Contrast enhancement for visualizing neuronal cytoarchitecture by propagation-based x-ray phase-contrast tomography

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ARTICLE INFO

Keywords:
3d neuronal cytoarchitecture
Phase-contrast x-ray tomography
Synchrotron-based x-ray imaging
Laboratory-based x-ray imaging
Embedding media for contrast enhancement

ABSTRACT

Knowledge of the three-dimensional (3D) neuronal cytoarchitecture is an important factor in order to understand the connection between tissue structure and function or to visualize pathological changes in neurodegenerative diseases or tumor development. The gold standard in neuropathology is histology, a technique which provides insights into the cellular organization based on sectioning of the sample. Conventional histology, however, misses the complete 3D information as only individual two-dimensional slices through the object are available. In this work, we use propagation-based phase-contrast x-ray tomography to perform 3D virtual histology on cerebellar tissue from mice. This technique enables us to non-invasively visualize the entire 3D density distribution of the examined samples at isotropic (sub-)cellular resolution. One central challenge, however, of the technique is the fact that contrast for important structural features can be easily lost due to small electron density differences, notably between the cells and surrounding tissue. Here, we evaluate the influence of different embedding media, which are intermediate steps in sample preparation for classical histology, on contrast formation and examine the applicability of the different sample preparations both at a synchrotron-based holotomography setup as well as a laboratory source.

1. Introduction

The gold standard to decipher the cytoarchitecture of neuronal tissue on the (sub-)cellular scale is histology, based on slicing the sample into thin sections in order to gain insights into the three-dimensional (3D) cellular distribution (Li et al., 2010). It is a time-consuming technique which consists of several steps of tissue preparation and processing, including, e.g., fixation, dehydration and embedding of the tissue, followed by the mechanical slicing procedure, staining of features of interest and observation of the individual sections under a light microscope. While it provides excellent results in 2D, the resolution in the third dimension is limited by the slice thickness, leading to an anisotropic reconstruction of the 3D cytoarchitecture, and the sample is destroyed afterwards. Furthermore, artifacts can easily occur due to the mechanical slicing procedure. Computed tomography (CT) offers a high potential for non-destructive 3D virtual histology, without any need for slicing due to the high penetration depth of x-rays through tissue. It provides isotropic reconstruction of the 3D cytoarchitecture based on differences in absorption coefficient (absorption contrast) or electron density (phase contrast). As the entire 3D information is available, virtual slices can be generated in any direction, which is one of the many advantages of virtual histology. In this way, slice orientation can be selected a posteriori to visualize specific features of the sample. In order to achieve sufficient contrast to resolve single cells within the tissue, the phase shift induced by the sample can be exploited for image formation instead of the absorption contrast, on which clinical CT is based. As this phase shift cannot be measured directly, it has to be made visible in the intensity images, e.g., via Talbot interferometry (Momose et al., 1996; Weitkamp et al., 2005; Pfeiffer et al., 2008), edge illumination (Munro et al., 2012; Hagen et al., 2014) or speckle-based imaging (Morgan et al., 2012; Zdora et al., 2017).

In this work, we use free space propagation between the object and the detector (Nugent et al., 1996; Paganin and Nugent, 1998; Cloetens et al., 1999; Bartels et al., 2015a), as this form of phase contrast imaging...
provides the high resolution needed to visualize single cells within the reconstructed volume (Zanette et al., 2013; Lang et al., 2014; Töpperwien et al., 2018). Depending on the Fresnel number $F = \frac{2r}{p}$, where $p$ is the pixel size, $r$ the wave length and $z$ the distance between the object and the detector, image formation can be classified to be in the direct-contrast regime ($F > 1$), in which phase contrast is visible as edge enhancement, or in the holographic regime ($F < 1$), in which contrast transfer is higher and multiple interference fringes can be observed. In both cases, phase retrieval has to be performed in order to retrieve quantitative information about the object’s phase distribution (Cloetens et al., 1999; Paganin et al., 2002; Witte et al., 2009; Hagemann et al., 2018).

The potential of propagation-based x-ray phase-contrast tomography as a novel large-scale, label-free, 3D neuroimaging technique, has been tested in a number of recent studies. The cerebral angio-architecture was visualized within large brain regions of rats in Zhang et al. (2015). Ex vivo whole brain scans of small animal models were evaluated in view of brain tissue morphology for both normal and cancerous tissue, and the treatment effects of X-Ray Microbeam Radiation Therapy on nervous tissue were investigated (Barbone et al., 2018). Importantly, resolution and contrast was demonstrated to be sufficient for single cell identification in larger volumes both for rodents (Fratini et al., 2015; Bukreeva et al., 2017), as well as for human tissues (Hieber et al., 2016; Khimchenko et al., 2018; Töpperwien et al., 2018). The density and spatial distribution of neurons, for example, is of interest as a structural marker in physiology and pathology. To this end, it is particularly important that automated segmentation can be achieved for unstained tissue, as shown in Hieber et al. (2016), Bukreeva et al. (2017), Töpperwien et al. (2018). 3D resolution beyond the optical limit was first demonstrated by Khimchenko and co-workers (Khimchenko et al., 2018). Tissue alterations in different disease states, for example vascular alterations and neuronal loss in a multiple sclerosis model (Cedola et al., 2017), or plaque formation in Alzheimer’s disease models (Astolfi et al., 2018; Massimi et al., 2019) have also been addressed. Finally, in view of a broader accessibility, it is important to note that phase-contrast tomography of neuronal tissues with sub-micron resolution has also been translated to compact laboratory x-ray sources (Töpperwien et al., 2017, 2018).

In all of the recent studies mentioned above, sufficient contrast for small soft tissue features was the key advantage of the technique with respect to conventional CT. Phase-contrast CT is capable to exploit the small signals of electron density differences between cells and the surrounding tissue, but in the case of hydrated samples this can easily fall below the detection threshold. Optimization of the sample preparation becomes crucial in view of maintaining or increasing the contrast levels. One possibility is to use radiocontrast agents such as metals in order to stain specific features of interest within the sample (Bartels et al., 2015b; Krenkel et al., 2015; Töpperwien et al., 2016a; Strotton et al., 2018). Another possibility is to embed the sample in a medium with an electron density differing from the main tissue components, resulting in a more global contrast increase (Hieber et al., 2016; Bukreeva et al., 2017; Khimchenko et al., 2018; Töpperwien et al., 2018). In Strotton et al. (2018), different mounting, staining and experimental parameters were evaluated in terms of stability, contrast and image quality for imaging of spinal cord tissue at a synchrotron-based microtomography setup.

In this work, we study the effect of the embedding media on contrast formation on the cellular and sub-cellular level in unstained tissue. To this end, we focus on cerebellar tissue from mice. The cerebellum represents a perfect natural test object, as it exhibits features of different length scales (the large Purkinje cells with a soma size of 20–40 μm and the small cells in the granular layer with sizes of 5–8 μm in humans (Shepherd, 2004)) and of different densities, e.g. the low-cell molecular layer as opposed to the cell-dense granular layer. In earlier work, we have demonstrated that drying of tissue according to the evaporation-of-solvent method leads to reconstructions with a high contrast, allowing for the unambiguous identification of single cells within the volume (Töpperwien et al., 2017). The applicability of this preparation to human tissue, however, still needs experimental validation. Further, it is accompanied by considerable shrinkage. Here, we concentrate on less invasive embedding media, which are furthermore all well established as intermediate steps in a paraffin-embedding sequence and are therefore of standard use in the preparation of human brain samples in pathology, namely PBS (electron density: ∼337 μ/m3), ethanol (∼269 μ/m3) and the wax itself (∼311 μ/m3) (Khimchenko et al., 2016, 2018; Töpperwien et al., 2018). This offers the additional advantage that the tissue is also well suited for a subsequent histological examination without the need for rehydration and stain removal and reembedding of the samples (Holme et al., 2014; Hieber et al., 2016; Khimchenko et al., 2018). The experiments were performed both at a high-resolution synchrotron-based holo-tomography endstation (Salditt et al., 2015; Töpperwien et al., 2018) and at a home-built laboratory setup (Töpperwien et al., 2017, 2018). This enables imaging of the sample on multiple length scales and fields of view.

2. Materials and methods

2.1. Sample preparation

Wild-type mice were either perfused with 10% sucrose solution for 10 min, followed by the dissection of the brain and fixation overnight in a solution containing 1% paraformaldehyde and 1% glutaraldehyde, or sacrificed with CO2 followed by the dissection of the brain and fixation in 10% formalin for 24 h.

Hydrated samples. In a first step, the fixed brain was cut into 500 μm or 1 mm thick slices. A liquid chamber was built with two aluminum rings with a diameter of 5 cm and a width of 500 μm which were covered with polypropylene foil on one side. Placing the brain slice together with additional PBS onto one of the foils and gluing a second ring on top of it results in a closed chamber around the sample (cf. Fig. 1(a)). The orientation of the aluminum rings determines the width of the chamber, as they can act as spacers between the polypropylene foils. In order to mount the sample stably in the setup, the width of the chamber equaled the width of the brain slice so that it was kept in place by the pressure of the polypropylene foils. In a last step, the resulting liquid chamber was clamped into a brass sample holder for mounting in the setup. As the measurements at the laboratory required a smaller sample-to-detector distance, the sample size was restricted and the aluminum rings could not be used. In this case, the sample was prepared similar to the ethanol-embedded case, as described in the following.

Ethanol-embedded samples. The fixed brain was sliced into 500 μm or 1 mm thick sections. For the exchange of water content, an ascending ethanol series was performed (70%: 1 × 15 min, 90%: 1 × 15 min, 100%: 2 × 15 min, 100%: 1 × 30 min, 100%: 1 × 45 min). Subsequently, a 1 mm punch was taken from the brain slice in order to reduce absorption of parts of the sample which lie outside the field of view and squeezed into a Kapton tube with 1 mm diameter, which was glued to a sample holder. The punch was positioned based on visual inspection such that all layers of the cerebellum were included in the respective volume. In order to maintain experimental conditions and prevent the sample from drying, the tube was filled with additional ethanol and sealed with 2-component glue (cf. Fig. 1(b)). For long measurements, as necessary at the laboratory, an additional layer of haematocrit sealing compound (Brand, Germany) was inserted between the ethanol and glue, since this proved to be more stable with regard to evaporation of the solvent.

Paraffin-embedded samples. After fixation, the entire mouse brain was embedded in paraffin. To this end, water content was removed via an ascending ethanol series (60%: 1 × 1.5 h, 75%: 2 × 1.5 h, 96%: 2 × 1.5 h, 100%: 2 × 1.5 h). Subsequently the ethanol was exchanged by xylene (2 × 1.5 h) and the sample was transferred to molten paraffin wax (∼60°C). After complete infiltration (2 × 1.5 h), the tissue was embedded in a fresh batch of paraffin, which was subsequently hardened. For the experiments, a 1 mm biopsy punch was taken and squeezed into a 1 mm
Kapton tube glued to a sample holder (cf. Fig. 1(c)). As the wax is hard at room temperature, no additional sealing of the Kapton tube was necessary as opposed to the preparations in ethanol or PBS.

### 2.2. Experimental setups

**Synchrotron setup.** The experiments were performed at the Göttingen Instrument for Nano-Imaging with X-Rays (GINIX) which is installed at the P10 beamline of the storage ring PETRAIII at DESY in Hamburg (Salditt et al., 2015; Töpperwien et al., 2018). The photons were monochromatized by a Si(111) channelcut-monochromator to an energy of 8 keV and prefocused by a set of Kirkpatrick-Baez mirrors to a focal spot of approximately $300 \times 300 \text{nm}^2$. To reduce high-frequency artifacts caused by inhomogeneities on the mirror surfaces, as well as to decrease the focus size and to obtain a higher degree of spatial coherence, a waveguide was placed into the focal plane of the mirrors (Neubauer et al., 2014; Chen et al., 2015; Hoffmann-Urba, et al., 2016). At a distance $z_{01}$ behind the waveguide, the sample was positioned on a fully motorized sample stage, allowing for a precise alignment of both the rotation axis with respect to the beam as well as the sample with respect to the field of view. Approximately $5 \text{mm}$ behind the sample, a scintillator-based fiber-coupled sCMOS detector with a Gadox powder scintillator and a pixel size $p = 6.5 \mu \text{m}$ (Photonic Science, UK) was located. Image formation by free space propagation for these experimental parameters results in the so-called holographic regime. Note that the divergent beam geometry leads to a selectable geometrical magnification $M = \frac{p_{\text{det}}}{p_{\text{sub}}} = \frac{q_{\text{sub}}}{q_{\text{det}}}$, with the source-to-detector distance $z_{02}$, resulting in an image with effective pixel size $p_{\text{eff}}$ $= \frac{p}{M}$. In this way, propagation imaging is enabled at the nanoscale. The parameters used for the experiments are listed in Table 1.

**Laboratory setup.** In the laboratory setup (Töpperwien et al., 2017, 2018), x-rays were generated by a liquid-metal jet microfocus source (Excillum, Sweden), consisting of GaIn with the main energy peak at the Ga-$K_\alpha$ line at 9.25 keV. The liquid-metal anode enabled sufficient spatial coherence while providing a comparably large photon flux. As in the synchrotron setup, the sample was placed on a fully motorized sample stage and the photons were detected further downstream. Due to lower magnification and smaller propagation distance compared to the synchrotron setup, image formation is now in the so-called direct-contrast regime. This is well suited for laboratory measurements, since this regime is more robust with respect to low spatial and temporal coherence. In order to reach (sub-)cellular resolution, the setup was operated in the ‘inverse’ geometry, i.e., the sample was placed close to the detector, leading to a magnification $M \approx 1$, and a high-resolution detector with a pixel size of $0.54 \mu \text{m}$ (XSi8on micron, Rigaku, Czech Republic) was used (Töpperwien et al., 2017, 2018). A detailed list of the experimental parameters is given in Table 1.

### 2.3. Data analysis

**Synchrotron measurements.** Phase retrieval was performed with the contrast transfer function (CTF)-based reconstruction algorithm introduced by Cloetens et al. (Cloetens et al., 1999; Turner et al., 2004; Zabler et al., 2005) on all empty-beam corrected projections (cf. Fig. 2(a) and b), 3 (a,b) and 4 (a,b). In the experiments on the ethanol- and paraffin-embedded samples, projections were recorded at several propagation distances to avoid artifacts due to the zero crossings of the CTF. In this case, the cone-beam geometry of the setup leads to a varying magnification and field of view in the single projections. To account for this, the corresponding projections were scaled to the pixel size with the highest magnification, aligned via a cross-correlation in Fourier space (Guizar-Sicairos et al., 2008) and cropped to the same field of view. An additional high-pass filter was applied on the empty-beam corrected projections to reduce low-frequency artifacts caused by an unstable illumination during the measurement. Ring removal was performed on the individual sinograms prior to tomographic reconstruction, either with a wavelet-based technique (Münch et al., 2009) (hydrated) or a simpler approach based on integration of the sinogram along the angle and subsequent filtering (Ketcham, 2006) (ethanol/paraffin). Tomographic reconstruction was carried out with the Matlab implementation of the filtered backprojection with a standard Ram-Lak filter. In order to obtain a higher signal-to-noise ratio (SNR), all slices were filtered with a Gaussian function with a standard deviation of 0.7 pixels (hydrated) or 1 pixel (ethanol/paraffin), leading to a denoising of the images at the cost of a slight image blur. Segmentation of the large Purkinje cells was performed in Avizo 9 (Thermo Fisher Scientific, USA), using a gray-value based region growing tool with manually defined seeding points. In order to also segment smaller features with a low signal-to-noise ratio (SNR), the segmentation was subsequently manually refined.

**Laboratory measurements.** Phase reconstruction was performed on the

### Table 1

| Parameters for the experiments at the synchrotron as well as laboratory setup. | synchrotron | laboratory |
|---|---|---|
| | hydrated | ethanol/paraffin | all samples |
| energy (keV) | 8 | 8 | 0.40/9.25 |
| $x_{01}$ (mm) | 141 | 145 | 158.75 |
| $x_{02}$ (m) | 5.1 | 5.1 | 0.185 |
| $p_{\text{eff}}$ (nm) | 178 | 187 | 463 |
| field of view (mm²) | 0.365 × 0.365 | 0.383 × 0.383 | 1.15 × 1.53 |
| number of distances | 1 | 4 | 1 |
| number of projections | 1662 | 1500 | 1000 |
| angular range (°) | [0,180] | [0,180] | [0,180] |
| exposure time (s) | 0.2 | 0.15/0.1 | 50 |
empty-beam corrected projections using the Bronnikov-aided correction (BAC) algorithm (Witte et al., 2009; Tøpperwien et al., 2016b). In order to increase the SNR, all projections were resampled by a factor 2. Tomographic reconstruction was carried out with the cone-beam implementation of the ASTRA toolbox (Palenstijn et al., 2011; van Aarle et al., 2015, 2016). Prior to the tomographic reconstruction, the simple ring removal approach was performed on the individual sinograms. By applying a Gaussian filter with a standard deviation of 1 pixel on the reconstructed slices, the SNR was further increased, though at the cost of a slight blurring of sample features.

3. Results

3.1. Synchrotron setup

Hydrated sample. Two exemplary slices through the reconstructed density distribution of the hydrated brain slice are depicted in Fig. 2(c). Note that the orientation of these virtual slices was chosen such that they lie parallel to the Purkinje cell layer. The typical layers of the cerebellar cortex, namely the cell-rich granular layer (GL), the low-cell molecular layer (ML) and the mono-cellular Purkinje cell layer (PCL), as well as the white matter (WM) consisting of large axon bundles, can be clearly distinguished. Within these layers, single cells can be resolved, especially the large Purkinje cells comprising a high contrast with respect to the surrounding tissue. Even sub-cellular details are to some extent visible as, e.g., inner structure of the granule cell nuclei or the nucleolus of the Purkinje cells (cf. Fig. 2(c), inset on the right). However, overall contrast is relatively low and a clear separation between single cells, especially in the cell-dense granular layer, is challenging. The contrast obtained for different features within the cerebellum can be quantified via the Weber contrast, defined as (Poli, 1990)

\[ C = \frac{I_{\text{target}} - I_{\text{background}}}{I_{\text{background}}} \]  

(1)

which is a measure for the contrast of specific targets compared to their background. In order to reveal the 3d cytoarchitecture of neuronal tissue, the exact localization of cells is necessary and hence, their contrast against the surrounding tissue is the main challenge. Due to the difference in cell types, contrast was determined for both the Purkinje cells as well as the granular layer. To this end, several cells with comparatively high contrast based on visual inspection were manually selected and their resulting contrast against the surrounding tissue was averaged, yielding \( C_{\text{PCL}} \approx 0.5 \) and \( C_{\text{GL}} \approx 0.19 \), respectively. Note that for better comparability, the gray values of the reconstructed volume were shifted so that the minimum is given by 0, as different offsets, e.g., caused by region-of-interest tomography, influence the absolute values for the Weber contrast. In order to visualize the 3d structure of the high-contrast Purkinje cell layer, selected cells were segmented, including the large cell body and the parts of the dendritic tree which could be well distinguished from background. The result is depicted in Fig. 2(d), with two individual Purkinje cells shown at the bottom. The typical shape of the Purkinje cells with a large and nearly two-dimensional dendritic tree, as well as their parallel arrangement within the entire layer can be well recognized. However, when considering the single cells it becomes clear that due to a lack of contrast, only the main part of the dendritic tree, close to the cell body where branches are thickest, is resolved. An estimate of the resolution can be either achieved by considering the Fourier shell correlation (FSC) (van Heel, 1987; Harauz and van Heel, 1986), yielding the overall quality of the experiment, or via an error function fit to the edge of a highly contrasted feature within the slices, measuring the system blur for the given experimental parameters. To generate two independent datasets for the calculation of the FSC, the tomographic scan was divided into two subsets, consisting of every second projection and starting with the first or second, respectively. The intersection point between the FSC of the central 1000 voxels of these reconstructions and the 1/2-bit threshold curve determines the spatial frequency of the smallest feature for which enough data was collected for interpretation (van Heel and Schatz, 2005), resulting in a half-period resolution of 809 nm. The resolution estimate via an error function fit to the profiles along manually selected edges between Purkinje cell bodies, exhibiting a comparably large contrast, and the surrounding tissue, yielded a FWHM of \( \sim 1.11 \mu \text{m} \) and hence a half-period resolution of \( \sim 555 \text{nm} \). The large difference between the two values indicates that noise and a weak contrast of the sample features, which have a large influence on the result obtained via
the FSC, significantly limit the (globally defined) resolution, notwithstanding higher resolution of local features with stronger contrast.

Ethanol-embedded sample. Two orthogonal virtual slices through the obtained 3D density distribution are shown in Fig. 3(c). The layers of the cerebellar cortex, including the single cells within these layers as well as the white matter are clearly resolved. Additionally, sub-cellular details as, e.g., the inner structure of the granule cell nuclei or the thick branches of the dendritic tree as well as to some extent the nucleus of the Purkinje cells can be recognized. The contrast of single cells against surrounding tissue was again determined via eqn. (1), yielding $C_{PCL} = 0.99$ for the Purkinje cell and $C_{GL} = 0.34$ for the granular layer. The 3D shape of the Purkinje cell layer is depicted in Fig. 3(d), showing the typical flat dendritic tree and the parallel orientation of the cells to each other. Although they exhibit a higher contrast compared to the hydrated tissue, the amount of detail within this segmentation is approximately the same, which is probably due to the shrinking of the sample upon dehydration which is further quantified below.

An estimate for the achieved resolution was gained both via the FSC criterion, and an error function fit to an edge between a high-contrast feature and surrounding tissue in the reconstructed slices. The FSC curve of the central 1000^3 voxels intersected the 1/2-bit threshold curve at 0.092 cycles/pixel, corresponding to a half period resolution of 1.02 μm. The fit of an error function to the edge between selected Purkinje cells and surrounding tissue yielded a FWHM of ~800 nm and hence a half-period resolution of ~400 nm. This again shows that the dataset has a relatively high noise level and low contrast in the bulk of the reconstructed density distribution, which significantly limit the (global) resolution.

Paraffin-embedded sample. Two orthogonal slices through the reconstructed density distribution are depicted in Fig. 4(c). Again, the typical layers of the cerebellum are resolved down to the (sub-)cellular level. Within the cells, especially the large Purkinje cells, details as the nucleolus and also the surrounding nuclei can be unambiguously distinguished whereas structures as the soma or dendritic tree of the Purkinje cells exhibit a relatively low contrast and can only be roughly visualized. This can be further quantified via the corresponding Weber contrast values, yielding $C_{PCL} = 0.2$ for the Purkinje cell layer and $C_{GL} = 0.56$ for the granular layer, respectively.

A segmentation of the Purkinje cell layer was performed on a selected number of cells, leading to the result depicted in Fig. 4(d). As in the case of the hydrated and ethanol-embedded sample, the typical flat shape and parallel orientation of the cells can be well recognized, though at lower detail due to the weak contrast of the dendritic tree in this preparation. The superior contrast of the cell nuclei, however, allows for an automatic segmentation of the small cells in the molecular and granular layer based on the Spherical Hough transform, as described in Topperwien et al. (2018) for human tissue. In Fig. 4(e) the result of this segmentation is shown both overlaid on a 2D slice and in 3D. The overlay of the segmentation on the corresponding virtual slice through the volume on the left shows that despite the larger cell density and hence denser packing of cells in mouse tissue, an automatic segmentation can be performed at high precision. This allows for a subsequent analysis of cellular distributions in 3D based on the exact locations of the cells within in the reconstructed volume.

Analogous to the hydrated and ethanol-embedded brain slices, the resolution of the scan was both estimated via the FSC criterion as well as an error function fit. The intersection between the FSC of the central 1000^3 voxels and the 1/2 threshold curve yielded a half-period resolution of 930 nm. For the determination of resolution via an error function fit, the edge between the high-contrast nucleolus of the large Purkinje cells and surrounding tissue was chosen, since the contrast of the previously considered cell bodies is comparatively low. This leads to a half-period resolution of ~310 nm, again indicating the large influence of noise and contrast on the estimated values.

Comparison. A comparison between the reconstructed densities for hydrated, ethanol-embedded and paraffin-embedded tissue is depicted in Fig. 5 and the obtained values for the Weber contrast as well as resolution are listed in Table 2. Embedding in ethanol leads to an overall increase in

Fig. 3. Ethanol-embedded mouse cerebellum at the synchrotron setup. (a) Exemplary empty-beam corrected projection. (b) Reconstructed projection using the CTF-based approach for homogeneous objects with four propagation distances. In these projections, the mono-cellular Purkinje cell layer with the large cell bodies as well as a clear density difference between the cell-rich granular layer and the low-cell molecular layer can already be recognized to some extent; hence even without tomographic reconstruction. (c) Orthogonal slices through the reconstructed density, showing the cell-rich granular layer (GL), the low-cell molecular layer (ML), the mono-cellular Purkinje cell layer (PCL) and the white matter (WM) at high contrast. Within the large Purkinje cell bodies, even sub-cellular details as the nucleolus can be recognized (inset on the right). (d) Segmentation of part of the Purkinje cell layer. The typical flat shape of these cells and their parallel arrangement as well as the to some extent the highly branched dendritic tree can be visualized in 3D. Scale bars: 50 μm.
Fig. 4. Paraffin-embedded mouse cerebellum at the synchrotron setup. (a) Exemplary empty-beam corrected projection, in which cracks within the paraffin can be recognized as bright features. Note that in order to reduce low-frequency artifacts due to a changing illumination, a high-pass filter was applied. (b) Reconstructed phase map obtained via the CTF-based algorithm for homogeneous objects using four propagation distances. (c) Orthogonal slices through the reconstructed density distribution, showing the layers of the cerebellar cortex (granular layer (GL), molecular layer (ML) and Purkinje cell layer (PCL)) as well as white matter (WM). Especially the nucleoli can be visualized at high contrast. (d) Segmentation of the Purkinje cell layer. The Purkinje cells show the expected two-dimensional shape and parallel arrangement. However, due to the relatively low contrast of sub-cellular details as the cell body or the dendritic tree, only a small part of these large cells can be visualized in 3d. (e) The high contrast of the nuclei in the granular and molecular layer, however, allows for their automatic detection (left), enabling the visualization of the cellular cytoarchitecture in 3d (right). Scale bars: 50 μm.

Fig. 5. Comparison between the different embedding media used for experiments at the synchrotron setup. Tissue contrast within the hydrated brain slice (a) is relatively low when regarding the small cells in the granular as well as molecular layer, and single cells cannot be unambiguously identified. The cell bodies and to some extent even the dendritic tree of the large Purkinje cells can, however, be visualized at comparably high contrast. The exchange of water content by ethanol (b) leads to an overall increase in tissue contrast, allowing for the identification of single cells in all layers of the cerebellum. In the paraffin-embedded tissue (c), contrast within sub-cellular details of the Purkinje cells, as, e.g., the cell bodies or the dendritic tree, is low with respect to the surrounding tissue. For the cell nuclei/nucleoli of the GL and ML, contrast is, however, much higher than in the other preparations. Scale bars: 50 μm (top) and 25 μm (bottom).
tissue contrast compared to a hydrated brain slice which can be recognized both by visual inspection and by regarding the Weber contrast values. Note, however, that unwanted experimental factors due to varying waveguide performance, sample motion in liquid, as well as small differences in data processing are not accounted for in this estimation. Hence, absolute values are only approximative and should better be regarded as a lower bound representing the current state of the art. The liquid embedding, in particular, may have been penalized in the comparison by motion artifacts. Within the reconstructed volume, subcellular structures as the dendritic tree and nucleolus of the Purkinje cells or the inner structure of the granule cell nucleus can be resolved at higher detail, when comparing hydrated to ethanol embedding. The latter also leads to an increase in resolution when regarding the higher detail, when comparing hydrated to ethanol embedding. The former measurement was performed at the synchrotron setup, whereas the latter was performed within our laboratory. The resolution achieved at the laboratory setup for the different sample preparations was estimated via the FSC criterion. In this case the intersection between the FSC for the central 1000³ voxels of the reconstructed volumes and the 1/2/2-bit threshold curve yielded half-period resolutions of 3.0 μm for the hydrated, 1.57 μm for the ethanol-embedded and 1.55 μm for the paraffin-embedded tissue. The Weber contrast values were estimated based on manually selected cells, yielding C_{GC} ≈ 0.34 for the Purkinje cell layer and C_{GL} ≈ 0.23 for the granular layer, respectively. This confirms the global increase of tissue contrast with respect to the hydrated brain slice.

In the paraffin-embedded tissue, the different layers of the cerebellar cortex, including the single cells within them, as well as the white matter can also be unambiguously identified due to a higher contrast of the cells against surrounding tissue. Similar to the synchrotron results, this increase is not global but the cell nuclei show a superior contrast compared to surrounding tissue, which can be recognized in Fig. 6(c), where the cell-dense granular layer is the dominating structure. In Fig. 7(d) this superior contrast is also visible on the (sub-)cellular scale, leading to a high visibility of single cells within the granular layer and the enclosed nucleolus and nucleus of the large Purkinje cells. This visual impression can be confirmed by the estimated Weber contrast values, yielding C_{GC} ≈ 0.19 for the Purkinje cell layer and C_{GL} ≈ 0.36 for the granular layer (see Table 3 for an overview of all contrast values).

The 3D cytoarchitecture can be visualized by volume renderings of the reconstructed volumes, as depicted in Fig. 8 for the ethanol- and paraffin-embedded samples. In both cases, the layers of the cerebellum are well distinguishable and single cells can be visualized in 3D. The differences in contrast, however, lead to a higher visibility of the cells in the molecular layer and the Purkinje cells in the case of the ethanol-embedded sample, whereas in the paraffin-embedded sample, the cells in the molecular and also the dense granular layer can be better visualized. Moreover, blood vessels can be recognized as well, which also allows for an examination of the vasculature in 3D.

The resolution achieved at the laboratory setup for the different sample preparations was estimated via the FSC criterion. In this case the experimental conditions were identical for all measurements. The intersection between the FSC for the central 1000³ voxels of the reconstructed volumes and the 1/2/2-bit threshold curve yielded half-period resolutions of 3.0 μm for the hydrated, 1.57 μm for the ethanol-embedded and 1.55 μm for the paraffin-embedded tissue. This proves the stability of the laboratory setup as well as the sample mounting, since a resolution of ~1.5 × the (resampled) pixel size was achieved despite the long exposure times. At the same time, this shows the negative influence of a low-contrast sample preparation (as in PBS), resulting in a significant loss of resolution.

4. Summary, conclusions and discussion

In summary, propagation-based phase-contrast tomography was performed both at the synchrotron and at a laboratory setup on mouse cerebellar tissue to visualize the underlying 3D cytoarchitecture and to evaluate the evolution of contrast when using different embedding media. At both setups, the typical layers of the cerebellum were visualized at a resolution high enough to identify individual cells and to some extent also sub-cellular details in all three preparations, hydrated as well as ethanol and paraffin embedding. The synchrotron setup provided results with a higher resolution in which details as the inner structure of the
The advantage of this preparation is that, apart from
and even at the laboratory, especially the large Purkinje cells. One major
However, single cells could still be recognized both at the synchrotron
setup, whereas cells within the cerebellar cortex were less visible.
comparably high contrast, most notably in the results of the laboratory
variation techniques provided results in which the overall cytoarchitecture of
the cerebellum was well represented, contrast of individual features
varied between the different embedding media. In hydrated tissue, white
matter, containing a large amount of axon bundles, can
be especially well recognized due to the insulating sheath of high-fat myelin around the axons. (b) Orthogonal slices through the reconstructed density of an ethanol-
embedded mouse cerebellum. Compared to the hydrated brain slice overall tissue contrast is increased and single cells can be identified in all layers of the cerebellar cortex. (c) Orthogonal slices through the reconstructed density of a paraffin-embedded mouse cerebellum. Tissue contrast is increased and cells can be unambiguously
identified throughout the cerebellar cortex. Similar to the synchrotron results, contrast of the nuclei is superior compared to the other preparation techniques whereas sub-cellular details as the cell bodies of the PCL are not as clearly visible. Scale bars: 200 μm.

Figure 6. Comparison of the laboratory results obtained for hydrated, ethanol- and paraffin-embedded tissue. (a) Exemplary slice through the reconstructed density
distribution of a hydrated punch from a mouse cerebellum. For a better SNR, the reconstructed slices were filtered with a Gaussian function with a 1 pixel (top) or 2
pixels (bottom) standard deviation. Despite the low contrast of the tissue against PBS, the layers of the cerebellar cortex, namely the cell rich granular layer (GL), the
low cell molecular layer (ML) and the mono-cellular Purkinje cell layer (PCL) can be distinguished. The white matter, containing a large amount of axon bundles, can
be especially well recognized due to the insulating sheath of high-fat myelin around the axons. (b) Orthogonal slices through the reconstructed density of an ethanol-
embedded mouse cerebellum. Compared to the hydrated brain slice overall tissue contrast is increased and single cells can be identified in all layers of the cerebellar cortex. (c) Orthogonal slices through the reconstructed density of a paraffin-embedded mouse cerebellum. Tissue contrast is increased and cells can be unambiguously
identified throughout the cerebellar cortex. Similar to the synchrotron results, contrast of the nuclei is superior compared to the other preparation techniques whereas sub-cellular details as the cell bodies of the PCL are not as clearly visible. Scale bars: 200 μm.

granule cell nuclei or the thicker branches of the Purkinje cell dendrites
could be resolved. The laboratory setup, on the other hand, allowed for a
larger volume to be probed and offers higher accessibility. All prepara-
tion techniques provided results in which the overall cytoarchitecture of
the cerebellum was well represented, contrast of individual features
varied between the different embedding media. In hydrated tissue, white
matter, containing a large amount of myelinated axon bundles, showed a
comparably high contrast, most notably in the results of the laboratory
setup, whereas cells within the cerebellar cortex were less visible.
However, single cells could still be recognized both at the synchrotron
and even at the laboratory, especially the large Purkinje cells. One major
advantage of this preparation is that, apart from fixation, no further tis-
sue alterations were performed. Hence, this technique is closest to the
native state as, e.g., tissue shrinkage due to the dehydration process can
be circumvented. Embedding in ethanol led to an overall contrast in-
crease in the cerebellar cortex, while the visibility of white matter was
reduced. Especially the large Purkinje cells, including its dendritic tree,
and the cells in the molecular layer were well visible, whereas the cells in
the granular layer showed a higher contrast compared to hydrated tissue,
but single cells could not be unambiguously identified. Paraffin embed-
ding resulted in a superior contrast of the cell nuclei, whereas sub-
cellular details as the dendritic tree of the Purkinje cells showed a
decreased visibility. In contrast to ethanol-embedded tissue, even single
cells within the dense granular layer could be identified. Hence, paraffin
embedding should be used for the visualization of neuronal cytoarchi-
tecture based on the position of the cell nuclei whereas ethanol-
embedding is the method of choice for imaging of the large Purkinje
cells, including sub-cellular details as their dendritic tree. Note, however,
that in both cases, this increased contrast comes at the price of a more
elaborate sample preparation which additionally leads to a noticeable
shrinking of the tissue.

It is particularly noteworthy, that all sample preparations are inter-
mediate steps in paraffin embedding, which is usually performed in order
to generate histological sections. Therefore, the tomographic results can
be easily combined with histological studies. These experiments could be
carried out as follows: (1) The overall 3d cyto-architecture is visualized by
propagation-based phase-contrast tomography based on the under-
lying electron density differences, probing a relatively large volume with
isotropic resolution. (2) For specific regions of interest within the sample,
chosen based on the results of the tomographic recordings, histological
sectioning with suitable staining agents is carried out to reveal specific
properties of the tissue as, e.g., different cell types or changes in their
pathological state. We also want to stress the relationship to prior work.
Changing the embedding medium for contrast variation is a powerful
extension of the discovery that paraffin-embedded unstained brain tissue
is amenable by phase contrast x-ray tomography (Hieber et al., 2016;
Khimchenko et al., 2016, 2018).

Next, we want to discuss the limitations challenges and extensions of
the present work. We first address the field of view (FOV), i.e. the vol-
umes which can be imaged. For the present proof-of-concept, all scans
were recorded from small 1 mm punches from fixed brains. Certainly, if
there was a strict requirement to obtain only such small samples from the
brain this would make the approach less attractive compared to imaging
of large brain regions or whole brain. It is important to note, however,
that by variation of photon energy and geometric parameters, the FOV is easily scalable, and the FOV can be increased up to the entire brain at the price of correspondingly smaller resolution. Since the technique is non-destructive large FOVs can be scanned first to identify regions-of-interest (ROI), which are then scanned at higher resolution. This will be especially important in the future, for example for studies of neurological diseases that affect specific brain regions and cellular morphologies within different regions. Controlled biopsy punches based on prior scans of large tissues scanned in overview CT has for example already been demonstrated in Töpperwien et al. (2019). Furthermore, the entire cellular cytoarchitecture could be probed by subsequent and densely sampled biopsy punches. A second important issue is the capability to reconstruct single neurons in 3d. In this work, we have demonstrated this only for the relatively easy task of segmenting the Purkinje cells (PC). In future, it will be important to extend this to other cell types. For this purpose, the larger volumes of neurons in human brain compared to mouse will certainly help. Regarding synchrotron radiation, we can anticipate that contrast can be increased and noise can be decreased by reducing the photon energy and increasing flux density. Already at present, different neuronal shapes can be identified visually, but automated segmentation often remains a challenge. To this end contrast variation based on different embedding media, as introduced here, will provide a valuable tool to highlight different neuron types. Potentially, one could even vary contrast in the same sample by exchange of solvent (e.g. ethanol-water mixtures), resulting in several gray values for the same voxel. This would significantly facilitate segmentation and classifications based on gray-value distributions in a higher dimensional space. Third, we must address the fact, that the current reconstructions fail to reveal the complete dendritic trees of the PCs. The main branches of the dendrites can be segmented, but contrast is not sufficient to visualize the entire elaborate structure, which may be limiting in studies of PC degeneration or development. By additionally increasing the contrast via metal staining of the features of interest, this could be significantly improved. Based on the rapid improvements in x-ray optics and source brilliance, however, we expect that these limits will also shift for unstained tissue, for which the physically limiting factor is a minimum of density difference between dendrite and environment. It is precisely this variable which can be controlled based on the embedding medium. Further, we want to briefly comment on the accessibility of the technique. The laboratory setup, in particular, offers permanent accessibility as no application for beamtime is necessary. This makes this setup useful for applications requiring a high throughout as well as short term availability. In this respect, it is important to stress that the laboratory setup used is a home-built instrument optimized for the purpose, but by no means restricted to groups with exclusive technical skills. All components, source, positioning units and detector are commercial, and the selection of geometry and parameters has been described in detail in Töpperwien (2018). We expect that commercial providers will soon offer a similar set of components and parameters. For the image quality obtained, the reconstruction algorithms are at least as important as the hardware. We have worked with our own numerical implementations
and algorithmic toolbox, which is again fully described in Töpperwien (2018), and referenced therein. We also make the full code available upon request. Regarding synchrotron holo-tomography, a dedicated endstation commissioned and operated by our group in collaboration with DESY has been used. The instrument is described in Salditt et al. (2015) and is available also to non-specialist users based on a proposal system.

Finally, we want to address the proper placement of the demonstrated method with respect to the portfolio of other techniques. We certainly do not expect that phase-contrast tomography of unstained tissues in different embedding media will replace conventional methods, such as classical histology, or more modern developments such as fluorescence light-sheet microscopy. We expect, however, that the approach will significantly complement and augment existing methods. By full 3D digitalization of scalable tissue volumes, it does not only fill a gap of non-destructive 3D imaging with isotropic and scalable resolution. It also provides a novel contrast based on electron density differences, which is to date not fully exploited. In contrast to interaction between visible light and tissue, this contrast is fully described by tractable quantifiable kinematic interactions, and hence ideally suited for automated and model-based analysis.

Acknowledgments

We thank Julia Scherber and Bärbel Heidrich for help in sample preparation and Michael Sprung for excellent support during the beamtime at DESY beamline P10. Financial support by the collaborative research center 755 Nanoscale Photonic Imaging as well as the Clusters of Excellence 171 Nanoscale Microscopy and Molecular Physiology of the Brain and 2067 Multiscale Bioimaging: From Molecular Machines to Networks of Excitable Cells of the German research foundation (DFG) is gratefully acknowledged.

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