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Original paper

High resolution hard X-ray 3D mapping of a *Macaca fascicularis* eye: A feasibility study without contrast agents

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ABSTRACT

Several complementary methods able to visualize the internal structures of eyes are used in the clinical practice in the diagnosis of pathologies affecting a specific zone of the eye. Despite the significant technological progress, the visualization of the entire eyeball at micrometric resolution is yet an unsolved task both in clinical diagnostics and in laboratory research. With this respect, high resolution 3D images of the eyeball would be extremely useful, in the study of various pathologies of the retina, the lens, and of the optic nerve. In this work we combined the state-of-the-art of micro computed tomography technology with phase-contrast imaging, a recent highly sensitive technique well adapted to investigate soft tissues without the use of contrast agents; we applied the technique in the post-mortem analysis of monkey eyes, which share several similitudes with the human organ. We reported here vascular, nervous and anatomical details of monkey eyes imaged with a $3.1 \times 3.1 \times 3.1\ \mu m^3$ voxel size as well as the first 3D visualisation of the entire globe of Macaca's fascicularis eye. Results have also been compared with, and validated by, histological analysis.

1. Introduction

The clinical procedures for the visualization of the internal structures of eyes include several complementary techniques like biomicroscopy, optical coherence tomography (OCT, [1]), confocal scanning laser ophthalmoscopy (cSLO, [2]), ultrasound scanning in the A- and B-mode (A- and B-scan), as well as ultrasound biomicroscopy (UBM), X-ray micro-computed tomography (X-ray microCT, [3]) and magnetic resonance imaging (MRI). A detailed description of all these techniques can be found in [4]. A complementary method to analyse tissues at (sub)microscopic resolutions is the histological analysis; however this procedure is destructive and is only carried out on excised organs ex-vivo. Moreover, during the preparation process, tissues can be altered by mechanical actions and chemical reagents, with consequent modification of their anatomy. Each clinical imaging method is particularly suited in the diagnosis of pathologies affecting a specific zone of the eye; all methods also present specific requirements for their application. In fact, both OCT and cSLO require a high optical transparency of the sample and can be used in the study of structures in the anterior segment of the eye or in the fundus. The application of UBM is instead limited to the cornea and the anterior chamber angle. B-scan, X-ray microCT and MRI have no optical transparency requirements and are the only techniques potentially allowing the visualization of the entire eyeball. Unfortunately, the resolution of the clinically available instruments, always exceeding 100 µm, is far from that necessary to provide a diagnostically significant image quality of the eye. High resolution images (particularly the ones that show cellular structure of tissue) of the eyeball would be extremely useful even in preclinical trials in the study of different pathologies of the retina, the lens and of the other structures including the optic nerve.

In order to overcome the present limitations encountered by preclinical ophthalmological research a completely different approach has been recently developed in laboratories. It consists in recording the phase variations of X-rays passing through the matter; this signal adds to the weak absorption signal always present in the images [5–7] and does not require the use of additional contrast agents. For a more detailed description of the technique we refer to the Material and Methods section.

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To our best knowledge, there are only four works in the literature describing the post-mortem application of X-ray to eye imaging either in radiography [8] or using microCT [9–11].

Kelly et al. [8] reported on the results of the study of formalin-fixed porcine eyes using a phase-contrast imaging technique. All images were acquired in radiographic mode, which suffers from structure overlap and does not allow a high resolution discrimination of the tissues. This was a seminal work, demonstrating the interest of applying the phase-contrast imaging techniques in the study of the eye anatomy. Yin et al. (in Chinese) [9] also discussed the use of phase-contrast imaging applied to the post-mortem visualization of the internal structures of New Zealand rabbit eye by microCT; the short paper does not report any detailed anatomical description of the findings. In the work of Hoshino et al. [10] the analysis focus on the optical properties of the eye lenses for different animal species by mean of Talbot interferometry. In the article of Ivanishko et al. [11], the results of 3D intra-cranial computed tomography of rabbit eyes has been reported; however no comparison with respect to histological data is reported. In this work we applied the state of the art high resolution microCT using monochromatic X-rays and the state of the art phase-contrast imaging data reconstruction methods we have developed in the past years [5,12–14].

We present here a full 3D acquisition of formalin-fixed monkey eyes (*Macaca fascicularis*) enucleated post-mortem from adult specimens at an isotropic voxel size of 3.1 μm^3. The results presented here aim at providing for the first time a high resolution 3D model of a monkey eye anatomy, showing the potential of phase-contrast imaging as a complementary preclinical diagnostic technique applied to the vision organ.

2. Methods

2.1. Phase-contrast imaging

In life sciences, samples consist very often of weakly absorbing tissues and structures composed by low Z elements (< 20). For such materials, the sensitivity of conventional radiography drastically decreases because of the small differences (few percent) in the X-ray absorption coefficients of the elements composing the different tissues (NIST database [15]).

The use of contrast agents, like iodine or gadolinium, both injected in vivo, can enhance the contrast generated by the vascular system; tissues can also be marked post-mortem using specific commercial markers or other heavy metal-based dyes [16]. A 3D imaging method able to image full organs at resolutions able to discriminate the smallest capillaries and/or the neuronal morphology, without the injection of invasive contrast agent (in vivo), or without applying an aggressive tissue preparation (post-mortem), is still required [17].

X-ray phase contrast imaging represents a novel way to overcome the limitations of conventional absorption-based imaging without the need of contrast agents. The behaviour of X-rays as they travel through an object can be described in terms of a complex index of refraction defined as $n = 1-\delta + i\beta$, whose real part, δ, and the imaginary part, β, are related to the X-ray phase shifts and attenuation in the object, respectively.

In the energy range typically used in X-ray diagnostics (15–100 keV), the phase term is orders of magnitude higher than the absorption one [18]; therefore radiographic techniques sensitive to variations of the δ term may potentially provide an increased image contrast with respect to those methods based only on the X-ray absorption process [6].

Several techniques have been developed for exploiting the phase effects in X-ray imaging. They include the propagation-based imaging technique (PBI) [19–21], the analyzer-based imaging technique [22,23], the grating interferometric [24,25] and the grating non-interferometric methods [26–28]. All modalities have been widely used in preclinical X-ray radiology; an extensive review of the theoretical basis, of the required technology as well as example of the application in different fields of medicine can be found in [6,29].

The phase-contrast technique applied in this study was the PBI, which makes use of a highly spatially coherent and quasi-monochromatic X-ray beam. Briefly, the X-ray wavefront distorted by traversing a sample, generate characteristic interference patters; at well determined directions, described by the Fresnel diffraction [30], these distortions are transformed in detectable intensity variations that can be recorded by a detector placed at a convenient distance from the sample [19,21]. These intensity variations highlight the borders of the sample or of the details embedded in the sample itself, therefore enhancing their visibility.

2.2. Experimental setup

The experiment was carried out at ID17 BioMedical beamline at European Synchrotron Radiation Facility (ESRF, France). A quasi-monochromatic ($\Delta E/E \sim 10^{-4}$) and quasi-parallel X-ray wave (divergence $< = 1$ mrad horizontally, and $\ll 0.1$ mrad, vertically) beam (energy 35 keV) was selected using a Si(1 1 1) double Laue crystal from a beam produced by a 21-pole wiggler [31]. The propagation distance between the sample and the detector was 11 m. The detection system was composed by a 350 μm thick YAG scintillator screen coupled with a 1:2× optics and a Scientific Complementary Metal-Oxide Semiconductor (sCMOS) PCO edge 5.5 camera [32]. The final pixel size was of 3.1 × 3.1 μm^2. In order to evaluate the real spatial resolution at which the images are obtained it is necessary the knowledge of the Modulation Transfer Function of the detection system. Previous measurements, reported in [32] show that the system used in this work shows an effective spatial resolution of about 8 μm.

2.3. Image acquisition and reconstruction

The computed tomography (CT) was performed by acquiring 5000 angular projections over 2π. Each angular projection presents an exposure time of 0.4 s. Due to the limited ID17 vertical beam size (7 mm) three different CTs were performed at different heights of the organ to acquire the whole volume. The overall image acquisition of a single vertical step lasted about 1 h; such long image acquisition time was linked to an un-optimized methodology applied in this pilot study.

However, as also reported in the discussion section, a fast acquisition procedure can be applied to reduce the acquisition time down to ~2 min for a single tomographic vertical step, without a significant loss of information in the regions of interest. Such times have been achieved by reducing the number of angular projections to 500 with an integration time of 0.1 s.

The extraction of the phase information was performed using the quasi-particle phase-retrieval approach described in [33,34]. The filtered-back projection algorithm [35] was then applied to perform the tomographic reconstruction and to create the 3D volume.

For histopathological examination, samples fixed in 10% neutral buffered formalin (NBF) for 24–48 h, were processed with a Tissue Processor Leica ASP300 S, and paraffin embedded (Embedding Center Leica EG1160). Four micron thick sections were cut, stained with Haematoxylin-Eosin (H&E) and evaluated under a light microscope (Leica DM 2500). Representative images were captured with a digital camera (Leica DFC310 FX).

2.4. Samples and ethical statement

In this experiment we used four formaline-fixed monkey eyes enucleated from adult (4–5 years old) *Macaca fascicularis*. During the imaging procedure the samples were embedded in an agar-agar preparation and included in a transparent cylindrical plastic container of 3 cm in diameter. The average diameter of the eye inside the container was about 1.5 cm. Samples did not undergo any other specific preparation and, in particular, were not in contact with any contrast agent