2013). The time restriction can be overcome by applying a serial optical coherence scanner (Wang et al., 2014), albeit spatial resolution in the third dimension still remains restricted to section thickness (Lang et al., 2014; Schulz et al., 2010a). Nonetheless, these methods are destructive and the same sample can often not be reused for subsequent examinations.

Synchrotron radiation-based micro and nano computed tomography (SRμCT) provides impressive 3D images of biological tissues on a (sub-)cellular level (Zehbe et al., 2010; Huang et al., 2015). For example, one can detect and image RNA/DNA-stained HEK 293 cell clusters (Müller et al., 2006), intracellular structures of dehydrated human cells (Guk et al., 2008), single endothelial cells labelled with iron oxide particles (Thimm et al., 2012), chondrocytes within the extracellular matrix of articular cartilages without metal staining (Zehbe et al., 2015), the lacuno-canalicular network and collagen fibres in human bone (Langer et al., 2012), osmium-stained individual ganglion cells (Lareida et al., 2009), unstained Purkinje cells (Schulz et al., 2010b) and freeze-dried neurons (Mokso et al., 2007). The limited accessibility of synchrotron radiation facilities, though, imposes severe restrictions on the user (Wenz et al., 2015).

In contrast to laboratory sources, the synchrotron radiation sources yield such a high photon flux, that a monochromator can be incorporated to pass about $10^{-4}$ of the photons and generate monochromatic light, avoiding beam hardening. Laboratory-based μCT systems have successfully been employed for 3D visualisation of higher density materials (Chappard et al., 2005; Blouin et al., 2006) and stained tissues (Metscher, 2009; de Crespigny et al., 2008; Ribi et al., 2008; Ashton et al., 2015). Recently μCT was successfully used for analysis of brain architecture of insect species (Sombke et al., 2015). Furthermore, μCT can achieve superb resolution, although performance for lower density materials is restricted due to limited contrast.

Consequently, we state that there is a paucity of methods to study the microstructure and morphology of large tissue components in 3D space with isotropic (sub-)cellular spatial resolution within a laboratory environment.

In this study, we evaluate the contrast of formalin-fixed paraffin-embedded (FFPE) tissue obtained with laboratory-based μCT. We aim to demonstrate the three-dimensional non-destructive visualisation of a human cerebellum sample with cellular resolution, with phoenix|xray nanotom® m laboratory-based μCT system.

In order to directly compare three-dimensional μCT data with histology, the counterpart of the histological section has to be localised within the three-dimensional μCT dataset (Stalder et al., 2014). Such registration enables the validation of structures within the μCT data by selected histological sections (Gambichler et al., 2007).

It was shown that synchrotron radiation-based computed tomography, using the phase-contrast mode, allows for identifying not only major blood vessels, but also *Stratum moleculare*, *Stratum granulosum* and white matter within formalin-fixed human cerebellum - even individual Purkinje cells are visualised (Schulz et al., 2010b). The question arises as to whether laboratory-based absorption-contrast μCT of an FFPE human cerebellum sample can provide comparable results. We investigate how μCT could become a complementary method to the microscopic examination of stained tissue slices, thereby extending its applicability to three-dimensional features.

## Materials and methods

### *Tissue preparation*

The specimen was extracted from the donated cadaveric brain of a 73-year-old man. Written consent for scientific use was documented. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethikkommission Nordwestschweiz. The brain was fixed in 4% histological-grade buffered formalin for two weeks prior to dissection. Tissue samples for histological work-up were excised by a scalpel. These specimens, approximately 15 mm × 15 mm × 4 mm, to fit into conventional histological embedding cassettes, were dehydrated and paraffin embedded according to surgical pathology procedures: 3× 1 h in 70% ethanol (EtOH) in $H_2O$ (v/v), 1 h in 80% EtOH in $H_2O$ (v/v), 2× 1 h in 96% EtOH in $H_2O$ (v/v), 2× 1 h 100% xylene followed by 3× 1 h paraffin/plastic mixture (Surgipath Paraplast ®, Leica Biosystems, Switzerland) at 60 °C. Next, samples within the molten paraffin were transferred to histological embedding moulds on a routine paraffin block-casting device. Here, the paraffin was cooled down to approximately −8 °C, to solidify and then removed from the casting moulds. Cylinders 6 mm in diameter were extracted from the FFPE tissue, using a metal punch, for μCT measurements. Typically, minimum formaldehyde fixation time is one hour, paraffin embedding takes approximately ten hours, casting blocks five minutes and punching five minutes.

### *Data acquisition and processing*

The tomography experiments were carried out using the absorption-contrast μCT system nanotom® m (phoenix|x-ray, GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany) equipped with a 180 kV–15 W high-power nano-focus® tube with W and Mo transmission targets (General Electric, Measurement and Control, 2014; Egbert and Brunke, 2011). The nanotom® m μCT-system allows measuring objects with up to 25 cm in diameter and height.

The μCT was performed with a voxel length of 3.5 $\mu m^3$ and a field of view of about 8.5 × 10.5 $mm^2$. For each acquisition, 1900 projections were recorded over 360°. Geometric magnification $M$ was maintained at 28.57 for selected focus-detector distances (FDD) and focus-object distances (FOD): $M$ = FDD/FOD. Measurements were taken in the tube operation mode "0" with an estimated source size of 2.7 μm.

Data acquisition and reconstruction were performed with datos|x 2.0 software (phoenix|x-ray, GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany). The average data acquisition time was 3.5 h, with the requirement of the user interaction in the beginning of the scan followed by the automated execution. Data reconstruction is based on Feldkamps cone beam reconstruction algorithm (Egbert and Brunke, 2011; Feldkamp et al., 1984) and can be automatically done within less than 15 min. The reconstructed slices were scaled from black to white within the range of three times the distance from the maximum to the minimum histogram peak position for each dataset.

The reconstructed datasets were compared for their contrast-to-noise ratio:

$$CNR = \frac{|I_1 - I_2|}{\sqrt{\sigma_1^2 + \sigma_2^2}} \quad (1)$$

where $I_1$ and $I_2$ indicate the mean intensities of homogeneous components within the specimen, and $\sigma_1$ and $\sigma_2$ the standard deviations. To this end, volumes of interest (VOI) were selected within the white matter (VOI = 18.200 voxels), *Stratum granulosum* (VOI = 5292 voxels), *Stratum moleculare* (VOI = 4032 voxels), and paraffin (VOI = 4704 voxels) of each dataset. To ensure comparability of the *CNR* values, VOIs were selected from the same location within each dataset.