Experimental Eye Research 167 (2018) 51–55

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Research article

Visualization of porcine eye anatomy by X-ray microtomography

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ARTICLE INFO

Keywords:
Micro-CT
Staining method
Anatomy
Image processing

ABSTRACT

The aim of our study is to obtain, as accurately as possible, porcine ocular tissue visualization using micro-tomography (micro-CT) method. We propose image contrast enhancement by different staining procedures with combination of micro-CT scanning. Porcine eye globes were investigated with Bruker-SkyScan 1172 micro-CT. We used 4F1G and Bouin's as sample fixation solutions and tincture of iodine, 100% Lugol, phosphotungstic acid and 1% osmium tetroxide solutions for staining. Quantitative and qualitative analysis was performed based on micro-CT reconstruction images histograms and 3D volume rendering models of investigated samples. This investigation showed that staining methods improved micro-CT image quality in case of ocular anatomy visualization. Characteristic profiles of the grey level distributions and quality of the cross-section and 3D volume rendering images confirmed the staining effect. Most significant contrast enhancement was obtained after 96 h staining in osmium tetroxide and Lugol solutions. The images of eye anatomical structures were characterized: cornea, lens, iris, ciliary body, vitreous, retina, choroid and sclera, vasculature and optic nerve. Staining of porcine eye globes used in this work leads to quality improvement of the micro-CT imaging. The most contrast images were obtained for Lugol and osmium tetroxide solutions. Different affinity of staining solutions to eye anatomical structures has been observed in the obtained images. Osmium tetroxide provides sharper image of conjunctiva, sclera, choroid, retina, iris and ciliary body structure. Lugol staining leads to more accurate vessels, cornea and optic nerve imagining.

1. Introduction

We are witnessing a real burst of novel diagnostic methods applied in medical basic research and clinical practice, that are used to investigate the distinctive and very complex anatomy of the eye globe and its pathologic changes. Imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI), ultrasonography (USG) and ultrasound biomicroscopy (UBM) or optical coherence tomography (OCT), play a vital role in these investigations. Each of abovementioned methods provides valuable ocular tissue data, but their utilities to comprehensively characterize eye globe structure and its pathological patterns, undergo some limitations. Clinically used CT and MRI imaging are capable to exhibit the anatomical relations in orbital and adnexal tissues, but do not provide sufficient spatial resolution for the examination of the globe itself on microscopic scale. On other hand, OCT method has a spatial resolution up to micrometer level but does not deliver an image of entire globe (Thomas et al., 2014). Only predefined parts of the globe, such as for instance macular region of the retina, optic nerve head, cornea and iridocorneal angle, can be visualized by OCT. In addition, this technique requires media transparency, i.e. cornea, lens, vitreous body must remain translucent. In the course of a pathological process, the degree of transparency might be very limited or even totally reduced. USG and UBM are, in fact, two-dimensional imaging methods, with no possibility of further processing the 2D data into a three-dimensional image. Apart from that, the image data are poorly repetitive because of the specific procedure applied for the image acquisition (Silverman, 2009).

On the other hand, considering medical research analysis of eye biopsy material, histopathologic examinations of enucleated eye, X-rays micro-computed tomography (Micro-CT) constitutes an attractive imaging technique for macro and microscopic characterization of the samples. This is a non-destructive method, designed to provide 3D information about small-sized samples, from hundreds of microns to a few centimeters. Micro-CT is powerful imaging tool also for pre-clinical research in which animal models are used, to study formation and course of pathologic processes in ocular tissues, under controlled

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https://doi.org/10.1016/j.exer.2017.11.004
Received 19 June 2017; Received in revised form 25 October 2017; Accepted 13 November 2017
Available online 22 November 2017
0014-4835/

conditions.

In this paper, studies on porcine ocular tissue visualization by X-ray micro-computed tomography are presented. The eye of the domestic pig (*Sus scrofa* domestica) is an ex vivo animal model often used in vision science research. In respect to size and globe anatomy, porcine eyes are similar to human eyes (Sanchez et al., 2011). Micro-CT offers a unique possibility of visualization of the entire eye globe with image resolution in a micrometer range, even through opaque media. The acquired images after processing procedures may undergo further detailed qualitative and quantitative assessment. Micro-CT enables to reconstruct spatial distribution of the linear attenuation coefficient of X-rays in an investigated object. The method is commonly applied for imaging highly calcified structures, for instance bones or pathological mineralization in soft tissues (Skrzat et al., 2013; Kozerska et al., 2013; Leszczyński et al., 2014; Orzechowska et al., 2014, 2017). Micro-CT imaging quality of soft non-mineralized tissues is strongly hampered because of very slight X-ray attenuation differences among soft tissue types and tissue components. To solve this problem, appropriate chemical compounds acting as agents improving the contrast of imaging are introduced into the tissue. Thanks to the application of staining procedures, micro-CT remains fully useful tool for high-contrast visualization of non-mineralized soft tissues as well.

The aim of our study is to obtain, as accurately as possible, porcine ocular tissue visualization using micro-CT method. To fulfil this goal, we tested a few staining procedures to determine the best one for high-contrast imaging of a specific ocular tissue of porcine eye. The obtained results stay in a close relation to our further pre-clinical research with animal models, regarding pathological changes in the ocular tissues, caused by various diseases expanding in animal body or by pathologic processes originating directly in the eye globe. Empirical knowledge of the staining protocol that provides the best 2D and 3D micro-CT visualization of the whole eye globe and its internal structure, is crucial for the planned studies.

2. Methods

For the experimental purpose, several porcine eye globes were obtained from the local abattoir. The globes were stored in the 0.9% saline solution immediately after enucleation and after that cooled down. The sample preparation consisted of two consecutive steps: globe fixation and staining procedure to enter a contrast agent into the globe.

2.1. Sample fixation

Two separate protocols were applied for fixation of porcine eye globes (Metscher, 2009). The first one is based on 4F1G solution: 4% formaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer (McDowell and Trump, 1976) and the second one utilized Bouin's solution which contains 5% acetic acid, 9% formaldehyde, 0.9% picric acid (Metscher, 2009). The globes were first fixed for 15 min in appropriate solution and then punctured about 3 mm from corneal limbus using a 23G needle to ease effective penetration of the selected fixation solution through the globes. The samples were afterwards again immersed in the fixation solutions for 48 h and stored at 5 °C in the refrigerator. Finally, the samples were in sequence dehydrated in 30% and then in 70% ethanol each for 15 min except samples planned for osmium tetroxide staining.

2.2. Staining protocols

Four common staining solutions were used (Metscher, 2009) to obtain high contrast images in micro-CT scanning: 1) 10% tincture of iodine in ethanol, 2) 100% Lugol's solution, 3) 1% osmium tetroxide in PBS and 4) PTA (1:9 Phosphotungstic acid (10%) $+H_2O$) × 30% + 70% Ethanol). The eye globes were soaked in 200 ml of staining solutions for 24, 48, 96 h. During this process, the eye globes were kept at 4 °C. Before micro-CT investigations started, the samples were washed in deionized water and drained. In case of osmium tetroxide solution, the staining procedure lasted 96 h.

2.3. Micro-CT scanning and image processing

Micro-CT scanner Bruker-SkyScan 1172 (Kontich, Belgium) was used in the presented investigations. The scanner is equipped with 80 keV X-ray tube with current of 100 μA. Porcine eye globe was placed in a 3D printed holder of appropriate shape and size. The holder walls were practically X-ray transparent. The cone-beam X-rays are passing through the sample and they are partly attenuated. The samples were rotating to 180° with a rotation step of 0.5°, 0.5 mm thick Al filter was applied. Image pixel size was set to 27 μm. For each rotation angle, the shadow projection was acquired by 11 megapixels CCD flat panel detector (Ritman, 2011). Despite the staining, signal to noise level was enhanced using 12 frames averaging. Scanning time was optimized to 70 min per sample, to prevent motion artifacts caused by sample drying.

After scanning procedure, collected 360 TIFF files were used in the computed 3D reconstruction process using four Dell Precision T5500 cluster computer equipped with NRecon, SkyScan software (Bruker microCT, Kontich, Belgium). This application is based on cone beam Feldkamp reconstruction algorithm (Feldkamp et al., 1984). The algorithm calculates 3D linear attenuation coefficient distribution rescaled into grey level images.

The result images visualization and 3D modeling were performed using volume rendering CTVox software (Bruker microCT, Kontich, Belgium). This software allows grey level transfer function to be modified making the vitreous transparent. We also applied RGB transfer functions adjustment for virtual coloration which enhanced fine details visibility (Leszczyński, 2016). Also 2D eye globe micro-CT cross sections were analyzed and all fine anatomical structures were identified.

3. Results

Both fixation methods, based on 4F1G and Bouin solution gave excellent tissue preservation making the eye globes stable. The grey level histograms of the images obtained with both fixation procedures were tested with Mann-Whitney statistical test (Mann and Whitney, 1947) and showed up no statistically significant difference in the grey level distributions ($p = 0.05$).

In Fig. 1, the graphs of grey level distribution are presented for each of the staining procedure applied. The distributions are normalized to the total volume of scanned porcine eye globe. As it is visible in the figure, a shape of distribution depends on the staining procedure used. Iodine and PTA procedures yield a single peak distribution, while for Lugol and OsO_4 staining, additionally, a shoulder appears on a large value side of the scale. This shoulder is especially well pronounced for 96 h Lugol and OsO_4 staining (Fig. 1D).

The single peak in the graphs comes from low contrasted tissues – mainly vitreous body. The shoulder appears due to highly stained parts of the ocular tissue and contains well contrasted anatomical structures of the sample such as cornea lens, ciliary body and globe walls. In general, all tested staining procedures yield the distributions which are shifted to a higher grey level in comparison to the distribution of unstained sample.

Few conclusions regarding an impact of the used staining on quality imaging of porcine eyes by microtomography emerge from findings given above. All kind of staining used has a positive influence on the image contrast. In general, the best quality images were obtained with staining lasting 96 h. Application of staining with 100% Lugol solution resulted in its effective penetration through the eye globe producing bright and well contrasted images. On the other hand, the image contrast improvement for PTA staining and tincture of iodine in ethanol solution staining, is significant as compared with unstained sample but