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Evaluation of heavy-metal stains and embedding media for phase-contrast tomography of neuronal tissue

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ABSTRACT

In the present work, we evaluate and compare the contrast and resolution obtained on neuronal tissues, i.e. human and mouse cerebellar tissue, with *propagation-based x-ray phase-contrast computed tomography* (PB-CT). At our laboratory-based liquid metal-jet setup, we obtain overview datasets at sub-micron resolution of mm³-sized volumes. In order to evaluate these parameters down to the sub-cellular level, we utilize the synchrotron endstation GINIX at P10, DESY. At this dedicated endstation developed and operated by our group, we utilize x-ray waveguide optics for high-resolution cone-beam scans at strong geometrical magnification. Exploiting this multi-scale approach, we investigate the image quality of cerebellum tissue treated with heavy-metal stainings based on OsO₄, PTA, UA, combinations thereof, and metallic mercuric sulfides (Golgi-Cox protocol). In addition, we study the electron density contrast in unstained tissues. Depending on the stain, epoxid resin or paraffin is utilized as embedding media, which also significantly affects contrast and image quality. With this work, we want to contribute to an optimized sample preparation to study the neuronal architecture of the brain tissue in greater detail in three dimensions.

Keywords: Propagation-based phase-contrast tomography, heavy-metal staining, contrast-optimized sample preparation, 3d histology of the cerebellum

1. INTRODUCTION

Phase-contrast micro-¹⁻³ and nanoCT^{4,5} of soft tissues and in particular biopsies and autopsies of the nervous system has been recently demonstrated to reveal cellular and sub-cellular structural details even in unstained tissue samples.⁵⁻⁷ However, it is long known that staining and labeling by heavy atoms can significantly increase contrast and signal of small structural features for electron and x-ray microscopy alike. This has been exploited since long for contrast variation in absorption-based radiography, in particular in angiography for visualisation of small capillaries,⁸ contrasted by dissolved BaSO₄, CaSO₄, or SrSO₄ salts. In the context of PB-CT, suitable stains and/or labels are also highly desirable for increased sensitivity to tissue component, for contrast variation and to obtain specific readouts. For small organisms, iodine and PTA stains are well established, see,^{9,10} but for soft tissue of mammals and in particular of human, suitability of phase-contrast agents is less well explored. Tissue components containing high concentrations of fatty acids can be contrasted by OsO₄,¹¹⁻¹⁴ based on protocols from EM. This has been exploited for μ - and nanoCT of myelinated nerve fibers.^{4,14,15} A novel eosin-based staining method for tissue blocks has been presented in.¹⁶ For neuronal tissue, volume stains for electron microscopy (EM) have recently gained increased interest in view of connectomics,^{17,18} notably *rOTO*.¹⁹⁻²¹

Generally, we are interested in the suitability of heavy-metal stains for tissue of the nervous system which can augment the capabilities of propagation-based x-ray phase-contrast tomography of neuronal tissue. For this investigation, we chose tissue from cerebellum, since it has a well-described morphology and cyto-architecture. Furthermore, unstained tissue of human cerebellum has previously been studied by absorption-²² and phase-contrast CT enabling detection of single Purkinje cells²³⁻²⁵ and subsequently also smaller cells such as granules,⁷

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showing impressive level of detail.

Analogous to comparable high-resolution imaging methods such as *scanning electron microscopy* (SEM) or light microscopy-based *histology*, the effect from staining on contrast is studied here. To this end, modified versions of the *rOTO*-protocol²⁶ are used, as detailed below.

The *rOTO* is a standard, OsO_4 -based staining procedure in three-dimensional (3d) SEM, a technique that requires enhanced heavy-metal impregnation for contrast formation using backscatter electron detection. It stands for “reduced osmium, *thiocarbohydrazide* (TCH), osmium”. The contrast-enhancing properties of osmium tetroxide OsO_4 (atomic weight: 190.2) are mainly due to aggregation of lower oxides of Os, and originate only to a minor degree from unreduced Os.²⁷ It binds to the phospholipid headgroups, hence contrasting plasma membranes, multi-vesicular structures, ribosomes and Golgi complexes. We hence expect in particular higher contrast for membrane-rich structures in the cerebellum such as fiber tracts. Notwithstanding contrast enhancement, OsO_4 ’s main purpose in EM is serving as a chemical fixative, enabling structure preservation by inactivation of osmolarity.²⁸ In *rOTO*, TCH is applied in between two consecutive OsO_4 immersions. TCH highlights carbohydrates in SEM or nerve tissue in light microscopy,²⁷ but its main purpose here is to increase OsO_4 ’s membrane penetration.

Another standard substance to increase feature contrast in OsO_4 -fixated tissue in SEM is *phosphotungstic acid* ($\text{H}_3\text{PO}_4 \cdot 12\text{WO}_3 \cdot 24\text{H}_2\text{O}$, PTA, atomic weight: 183.92).²⁷ This contrast agent functions via binding to hydroxyl groups due to its anionic nature. It selectively decolors compounds by exchanging their anions, and its performance is directly connected to the buffers’ pH level.²⁷ at $\text{pH} \leq 2.0$, it is sensitive for polysaccharides with a feature specificity depending on the exact pH value, while $\text{pH} \approx 3.0$ -3.5 turned out advantageous for nucleoproteins, and $\text{pH} \approx 4.0$ for glycogen and nucleoproteins. In general, for $\text{pH} \geq 7.3$, PTA stains inefficiently. Due to the requirement of low pH levels, PTA treatments usually demand adequate pre-fixation for tissue protection. Also, PTA itself may serve tissue fixation. Applied to brain tissue, PTA especially binds to synaptic complexes, fibres and membranous structures.²⁷

Uranyl acetate ($\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, UA), with an atomic weight of 238.07, is one of the heaviest stain used in EM.²⁷ Also for this stain, a low pH level is crucial, ideally ~ 1.0 -2.0. It binds particularly well to nucleic acids and phosphate groups, and less so to proteins depending on their charge, hence highlighting for example nucleohistones in DNA or membranous structures. Also, when combined with reduced Os from (brain) tissue pre-treatment, UA may function as a morphological stabilizer. Like OsO_4 and PTA, UA serves both staining and fixation. All three substances perform effectively when combined with prior glutaraldehyde treatment.²⁷

The primary goal of this work is to test different *rOTO*-derived heavy stains for their suitability in propagation-based x-ray phase-contrast tomography. Apart from these stains developed in the field of EM, we also include one of the oldest heavy atom stains of histology, notably the *Golgi-Cox* stain, which has already proven suitable for phase-contrast x-ray tomography.^{29,30} As reviewed in,³¹ it is a derivative of Camillo *Golgi*’s method, who discovered the penetration of metals in neurons in 1873.³²⁻³⁴ S.R. Cajal’s works are well-known for early images and studies of glial and dendritic architecture using Golgi’s protocol.³⁵ The key of the protocol’s success is its sparse staining of only ~ 3 -10% of all neuronal features in a structure-rich tissue. The Golgi protocol consists of sample treatment with a potassium chromate and potassium dichromate mixture, followed by silver nitrate. A further development has been made by Cox, who established addition of mercuric chloride to the potassium chromate and potassium dichromate mixture, changing its pH-level for a better tissue impregnation. This modification is referred to as Golgi-Cox method: In a first step, tissue forms compounds with mercuric chloride, which are then transformed to metallic mercuric sulfides (molar weight: 232.66g/mol ³⁶) in a later step. It is considered to be a structure-preserving staining technique for axons and dendrites.

In this work, we combine laboratory micro-PB-CT (lab- μCT)^{37,38} and high-resolution synchrotron radiation computed tomography (SR-CT) based on a focusing unit composed of Kirkpatrick-Baez mirrors and an x-ray waveguide.³⁹ For notational clarity, we next briefly introduce phase-contrast x-ray imaging by propagation, and the basics of phase retrieval as used here.

Phase contrast is governed by the real part decrement $\delta(\vec{r}, E)$ of the x-ray refractive index $n(\vec{r}, E) = 1 - \delta(\vec{r}, E) + i\beta(\vec{r}, E)$. The imaging regime is governed by the Fresnel number F . The lab- μCT data is recorded in the *direct contrast* or *edge enhancement regime*, defined by

$$F = \frac{px_{\text{eff}}^2}{z_{\text{eff}}\lambda} = \frac{px^2}{z_{12}M\lambda} \approx 1, \quad (1)$$

where $px_{\text{eff}} = px/M$ for the effective pixel size and $z_{\text{eff}} = z_{12}/M$ for the effective propagation distance have been used, based on the respective real values px and z_{12} , exploiting the Fresnel scaling theorem at geometrical magnification M .⁴⁰ In this regime, a reconstruction scheme known as *Bronnikov-aided correction* (BAC)⁴¹ can be used, which is compatible low coherence sources and which preserves image sharpness.^{37,38} BAC can be derived from the *transport of intensity equation* (TIE), which describes the propagation of a paraxial wave along $\vec{z}$ with intensity $I(\vec{r})$ and phase distribution $\phi(\vec{r})$: $\nabla_{\perp}(I(\vec{r}) \cdot \nabla_{\perp}\phi(\vec{r})) = -k\partial_z I(\vec{r})$, k being the wavenumber. For small propagation distances z_{eff} and a purely phase shifting object, the TIE can be linearized, and an approximated phase $\tilde{\phi}$ can be computed as^{42,43}

$$\tilde{\phi}(\vec{r}_{\perp}) = 2\pi F \cdot \mathcal{F}_{\perp}^{-1} \left[\frac{\mathcal{F}_{\perp} \left[\frac{I(\vec{r}_{\perp}, z)}{I_0} - 1 \right]}{|\vec{k}_{\perp}|^2 + \alpha} \right], \quad (2)$$

where $\mathcal{F}$ denotes the Fourier transform and I_0 the (uniform) intensity distribution. The parameter α serves to regularize the singularity at zero spatial frequency and consider residual absorption in the sample, and in practice is chosen such that edge enhancement is canceled. In a second step, the approximated phase $\tilde{\phi}$ is used to compute a corrected, sharp intensity distribution in the object exit plane, according to⁴¹

$$I(\vec{r}_{\perp}, z = 0) = \frac{I(\vec{r}_{\perp}, z)}{1 - \gamma \nabla_{\perp}^2 \tilde{\phi}(\vec{r}_{\perp})}. \quad (3)$$

The two regularization parameters α and γ are chosen such that the resulting 'hypothetical' intensity distribution contains contributions from intensity and phase contrast.

The SR-CT data is recorded in the *holographic regime* at $F \ll 1$, offering higher contrast for high spatial frequencies compared to the direct-contrast regime. For weakly absorbing and homogeneous objects, the *contrast-transfer function* (CTF) is given by⁴⁴⁻⁴⁶

$$\frac{I(\vec{k}_{\perp}, z)}{I_0} = 2\pi\delta_D(\vec{k}_{\perp}) + 2\sin(\chi) \cdot \phi(\vec{k}_{\perp}) - 2\cos(\chi) \cdot \xi(\vec{k}_{\perp}), \quad (4)$$

with $\chi = \frac{\lambda z k_{\perp}^2}{4\pi}$, the Dirac-delta function $\delta_D(\vec{k}_{\perp})$ and the absorption coefficient $\xi(\vec{k}_{\perp})$. From the CTF, an expression for the phase in the object plane can be derived:⁴⁶

$$\phi(\vec{r}_{\perp}) = \mathcal{F}_{\perp}^{-1} \left[\frac{\sum_{d=1}^N \left(\sin(\chi_d) + \frac{\delta}{\beta} \cos(\chi_d) \right) \cdot \mathcal{F}_{\perp} [I(\vec{r}_{\perp}, z_d) - 1]}{\sum_{d=1}^N 2 \left(\sin(\chi_d) + \frac{\delta}{\beta} \cos(\chi_d) \right)^2 + \alpha} \right], \quad (5)$$

where $\frac{\delta}{\beta}$ is assumed to be constant (homogeneous object approximation), and α is a regularization parameter. N is the number of exposures recorded at different defocus distances (Fresnel numbers) in order to sample all spatial frequencies at high contrast. For the exact implementation of the regularization scheme used here, see also.⁴⁷

2. METHODS

2.1 Experimental setups

2.1.1 Laboratory-based setup

Fig. 1a) depicts the geometry of the home-built laboratory microCT setup (lab- μ CT) operated at a liquid metal jet x-ray source (Excillum, Stockholm, Sweden).^{15,48} The anode material *Galinstan* (68.5% Ga, 21.5% In, 10% Sn) is liquid at room temperature, allowing for higher thermal load and hence a corresponding brilliance gain beneficial for phase contrast.⁴⁹ Phase-contrast tomography using this source has been presented in.⁵⁰⁻⁵² The source was operated with source size $10\mu\text{m}$, e^- -beam current 1.4mA , and 40kV voltage, resulting in corresponding cut-off photon energy of the bremsstrahlung. The dominant energy for phase contrast is given by the characteristic line $K_{\alpha, \text{Ga}} = 9.25\text{keV}$. The sample stage positioned at a distance z_{01} from the source is equipped with 6 motors

Figure 1. Illustrations of the experimental setups: (a) Lab- μ -CT setup, with liquid metal jet anode, high lens-coupled scintillator detector and magnification $M \simeq 1$, i.e. $z_{02} \ll z_{01}$. (c) SR-CT setup with KB/waveguide optics. KB-mirrors focus the monochromatized undulator beam onto the waveguide. The waveguide exit beam is used for holographic illumination at high M . (b & d) Examples of flat-field images for the two setups. Due to coherent multi-modal superposition, the waveguide flat-field shows modulations.

(1 rotation, 5 translations), allowing for alignment of the rotation axis and the sample. The detector is positioned along a rail at distance z_{02} , and can be translated on top along x and y . Adjustment of system resolution as a function of spot size, detector pixel size and geometry (z_{01} , z_{02}) for the setup has been discussed in.^{37,48,53} For most scans presented here, we have used the lens-coupled single-crystal scintillator-based CCD-detector *Xsight* (Rigaku, Tokyo, Japan) with 2504×3326 pixels ($h \times w$) with a $10\times$ -objective, resulting in $px = 0.54\mu m$. To reach a resolution below source size based on the high resolution capability of the detector, small geometrical magnification $M = \frac{z_{02}}{z_{01}} = \frac{176.6mm}{155.0mm} \approx 1$ was chosen.³⁷ Empty beam corrected data were corrected for hot pixels. To increase the signal-to-noise-ratio (SNR), pixels were 2×2 -binned. The acquisition parameters resulted in $F = 0.35$, corresponding to the direct contrast regime. Fig. 1b) gives a flat-field image of the setup, showing the influence of the objective on the illumination profile. X-ray phase-contrast tomography scans were recorded with 1001 angular positions at an acquisition time of 50s. For the overview scan of the Golgi-Cox stained mouse brain, a CMOS flat panel detector (Dexela, PerkinElmer, Waltham MA, USA) with 1536×1944 pixels ($h \times w$) and $px = 75\mu m$ was used. Its rather large sensitive area is advantageous for large FOV at moderate geometrical magnification, $M = \frac{z_{02}}{z_{01}} = \frac{1739.2mm}{127.5mm} \approx 14$ namely, providing $px_{eff} = 5.5\mu m$ and $F = 1.91$. As shown before, phase reconstruction with BAC (Eq. 3) gives excellent results for 3d histology.^{3,15,37} Prior to tomographic reconstruction based on,^{54,55} ring artifacts in the sinograms were mitigated.⁵⁶

2.1.2 Synchrotron radiation-based setup

After a first screening and overview scans with lab- μ CT, high resolution scans were performed at the GINIX nanofocus endstation³⁹ of the P10 beamline at the PETRAIII storage ring (DESY, Hamburg). As sketched in Fig. 1c), the undulator radiation is monochromatized by a double-crystal Si(111) monochromator to a photon energy $E = 7.9keV$, and focused by Kirkpatrick-Baez (KB) mirrors to about $400 \times 400\mu m^2$ (FWHM). The source spot size s relevant for holography was determined by *x-ray waveguides* positioned in the KB-focal plane. Apart from the reduction of s (and correspondingly increase of numerical aperture), waveguide optics increases spatial coherence by mode filtering⁵⁷ and delivers smooth illumination wavefronts for holography.^{58–60} For this experiment, air-filled channel waveguides in silicon or germanium were used, fabricated by e-beam lithography, reactive ion etching and wafer bonding.⁶¹ Channels with different lateral size and depth were used, in the order

Figure 2. Overview on the sample preparations involving (left) Golgi-Cox and (right) the *rOTO* protocol (with modifications), based on OsO_4 , PTA and UA.

of 10^2 nm. Note that the actual beam confinement is given by the width of the confined beam intensity, which is smaller than the dimensions of the rectangular channel cross section. This value for s can be retrieved by inversion of the far-field pattern.^{58,59} Scans were recorded at magnification $M = \frac{z_{02}}{z_{01}} = \frac{5126\text{mm}}{138\text{mm}} \approx 37 \gg 1$. A sCMOS camera with Gadox scintillator and 2048×2048 pixels with $px = 6.5\mu\text{m}$ (Photonic Science, UK) was used, resulting in $px_{\text{eff}} = 176\text{nm}$. Accordingly, data were recorded in the holographic regime with $F = 0.0015$, and phase reconstruction was performed by CTF (eq. 5). An exemplary flat-field image is given in Fig. 1d). Due to changes in the electron beam orbit, as well as drift in the beamline optical components (in particular due to cooling of the monochromator), the waveguide intensity and mode distribution changed, and frequent realignments were required. The acquisition time was $\sim 1\text{s}$ per projection. In order to achieve larger penetration depth, the setup was also adjusted for 13.8keV x-rays.

GINIX also allows for parallel beam tomography, which can be implemented in parallel to cone beam after slight rearrangement of optics, and used on the same sample, as we will report in detail elsewhere. This parallel-beam data (presented in Fig. 8) was recorded at $M = \frac{z_{02}}{z_{01}} = \frac{150\text{mm}}{138\text{mm}} \approx 1$ and $F = 0.0121$, and reconstructed by BAC. For data from both configurations, empty-beam-corrected images were filtered for hot pixels, and after phase reconstruction filtered for ring artifact mitigation using.^{56,62} If required, sinograms were further smoothed in prior to the tomographic reconstruction, which was based on the Matlab (The MathWorks, Inc., MA, USA)-integrated function *iradon* with a standard Ram-Lak filter.

2.2 Sample preparation

2.2.1 rOTO-based staining

As *rOTO* is an established protocol for large volume imaging by SEM, a method which requires enhanced contrast, its suitability for X-ray phase-contrast tomography has to be tested. Cerebellar tissue from the same adult C57/Bl6 wild type mouse was used for all samples with this stain. Animals were deeply anesthetized and perfused with *Karlsson-Schultz* (K&S) solution, consisting of 2.5% glutaraldehyde and 4% formaldehyde in phosphate buffer, following the procedures as described in.⁶³ The cerebellum was dissected and chemically post-fixed for 24h. The tissue block was then cut into 200-500 μm sagittal slices with a vibratome. In between each step, samples were washed for 15min. Before staining, the tissue was first placed into 0.1mol phosphate buffer for 15min. First incubation was carried out with reduced osmium (i.e. OsO_4 with added potassium ferrocyanide) for either 30 or 60min at room temperature. As *rOTO*²⁶ stands for “reduced osmium, TCH, osmium”, tissue was then placed into the osmiophilic TCH solution for 1h, followed by again 30 or 60min reduced osmium. OsO_4 does not only stain, but also chemically stabilizes the molecular tissue structure. For some tissue pieces, the protocol was continued with a 15min incubation step with 2%UA or 2%PTA at 4°C. To investigate the staining properties of solely UA or PTA, the *rOTO* protocol was modified: the OsO_4 steps were skipped for some samples. However, the fixation effect of OsO_4 's is crucial for brain tissue, such that three of these samples desintegrated during preparation, and only one sample stained with UA for 30min remained. Via increasing

acetone and then epoxid-resin-in-acetone series, tissue was dehydrated and fully penetrated by the resin. When kept at 60°C for 24h, the epoxid resin polymerizes. As indicated on the right of Fig. 2, samples were either prepared to be “flat” (0.8-2mm laterally, 30-60μm in depth) or trimmed with the microtome by using a 90° diamond knife to form “blocks” (360-530μm laterally, 170-500μm in depth). For the latter, sample surfaces were smoothened by applying another thin layer of epoxid resin.

2.2.2 Golgi-Cox-based staining

Apart from *rOTO*, which was the main focus in this work, we also used Golgi-Cox staining. The Golgi-Cox method was developed to give specific contrast to neurons, including their dendrites and spines. For this purpose, we used the kit provided by FD NeuroTechnologies’ (Columbia MD, USA) *FD Rapid GolgiStain Kit*, which is based on the protocol by Ramón-Moliner⁶⁴ and Glaser and Van der Loos.⁶⁵ 24h prior to the start of the preparation, solution *A* and *B* are mixed 1:1. They involve mercury, potassium chromate and potassium dichromate, a highly toxic mixture which may be fatal in case of misuse. The top part of the mixture is sucked off and used as stain, avoiding the solid particles which settle at the bottom. As this staining procedure also includes the required chemical fixation, the cerebellum tissue was freshly removed from the animal, carefully rinsed with ultrapure water (Milli-Q), and placed into the mixture, and subsequently always stored in the dark. For one mouse brain, ~10ml was used and replaced after 6h or the next day. After 14 days at room temperature, the tissue was transferred to solution *C* for 72h at room temperature, and renewed the next day. Then, tissue blocks were washed with Milli-Q twice for 10-20min. Then, sections were put into a 1:1:2 mixture of solution *D*, *E* and Milli-Q for ~30min. This is a crucial step, since it serves for the metal-formation of the stain (cf. section 1). Again, the tissue is washed twice with Milli-Q for 10-20min. The stained tissue blocks were then paraffin-embedded, involving an ethanol-based dehydration series, washing with xylene and full tissue penetration series with paraffin at 60°C, turning solid at room temperature. As shown in Fig. 2, the stained cerebellum blocks were mounted on paraffin-embedding cassettes. Using a 1mm biopsy punch, smaller cylindrical samples were extracted and inserted into Kapton polyimide tubes (Professional Plastics, Fullerton CA, USA) for high-resolution scans.

2.2.3 Preparation of control samples

In order to clearly evaluate whether observations are made based on the respective heavy-metal stain, on the tissues itself or the experimental setup, unstained control samples were included: Firstly from a previous study,⁷ an unstained human cerebellum autopsy, 10% PFA-fixated and paraffin-embedded, provided by C. Stadelmann (Institute for Neuropathology, UMG, Göttingen). In addition, a K&S-fixated and paraffin-embedded mouse cerebellum was included. Autopsy samples were extracted using a 1mm biopsy punch and inserted into polyimide tubes.

3. RESULTS

Fig. 3 presents lab-μCT results for the unstained controls of cerebellum tissue (mouse and human). (a) illustrates its basic anatomy with a more detailed zoom-in view. The *white matter* (WM) contains myelinated neuronal structures such as *axons*, forming *fiber tracts* (FT). Fibers originating in the WM traverse the *granular layer* (GL) unmyelinated, consisting of *granular cells* (GC) and *Golgi cells* (GgC). A single layer of *Purkinje cells* (PC, PCL) is located in direct proximity of the GL, with sparse occurrences of *basket cells* (BC). PCs’ long and widely branched dendritic trees penetrate into the *molecular layer* (ML), which distinguishes itself from the GL by its larger and more sparsely spread cells, the *molecular cells* (MC). The *external granular layer* (EGL) borders the ML. The EGL is a characteristic structure for brain development.

3.1 rOTO-based & control preparations

Prior to valuable synchrotron beam time, samples were screened with the Xsight-based lab-μCT presented in section 2.1.1. Fig. 3 presents virtual slices through the reconstructed 3d volumes of the unstained and paraffin-embedded autopsies. For phase-retrieval, (human) $\alpha_{\text{BAC}} = 0.05$ and $\gamma_{\text{BAC}} = 0.17$ and (mouse) $\alpha_{\text{BAC}} = 0.008$ and $\gamma_{\text{BAC}} = 0.16$ were chosen based on visual inspection. For both preparations, the characteristic tissue regions (WM, GL, PCL and ML) with their corresponding cells (GC, PC and MC) are clearly recognized. However, in particular for PCs, there appears to be a feature contrast gradient within the mouse tissue, indicating fixation

Figure 3. (a) Schematic drawing of the cellular anatomy of the cerebellum.

Overview on data achieved from unstained, paraffin-embedded cerebellum tissues with the lab- μ CT setup, presented as effective absorption coefficient per voxel. Virtual slices in the yz-plane through data from (b) human and (c) mouse tissue. Scale bars: 200 μ m.

inhomogeneities. Note the difference in size of the tissue components between human and mouse, in particular the PCs, which also exhibit different shapes and contrast.

Fig. 4 gives an overview of typical contrast properties of the different *rOTO*-derived stains, as they appear in lab- μ CT. Preparations involving OsO_4 show particularly high contrast in the WM. Single cellular features emerge to much lower extent than in the data in Fig. 3. Only for OsO_4 without additional stain (PTA, UA), some granularity in the GL is visible. Also, the PCL appears to be stained negatively by OsO_4 . As described in section 1, this may be attributed to the lower density of ribosomes in PCs compared to GC, for instance. When combining OsO_4 with PTA or UA, GL and ML also differ in contrast, however without a clear distinction of the PCL. A special case of contrast is noticed for UA-only staining: there is no dominant appearance for the WM, which becomes indistinguishable from the GL. Some granularity is recognized for the ML in this case. The most striking feature is the high contrast of the EGL. As motivated earlier, this structure is predominantly expressed during brain development. From the characteristics of UA binding to membranous and DNA-structures (cf. 1), particular staining behavior of structures at maturation stages becomes conclusive. Given the fact that all the *rOTO*-derived preparations originate from the very same animal, this structure has not been observed for any of the other staining preparations, in particular not for the combination of OsO_4 with UA. However, due to the brittle architecture of cerebellum when not OsO_4 -treated, this observation is rather wary.

Next, samples were imaged at sub- μ m resolution using the SR-CT setup introduced in 2.1.2. The δ/β -ratios in CTF-reconstruction were chosen based on physical properties and fine-tuned from visual inspection. Fig. 5 shows the unstained controls, and Tab. 1 the corresponding quality measures. The evaluation of the resolution based on the Fourier-Shell-Correlation (FSC), which is also affected by the overall noise, gives a value

Figure 4. Overview of contrast obtained with different stains (mouse cerebellum) at the lab- μ CT setup, given as effective absorption coefficient per voxel. The tissue was stained with (a) OsO_4 (30min), (b) OsO_4 & PTA (30min), (c) OsO_4 & UA (60min) and (d) UA, and epon-embedded in “flat” geometry. Tab. 2 gives further parameters of preparation and data quality. Scale bars: 200 μm .

Figure 5. Overview of SR-CT results in terms of phase shift per voxel for the unstained, paraffin-embedded samples, shown for (a) human and (b) mouse cerebellum. Circles mark PCs. Quantitative quality measures are presented in Tab. 1. Scale bars: $90\mu\text{m}$.

of $0.7\mu\text{m}$, steepness-of-edge at a blood vessel profile about $0.5\mu\text{m}$. The SNRs of different features in the unstained cerebellum are also given. Note that the background signal for PC nucleus was chosen to be outside-the-PC, not inside-the-PC's body. Visual and quantitative assessment of the PCs' contrast confirm the observations made for the lab- μCT data (Fig. 3). Especially the SNR for the mouse PC body data stands out. The appearance of the PCs' dendritic trees for the mouse tissue sample is particularly remarkable, and may eventually be explained by the superior structure preserving property of K&S-fixation. Nevertheless, their nuclei turn out fuzzier. While the GC emerge stronger contrasted for mouse tissue with lab- μCT , same is true for the GC in human cerebellum with the synchrotron setup. For both data sets, also blood vessels appear comparably strong due to blood remains.

Fig. 6 presents the results for *rOTO*-preparations, recorded with the SR-CT setup. Directly visible (already without zoom-in) are the blood vessels which appear in negative contrast, since they were emptied by perfusion of the animals before staining. In the case of unstained and paraffin-embedded samples, identification and tracking of blood vessels is also possible, but since tissues were not perfused but chemically fixated by diffusion only, blood remains would complicate the automatic segmentation. Also, the overall contrast between empty vessels and stained tissue is of course huge for all of the stained samples. Next we note, there is no strong overall contrast of the WM, as in the respective lab- μCT data, but the discrimination of the different layers and features emerges on a cellular level. MC bodies and nuclei, for example, are contrasted in the OsO_4 & PTA data. As expected, the OsO_4 -preparations reveal particular contrast for myelinated axons in the FT. Still, SNR-values are lower than for the data from unstained and paraffin-embedded mouse tissue. Same is true for PCs' nuclei. Compared to the other *rOTO*-derived preparations, PC bodies appear slightly stronger contrasted when PTA is involved. Concerning resolution, steepness-of-edge values are at about the same level for all synchrotron data sets. Contrarily, differences are observed for the more overall-preparation-mirroring FSC-values. For all "flat" preparations, the FSC-resolution is $\approx 2\mu\text{m}$ and far below capabilities of the instrument, the stains and the tissue. From comparison with the steepness-of-edge, an increased noise level seems to affect the data quality. This can be attributed to artifacts related to facets and in part also to higher absorption for some angles. The "block" geometry, however, does not suffer from these artifacts and approaches the results of the unstained preparations. When OsO_4 is combined with PTA or UA, resolution is slightly better than for the unstained samples ($0.55\mu\text{m}$ and $0.56\mu\text{m}$ vs. $\sim 0.65\mu\text{m}$). For the combination of OsO_4 with PTA, MC bodies and nuclei are recognized as for no other preparation discussed here.

Since the 60min- OsO_4 "flat" preparation of the OsO_4 & PTA-stained tissue resulted in comparably strong

Table 1. SR-CT data quality metrics for unstained, paraffin-embedded samples. FSC is based on volumes of $200 \times 200 \times 200$ voxels except for ⁺ ($150 \times 150 \times 150$), and computed with with a Kaiser-Bessel window of *7pixels*.⁶⁶ Steepness-of-edge has been evaluated for blood vessels. The SNR was calculated as $(\mu_{ft} - \mu_{bg})/\sigma_{bg}$ on neuronal fibers in white matter, Purkinje cells' body and nucleus and blood vessels, respectively. Annotations on SNR marked with * give the number of features evaluated. Empty beam intensities are given in analog-digital-units (ADU). In the case of the human tissue, the position of the WM region was located too close to the edge of the scan volume to perform reasonable evaluation of the axons' contrast, hence "n.a." (not applicable).

	Unstained cerebellum	
	human	mouse
δ/β (CTF)	35	55
Resolution		
FSC 1/2bit (μm)	0.69	0.65
Steepness-of-edge (μm)	0.44	0.53
SNR		
Axon	n.a.	3.75* ⁵
PC nucleus	6.87	8.21* ³
PC body	0.99* ³	4.23* ³
Blood vessel	4.54* ²	4.54* ²
Total empty beam intensity per projection before/after scan ($\cdot 10^{10}$, ADU)	5.85/2.14	2.04/2.03

Figure 6. Overview of SR-CT results for the stained samples, given as phase shift per voxel. Stains are (a to c): OsO₄ (30min, “flat”), OsO₄ & PTA (60min, “block” & “flat”, respectively), OsO₄ & UA (60min, “block”). Circles mark PC. Preparation parameters and quality measures are tabulated in Tab. 2. Scale bars: 90 μm .

Table 2. Metrics for data quality for the stained samples. “b” stands for “block”, “f” for “flat”. Except for ⁺ ($150 \times 150 \times 150$), FSC is based on volumes of $200 \times 200 \times 200$ voxels, with a Kaiser-Bessel window of 7pixels .⁶⁶ Steepness-of-edge calculated on blood vessels. The SNR was calculated as $(\mu_{\text{ft}} - \mu_{\text{bg}})/\sigma_{\text{bg}}$ on neuronal fibers in white matter, Purkinje cells’ body and nucleus and blood vessels, respectively. Annotations on SNR marked with * give number of features the number is based on. Empty beam intensities are given in analog-digital-units (ADU).

	<i>x</i> min. OsO ₄			<i>x</i> min. OsO ₄ & 15 min. PTA			<i>x</i> min. OsO ₄ & 15 min. UA		
	60 min., b	30 min., f	60 min., f	60 min., b	30 min., f	60 min., f	60 min., b	30 min., f	60 min., f
δ/β	15	10	10	18	15	15	12	18	18
Resolution									
FSC 1/2bit (μm)	0.92 ⁺	1.81	1.99 ⁺	0.55	1.40	2.26	0.56	1.80	2.55
Steepness-of-edge (μm)	0.45	0.49	0.41	0.54	0.47	0.60	0.82	0.60	0.74
SNR									
Axon	n.a.	2.45* ⁵	2.27* ⁵	n.a.	1.75* ⁵	2.65* ⁵	1.29* ⁵	n.a.	n.a.
PC nucleus	n.a.	3.18* ¹	n.a.	n.a.	n.a.	2.63* ³	2.08* ³	n.a.	n.a.
PC body	n.a.	0.64* ¹	n.a.	n.a.	n.a.	1.04* ³	0.78* ³	n.a.	n.a.
Blood vessel	4.16* ²	2.99* ²	3.57* ²	7.03* ²	6.37* ²	6.61* ²	6.87* ²	4.29* ²	5.12* ²
Total empty beam intensity per projection before/after scan ($\cdot 10^{10}$, ADU)	4.60/0.63	7.76/6.35	5.57/4.92	5.65/5.27	3.74/3.90	4.15/3.87	5.29/5.14	4.40/3.38	4.14/4.29

SNR, this sample was scanned also at higher geometric magnification, resulting in $px_{\text{eff}} = 46.8\text{nm}$. In addition, the sample was used for segmentation of blood vessels. Fig. 7 shows slices through both reconstructions, the $px_{\text{eff}} = 176\text{nm}$ and $px_{\text{eff}} = 46.8\text{nm}$ datasets. The high M dataset shows impressive level of detail concerning axons in the FT. It is difficult to appreciate the structure based on one or two slices, but to date we have not yet a segmentation workflow at hand. Instead we illustrate 3d segmentation for (the much easier) case of the blood vessels, which appear as a 3d network (red) after semi-automatic segmentation, using e.g. Avizo (Thermo Fisher Scientific, Waltham MA, USA). As mentioned above, the negative staining of blood vessels permits a very clear visualization of the vessel network. A first attempt of manual segmentation of fibers is shown in (green).

3.2 Golgi-Cox-based preparations

A mouse brain was stained according to the Golgi-Cox protocol described in section 2.2.2. Prior to the extraction of punches, the full mouse brain was imaged with the laboratory setup. BAC-phase retrieval was carried out with $\alpha_{\text{BAC}} = 0.1$ and $\gamma_{\text{BAC}} = 0.16$. Fig. 8 (a) confirms that the mercury stain penetrated the tissue to full extent. Yet, at the edges of the tissue, low staining contrast emerges, presumably since the metal has dissolved again. In parallel-beam geometry and at 13.8keV x-rays at the GINIX instrument, a 1mm -sample was obtained as described and scanned. $\alpha_{\text{BAC}} = 0.002$ and $\gamma_{\text{BAC}} = 0.16$ has been used for phase retrieval of this dataset. The semi-automatic rendering of PCs and their dendrites and also the maximum intensity projection demonstrate the particular contrasting of these features. Accordingly, the orientation in a 3d space can be assessed. Further features are contrasted, such as fibers in the GL and WM. At the same time, data also show fractionated staining of features, pointing towards preparation issues. The root of this was examined systematically in further trials. As the impregnation with the solution-D&E-mixture causes the formation of metallic mercuric sulfides, cerebellum blocks were first cut into 1mm -slices to then allow for better penetration of the reagent. In addition, the impregnation was prolonged to 1h . According to our first laboratory tests, this was found to benefit staining of single neuronal structures. Moreover, increased surrounding temperature to 60°C for hours during paraffin embedding may lead to the contrast gradient reported in section 3.2. Keeping the sample hydrated, no such defect was observed in first tests.

4. DISCUSSION AND OUTLOOK

In summary, we have presented a first test of different *rOTO*-derived heavy-metal stains for their suitability in x-ray propagation-based phase-contrast tomography. The overall result for contrast of the cytoarchitecture was

Figure 7. Detailed inspection of the 60min OsO_4 & 15min PTA “flat” preparation. (a) virtual slice through the 3d volume of the standard synchrotron scan (as in Fig. 6). The orange square marks the region of the close-up scan shown in (c) and in a different position in (d). Data are presented as phase shift per voxel. (b) Volume rendering of manually segmented features: (red) blood vessels, (violet) PC bodies as also marked in (a), (green) axon fiber. Scale bars: (a & b) $90\mu\text{m}$, (c & d) $40\mu\text{m}$.

Figure 8. Golgi-Cox-stained mouse cerebellum. (a) Lab- μ CT result, presenting the effective absorption coefficient per voxel. The red circle indicates the position of the 1mm-biopsy punch. (b) Volume rendering of the SR-CT result after semi-automatic segmentation of the PCs with the corresponding dendritic trees; the gray slice underneath permits anatomic correlation to the position marked in the overview data. (c) Maximum intensity projection. Scale bars: (a) 1mm, (b & c) 200 μ m.

rather disappointing on the cellular level, while contrasting of larger areas of the cerebellum (e.g. ML versus GL) worked well for large overviews obtained at lab- μ CT. The situation of heavy-metal stains versus unstained tissue seems to be reciprocal in EM and phase-contrast x-ray microscopy. While the latter gives already excellent results for unstained tissue, as also demonstrated for (high-resolution) SR-CT scans of human cerebellar tissue,⁵ in particular regarding the cytoarchitecture, the use of heavy-metal stains even degrades feature visibility to some extent, whereas most EM techniques are almost useless for unstained tissue. Certainly, this may be explained by current deficits of the protocols as well as not-yet identified small flaws in the preparation. Notwithstanding important further modifications and extensions, we still face an 'overstaining' for x-ray phase contrast. The β/δ -ratio may change in a non-optimal way, since presently used stains affect absorption much more than the overall electron density and hence the phase shift. Despite the enhanced absorption, we did not observe motion artifacts due to heating for the present experimental setting as reported in.⁵ Optimizing the β/δ -ratio also calls for more rigorous simulations of the optical properties based on stoichiometry models and the associated concentration levels.

Preparation details including fixation, embedding and mounting of the samples and the choice of photon energy should be carefully reconsidered, since feature contrasting is not solely based on contrast agents, but can also largely be determined by chemical fixation and tissue embedding media. This also calls for a thorough reinvestigation of the best staining strategy. New approaches like the one in¹⁶ based on atoms with intermediate Z such as Bromide may point to the right direction. At the same time we must acknowledge that OsO₄-based staining has already given excellent results for phase-contrast tomography of the peripheral nervous system (PNS),⁴ and that PTA and iodine stains has become a gold standard for small animal tomography, see the review of.⁶⁷ Golgi-Cox, also delivers according to expectation, but of course with its characteristic limitations associated with statistical and sparse labeling. For CNS, where contrast for dendrites and synapses in large volume would be a game-changer, we therefore do not yet have a solution at hand. The obvious extension of this work, is to investigate the quality loss/gain situation step-by-step for the protocol and to systematically vary the relevant parameters. While we await such results, we can only recommend phase-contrast imaging of unstained tissues, with proper choice of embedding medium for contrast variation.⁶⁸ To this end, we also stress that the K&S fixated unstained sample scanned in this work, shows particularly convincing structure preservation, underlining the importance of the fixative.

We do not want to end the report, without a note in passing: as expected, OsO₄ does convincingly increases contrast for myelinated neuronal structures in the fibre tracts of cerebellum. As an outlook, we anticipate that this could be propably refined and exploited in future to study the myeloarchitecture, and hence could uniquely contribute to connectomics on larger scales.

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