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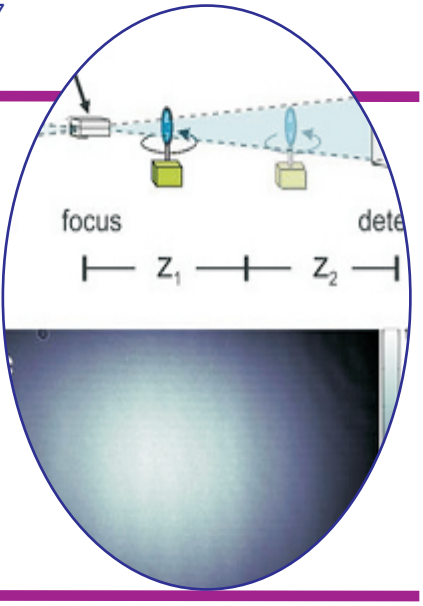
X-RAY PHASE CONTRAST TOMOGRAPHY FROM WHOLE ORGAN DOWN TO SINGLE CELLS



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ABSTRACT :

We use propagation based hard x-ray phase contrast tomography to explore the three dimensional structure of neuronal tissues from the organ down to sub-cellular level, based on combinations of synchrotron radiation and laboratory sources. To this end a laboratory based micro focus tomography setup has been built in which the geometry was optimized for phase contrast imaging and tomography. By utilizing phase retrieval algorithms, quantitative reconstructions can be obtained that enable automatic renderings without edge artifacts. A high brightness liquid metal micro focus x-ray source in combination with a high resolution detector yielding a resolution down to 1.5 m. To extend the method to Nano scale resolution we use a divergent x-ray waveguide beam geometry at the synchrotron. Thus, the magnification can be easily tuned by placing the sample at different defocus distances. Due to the small Fresnel numbers in this geometry the measured images are of holographic nature which poses a challenge in phase retrieval.

KEYWORDS : *X-ray, phase contrast, tomography, in-line holography, propagation based phase contrast, phase.*

INTRODUCTION:

The three-dimensional structure of tissue of the central nervous system (CNS) is commonly assessed in form of thin sections in combination with histological staining and imaging techniques. A non-destructive survey of the small animal brain from the organ to the cellular level is to date beyond our capabilities, but extremely desirable for neuroscience, in particular if this could be combined with high throughput. X-ray imaging is a powerful technique to reveal internal structures of objects and tissues. With modern x-ray sources and optics, the imaging approach can be easily scaled over a wide range of resolutions, from the patient scale in the hospital down to nanometer resolution in x-ray microscopy. However, the biggest advantage of x-rays, i.e. their weak interaction and thus large penetration depth in tissue, can also be a significant disadvantage. Especially in case of imaging small structures and especially soft tissue, since low attenuation means low contrast, at least in conventional absorption imaging. One possibility to overcome this limitation is to use the stronger phase shift interaction of the object. The phase shift depends on the decrement d in the complex refractive index $n = 1 - d + i\beta$ which is up to 1000 times larger than ß for hard x-rays thus providing a huge potential contrast gain. There are several phase contrast x-ray imaging techniques which convert the phase shift, imposed by an object on the x-ray wave, into measurable intensity variations.

In this paper we use propagation based phase contrast tomography to image soft biological tissue of the

CNS in mouse and Venousleaves tadpoles, in order to provide a benchmark and proof-of-concept study. With regard to previous work, we mainly explore two directions: (A) The use of compact laboratory micro focus x-ray sources as a tool for neuron scientists circumvents the need for synchrotron radiation, which is considered to be less accessible. To realize tomography of CNS tissue at micro focus source, we employ novel liquid jet high brightness anodes,10 a range of state-of-the art detectors, and an optimized imaging geometry.11 (B) Tomography of unstained CNS tissue in the hydrated state. Contrast of soft tissue can be generated based on the mentioned phase shift of the x-ray wave with respect to the empty beam going through air. However, in the hydrated state, it is not the phase shift of tissue with respect to air, but the much weaker difference to water. Without any labeling with contrast agents, the native internal density distribution can be used for contrast formation. Today most synchrotron setups use propagation based phase contrast only in the so called direct contrast regime,?, 12 where the potential of the phase shift is not fully exploited. However, in the holographic regime more details can be observed due to a better contrast transfer. As the observed images do not show the structures of interest but a holographic version of it, phase retrieval is an indispensable step in this regime but a high quality illumination is needed to circumvent reconstruction artifacts. To this end we use x-ray waveguide optics in this work as a highly controlled secondary source for imaging.? Apart from exceptional spatial coherence, x-ray waveguides also provide small source sizes down to below 10 nm and wave fronts of high quality (low aberrations), meeting the idealized requirements of perfect illumination by a plane or spherical wave, which is ubiquitous in propagation imaging. The next two sections start with a brief discussion of suitable phase retrieval algorithms followed by a demonstration of the experimental applications to CNS tissue.

IMAGING MODES AND PHASE RETRIEVAL

1. Propagation based phase contrast

By free space propagation, structural information of an object, encoded in the phase of the exit wave, is transformed to a measurable intensity distribution as the wave perturbed by the object interferes with itself. The starting point to understand the image formation in this so called propagation based phase contrast method and the corresponding phase retrieval, is the wave equation. For hard x-rays, the paraxial scalar wave equation provides a suitable framework, if polarization effects and scattering to high angles can be neglected. This approximation is justified if the interaction of the object can be described on length scales much larger than the x-ray wavelength ?, i.e. if a continuous and slowly varying index of refraction n(r)=1 - d(r) - ß(r) is a suitable description for the object. A solution for the propagated exit wave described by the paraxial (parabolic) wave equation can then be derived by the angular spectrum method which leads to the following expression describing the evolution of a wave in free space13

2. Phase retrieval :

To obtain a quantitative image of the object from measured holographic intensity distributions, different phase retrieval algorithms are employed. As for the image formation, only the Fresnel number F is needed as a parameter to reconstruct the image, the combination of ?, z and p in terms of F is sufficient. In this work we use two different phase retrieval algorithms for different imaging regimes.

3. Bronnikov Aided Correction :

One of the two phase retrieval algorithms used below is denoted as Bronnikov Aided Correction (BAC). It is based on a linearization of the transport of intensity equation (TIE)14, 15 and is valid for weakly absorbing objects and small propagation distances, thus large Fresnel numbers. In this so called direct contrast regime, the phase contrast image will show bright and dark fringes especially around edges of the object, as given by the two-dimensional (2D) Laplacian of the projected phase. Under these assumptions, a simplified form of the TIE can be written in the following form I(r⊥ Δz) = I0 1 - Δz k∇2⊥ φ(x, y) .

4.Contrast Transfer Function

For smaller Fresnel-numbers F the image will not just show edge enhancement effects, but multiple

fringes will appear and the image loses resemblance to the real structure. In fact, an in-line hologram of the object is recorded as originally proposed for electron beam holography by Gabor.16 In this imaging regime, phase retrieval is not only needed to get quantitative contrast values from the measured images but also to reconstruct in particular high frequency spatial information, which has been blurred by diffraction. One deterministic method, which is very robust to experimental imperfections and noise, is based on the contrast transfer function (CTF). It can be derived by again assuming a weakly absorbing object and a slowly varying phase but making no assumption to the propagation distance.17–19 Writing the complex valued field in dependence of the attenuation μand phase distribution ϕof the object and using Eq. (1) the CTF can be derived by a first order expansion ~I(kx, ky, z) = δD + 2ϕ sin χ?μ cos χ.

EXPERIMENTAL REALIZATION

1.Supercritical dried tadpole heads :

For the laboratory experiments, the olfactory system of Xenopuslaevis tadpoles of about stage 5421 are used as they are a well known model organism for neuronal studies concerning the olfactory system.22 As the contrast of soft tissue to water is rather weak, dried tadpole heads were used. To preserve the ultra structure of the samples and prevent it from shrinking the samples are supercritically dried. The finally dried heads are mounted to a sample holder. A photograph of the final tadpole head specimen is shown in Fig. 2 (a). The dashed rectangle indicated the position used for the tomographic measurements.

2. Brain slice:

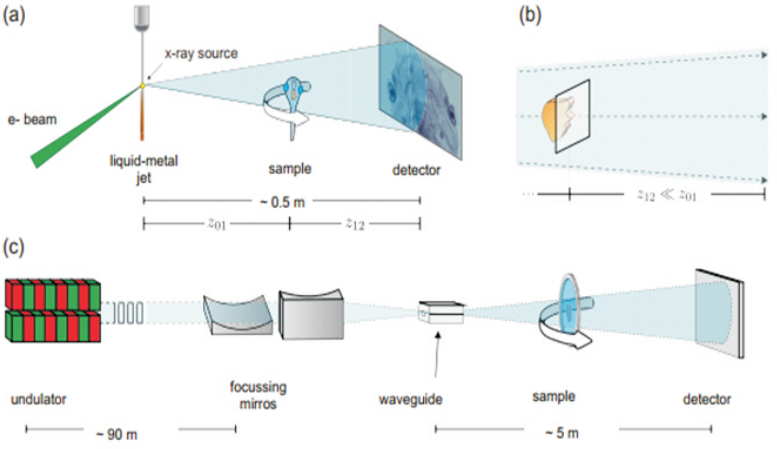
For synchrotron experiments it is also possible to investigate hydrated samples as the illuminating beam has a higher coherence and a much smaller bandwidth, thus enabling the increase of contrast by choosing smaller Fresnel numbers. As a proof of concept a thick slice of a mouse brain was prepared and mounted in a liquid chamber. A wild type mouse was sacrificed with CO2 and its brain perfused with 4% para-formaldehyde (PFA) to fix the tissue. Afterwards the brain was dissected and fixed in PFA over night. The fully fixed brain was cut into 1 mm thick slices, which were stored in phosphate buffered solution (PBS) until the experiment. Directly before the measurements one slice was mounted in PBS between two polypropylene foils on aluminum rings forming a liquid chamber.23 The rings were glued together and mounted on a sample holder. Figure 5 (b) shows a photograph of a final preparation with the brain slice kept in PBS between the two rings.

3. Single neuronal cells :

In addition to the fixed brain slices, also primary neurons from cell culture have been examined. To this end, the hippocampus from 18 days old rat embryos was extracted. By adding trypsin for the digestion of the tissue and DNAse to dissolve the thereby released DNA, the neurons can be extracted. The digestion of the tissue is stopped by adding bovine serum. These neurons are then given onto a silicon nitride membrane which was sterilized before. For better adhesion of the neurons, the membrane was coated in Poly-L-ornithine solution for about four hours. Growth of the neuron cell culture was enabled by incubating the membrane in medium for one week. As a final step of the preparation, the cells were fixed with PFA and stored in PBS. Prior to the measurements, a second silicon nitride membrane is glued onto the first one to create a closed liquid chamber which is then mounted on a sample holder.

4. Laboratory phase contrast imaging with micro focus x-ray sources

By using micro focus sources together with an optimized geometry and high resolution detectors, x-ray phase contrast imaging can be realized in the laboratory. Here we use a liquid metal jet x-ray source (Excellus) which allows an up to ten times higher flux density for small source sizes.24 A metal alloy based on gallium, indium and tin (Glisten) which is liquid at room temperature is used for broadband x-ray emission with a peak at the K-alpha line of gallium at 9.25 keV. Additionally, a solid molybdenum target can be used for the measurements with a peak energy at 17.5 k

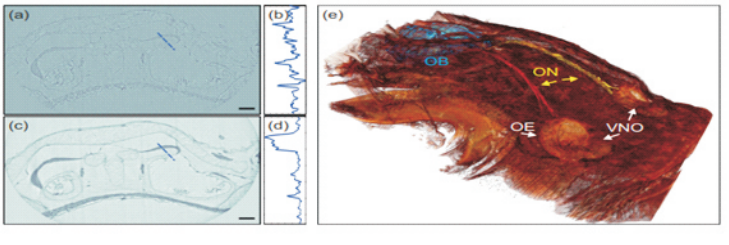


5. Magnifying geometry :

Figure 1 (a) shows the schematic of a magnifying imaging mode. This “classical” mode is based on using an efficient x-ray detector with pixels larger than the desired resolution and employing the effect of geometric magnification. Thus zooming is easily realized by moving the sample to different positions in the divergent beam. Two different detectors are used in this mode. The first one is a camera in which a Gadox scintillator is coupled by a fiber optic plate to a CCD camera (FDI, Photonic Science) with a pixel size of 6.54 m. The second detector is a directly illuminated CCD camera with 20 m pixel size (LCX, Princeton Instruments). The x-ray source size limits the resolution in this mode, which is in the range of about 3 to 4 m as confirmed by a resolution test pattern. Figure 2 (b) and (c) show exemplary phase contrast projections of a dried tadpole head measured at two different magnifications with the different detectors, showing the entire head in Fig 2 (b) and a smaller region of interest (ROI) with the olfactory system in Fig. 2 (c). In both cases the edges of the image are enhanced by interference effects improving the contrast of the soft tissue. The relevant experimental parameters are listed in Tab. 1. By rotating the sample and recording images for several angles in the range of 0 to 180 degree, a tomographic dataset can be recorded. This is done in the configuration shown in Fig. 2 (c).

• Inverse geometry :

To further increase the resolution, a different experimental geometry can be used for phase contrast imaging.? Figure 1 (b) schematically shows the arrangement of sample and detector in this “inverse” geometry. By moving the sample rather close to the detector, the resolution is limited mainly by the detector. To this end, a very high resolution detector is used which is based on a 20 m thin single crystal LuAG:Ce scintillator (Crytur) which is viewed by an optical microscope consisting of a 10-fold magnification and a cooled CCD camera (pco.2000, PCO). This kind of detector is usually used at synchrotron sources where a resolution down to below 1 m is possible.25 The optical system behind the scintillator transmits only a small part of the generated light to the camera, but the high flux provided by the liquid metal source enables the use of this kind of detectors also in the laboratory with reasonable exposure times. With the detector used here, a resolution of about 1.5 m could be achieved. Due to the high resolution small Fresnel numbers and thus phase contrast images can be recorded even for rather short propagation distances in a range of e.g. 20 mm between object and detector



• X-ray waveguide imaging for high resolution phase contrast tomography :

The "inverse geometry" is widely used in synchrotron imaging as it can be used without any optics. Moreover, the low divergence of synchrotron beams allows for a large range of propagation distances to choose the Fresnel number according to the contrast of the sample. For the high resolution detectors described before the resolution is limited by the diffraction limit of the visible light created in the scintillator, thus being in the range of about 300 to 500 nm. However, the detection efficiency in this scheme is rather low. Another promising approach is to again use a magnifying setup in which the resolution is limited by the (effective) source size. As the synchrotron radiation is very brilliant, it can be focused to small spots with large flux densities. This enables the use of x-ray waveguides (WG) consisting of low density guiding channels and a high density cladding, which have to be precisely aligned in a focused beam. The general idea is sketched in Fig. 1 (c). Here we have used a crossed multilayer WG with 59 nm guiding layer over a length of about 0.7 mm as the illumination forming optic. Waveguides of this kind are described in detail e.g. by Krugeret. al.? Based on total reflection of the x-rays inside these channels, the radiation is confined and it can be shown that only a few optical modes can propagate through the channels. This increases the spatial coherence,26 significantly improves the quality of the illumination wave front,27 and leads to a smaller effective source size. The WG used here produces a source size of 16 nm according to finite difference simulations which is confirmed by reconstructions of the measured far field intensity distribution. Using this WG, resolution values below 30 nm were achieved previously, as demonstrated by two-dimensional test patterns.27 Figure 5 shows the results obtained for a hydrated thick brain slice imaged in an x-ray waveguide setup. In Fig. 5 (a) an empty beam-corrected phase contrast projection is shown, which is not in the direct contrast regime anymore. Hence the CTF-based phase retrieval algorithm described above is used.

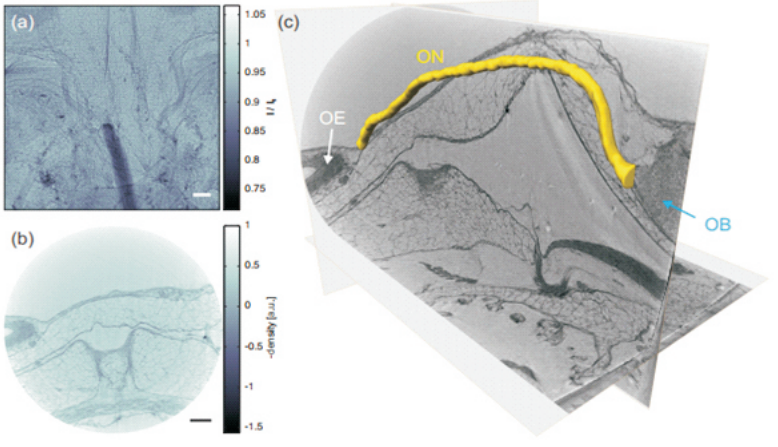


Figure 4. (Color online) Phase contrast tomography with the high resolution "inverse geometry" mode. (a) Phase contrast projection of the olfactory system recorded with a high resolution detector at a liquid-metal x-ray source. (b) Reconstructed slice (BAC phase retrieval + FBP) of a corresponding tomographic measurement. (c) 3D Rendering showing three perpendicular orthoslices together with a manually segmented olfactory nerve (ON). Scalebars denote 100 m.

CONCLUSION :

We have demonstrated the potential and the challenges in phase contrast tomography of unstained neuronal tissue. First, we have used a liquid metal micro focus laboratory source for phase contrast tomography of tadpole heads, thereby revealing the internal structure of the olfactory system on a micrometer resolution scale in the laboratory. The results have been obtained on a specimen prepared by critical point drying,

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