Polarization properties of single layers in the posterior eyes of mice and rats investigated using high resolution polarization sensitive optical coherence tomography

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Abstract: We present a high resolution polarization sensitive optical coherence tomography (PS-OCT) system for ocular imaging in rodents. The system operates at 840 nm and uses a broadband superluminescent diode providing an axial resolution of 5.1 µm in air. PS-OCT data was acquired at 83 kHz A-scan rate by two identical custom-made spectrometers for orthogonal polarization states. Pigmented (Brown Norway, Long Evans) and non-pigmented (Sprague Dawley) rats as well as pigmented mice (C57BL/6) were imaged. Melanin pigment related depolarization was analyzed in the retinal pigment epithelium (RPE) and choroid of these animals using the degree of polarization uniformity (DOPU). For all rat strains, significant differences between RPE and choroidal depolarization were observed. In contrast, DOPU characteristics of RPE and choroid were similar for C57BL/6 mice. Moreover, the depolarization within the same tissue type varied significantly between different rodent strains. Retinal nerve fiber layer thickness, phase retardation, and birefringence were mapped and quantitatively measured in Long Evans rats in vivo for the first time. In a circumpapillary annulus, retinal nerve fiber layer birefringence amounted to 0.16°/µm ± 0.02°/µm and 0.17°/µm ± 0.01°/µm for the left and right eyes, respectively.

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1. Introduction

Optical coherence tomography (OCT) has emerged as a powerful tool in ophthalmology since its introduction at the end of the twentieth century [1]. As a non-invasive imaging modality, OCT provides single cross sectional images (B-scans) or 3D data sets of the sample (Fig. 1(A)). OCT is often referred to as optical biopsy. Similarly to regular biopsy and its histological sections, OCT can be used for diagnosis or to study disease progression [2]. Because many retinal diseases are not yet fully understood, OCT is also a valuable tool in basic research of their pathophysiology [3]. Besides standard OCT, which produces images based on the intensity of light backscattered by sample structures, image contrast can be
extended by additional modalities. Doppler OCT can detect and quantify blood flow [4–6]. Spectroscopic OCT [7] can measure quantities like oxygen saturation [8].

![Figure 1](image)

Fig. 1. 3D rendering of OCT data set and sketch of the high-resolution PS-OCT system. (A) 3D rendering of a data set acquired from a Sprague Dawley rat retina (field of view 11° × 11°) together with the sketch of the rat eye to pinpoint the location. (B) Sketch of the OCT system, SLD - superluminescent diode, PC - polarization controller, SMF - single mode fiber, PMF - polarization maintaining fiber, GM - galvanometer mirrors, QWP - quarter wave plate, HWP - half wave plate, NPB - non-polarizing beamsplitter, GTP - Glan-Thomson polarizer, PB - polarizing beamsplitter, ND filter - neutral density filter.

Polarization sensitive OCT (PS-OCT) can detect birefringence or other polarization properties of the sample [9–11]. Depending on tissue microstructure, different polarization properties can be detected by PS-OCT:

- Preservation of the polarization state in tissues that do not change the polarization of the light
- Birefringence in tissues, in which the index of refraction depends on the polarization state of incoming light
- Scrambling of the polarization state, or depolarization, in tissues that are changing the polarization state of the incoming light randomly

Since several tissues in the eye affect the polarization state of the light [11], PS-OCT can be used to help to identify them [12], segment them [13] or to study them under different experimental conditions [14]. Tissues containing melanin pigments can scramble the polarization state of the light [15] and thus can be identified as depolarizing tissues (retinal pigment epithelium - RPE, choroid). Tissues with organized, fibrous structure (collagen fibers, nerve fibers) are birefringent [16] (sclera, retinal nerve fiber layer - RNFL). Previous studies have shown that the sclera and RNFL can play key roles in glaucoma [17–21]. On the other hand, pigmented layers play important roles in age-related macular degeneration (AMD) [22–24].
Fig. 2. Comparison of the human, rat, and mouse eyes and retinas. (A) Sketch of human, rat, and mouse eye. Rodent eyes shown both in natural size and scaled to the size of the human eye. (B) Retinal OCT scan of human, rat, and mouse retina (all pigmented). Human retina scanned near the fovea. The rodent eyes are much smaller than the human eye. The rodent retina is thinner than the human retina, but not proportionally to the size of the eye. Note that the layered structure of the retina is resolved in all eyes. However, higher axial resolution is required for rodent imaging in order to distinguish all the layers. The human retina was imaged using a clinical PS-OCT system described previously [25].

Since PS-OCT can be used to gain more information and study these tissues in vivo, it promises to be a very useful tool in basic research using animal models. Rats and mice are animal models frequently used in preclinical research because of their short lifespan, high breeding ability, and wide range of genetic variations. Retinal structures in rodents are similar to those in humans (Fig. 2), and rat and mouse models of a large variety of retinal diseases, that are major causes of blindness in humans, have been created and are available for study [26,27]. Complementary to ongoing PS-OCT studies in human eyes [28–30], in vivo imaging studies in rodents may provide additional insight into disease etiology. Ex vivo studies of the tissue polarization properties have already been performed, for example in the RNFL and sclera [31,32]. Moreover, PS-OCT has been used in vivo to investigate peripapillary sclera and RNFL in healthy albino rats [33] and in non-human primates [34], with an axial resolution of 7.6 μm and 12 μm, respectively.

In this article, we (1) present high resolution PS-OCT imaging in the posterior eye segments of healthy pigmented and albino rats along with images of pigmented mice, (2) quantify depolarization characteristics of RPE and choroid, and (3) measure birefringence of the RNFL in rodents. All results were obtained using a high resolution PS-OCT system dedicated for preclinical imaging in rodent eyes. In contrast to an earlier system for rodent imaging [33], this system offers a higher A-scan rate of 83 kHz and higher resolution of ~3.8 μm in tissue. Therefore high resolution images can be acquired faster and motion artifacts are suppressed. High resolution is enabling to delineate the RPE from the choroid and measure RNFL birefringence. This should support the detection of clinically relevant features and lesions during in vivo studies concerning these layers, for instance in AMD models [27,35] and glaucoma models [26].

2. Methodology

2.1 High resolution polarization sensitive OCT

The preclinical high resolution PS-OCT prototype was designed based on polarization sensitive low coherence interferometry [36] in a spectral domain OCT modification [37,38].
enabling measurements of reflectivity, phase retardation, fast axis orientation and degree of polarization uniformity (DOPU). A sketch of the system used in this article is shown in Fig. 1(B). A multiplexed superluminescent diode (SLD, Superlum) with central wavelength 840 nm and a bandwidth of 100 nm (full width at half maximum) was used as a light source. The broad bandwidth of this light source results in a measured axial resolution of 5.1 µm in air (~3.8 µm in tissue with n = 1.35). The single mode fiber output from the SLD was connected to a collimator after which the beam was vertically polarized by a Glan-Thomson polarizer. In the Michelson interferometer, a non-polarizing beam splitter (50/50) split the beam into the reference and the sample beams. In each arm, there was a quarter wave plate (QWP) with its fast axis oriented differently with respect to the horizontal axis. In the reference arm, it was oriented at 22.5° to provide equal power in the two orthogonal polarization states after double pass. Glass prisms were added for dispersion compensation and a neutral density filter for light attenuation in the reference arm. In the sample arm, the QWP was oriented at 45° to provide sample illumination with circularly polarized light. When the light backscattered from the sample passes the QWP the second time, it is in an elliptical polarization state in general and contains information about the phase retardation and fast birefringent axis orientation of the sample [10]. At the non-polarizing beam splitter, the sample and reference beams were recombined and passed through a half wave plate (HWP) to the exit collimator. Using the HWP, the horizontal and vertical polarization axes of the free space beam and those of the polarization maintaining fiber were matched. The collected light was directed to the polarizing beam splitter by a polarization maintaining fiber and was split into two perpendicular polarization channels. Each channel was directed into one of two custom built spectrometers consisting of transmission gratings (1200 lines/mm), f-theta lens (focal length 160 mm) and Basler Sprint cameras (with 4096 pixels, in order to increase line acquisition speed 3072 pixels were used). The line speed of the cameras was 83 kHz, system sensitivity was 96 dB, the imaging depth range was 3.93 mm in air (2.91 mm in tissue) and the 10 dB roll off depth was 1.87 mm in air (1.38 mm in tissue). Due to the smaller pupil size, the beam diameter needs to be smaller for imaging in rodents than for imaging in humans. Therefore, a demagnifying telescope comprising three achromatic doublets (f = 50 mm) was used in the sample arm (two were combined to make a lens with f = 25 mm). The beam diameter was reduced from 1.6 mm to 0.8 mm and the power of the beam at the cornea was 2.85 mW. Homemade acquisition software (LabVIEW, National Instruments, Austin, TX, USA) was used to acquire data during measurements.

From the acquired spectral data, reflectivity, phase retardation and fast axis orientation images were calculated [10,37]. In addition, DOPU images were computed [13]. For averaging PS-OCT images, a method presented earlier was used [30]. DOPU cross sectional images were computed using 2D spatial evaluation kernels as well as 2D kernels averaged over repeated acquisitions. For the latter, DOPU images were calculated using a floating window extending over 4 × 5 pixels in x-z direction over 10 images acquired at one position [39]. Thereby DOPU was only calculated when the number of pixels with intensities 7 dB above noise level was > 15% in the evaluation window (volume). Pixels that did not meet this threshold were displayed in gray. These criteria are based on [39] and were adjusted empirically for the system and imaging parameters used in this work. Processing of the data took ~14 s per cross sectional image.

The PS-OCT prototype offers high acquisition speeds. Since the acquisition time for a 3D data set was less than 4 seconds and animals were anesthetized, motion artifacts during measurements were minimal.

2.2 Quantitative depolarization analysis

In addition to qualitative imaging, we also performed a quantitative DOPU analysis. The depolarization characteristics of pigmented posterior eye tissues were analyzed in two image slabs containing the RPE and the choroid, respectively. In order to assess melanin-related
depolarization in a quantitative manner, 3D PS-OCT data sets consisting of \(200 \times 1024\) A-scans and \(400 \times 512\) A-scans covering a field of view of \(28^\circ \times 28^\circ\) were acquired in rats and mice, respectively. PS-OCT images were computed as outlined above. DOPU images were generated using window sizes of \(25 \times 2\) pixels (\(38 \mu m \times 3.8 \mu m\)) and \(15 \times 2\) pixels (\(28 \mu m \times 3.8 \mu m\)) from the rat and mouse data, respectively. Depolarization analysis included the following steps involving both reflectivity and DOPU images. First, Bruch’s membrane (BM) was segmented in the reflectivity B-scan images of each 3D data set using an A-scan template crafted from 30 A-lines of a rat retina scan. The template was axially matched to each A-scan in the data sets using a least squares approach. An example of this segmentation is shown in Fig. 5. In the next step, pixels in the regions of interest in the RPE and choroid were extracted from the DOPU images. Using the location of Bruch’s membrane as a backbone, the slab for RPE analysis was defined as six pixels anterior to one pixel posterior to the BM position. Choroidal DOPU was assessed in a slab extending over 40 pixels posterior to the BM position. Within each of the two slabs, the average DOPU value (DOPU_{mean}) and the minimum DOPU value (DOPU_{min}) along each A-line were computed and plotted in fundus depolarization maps. Statistical comparison between average DOPU in the left and right eyes of different animals and strains was performed using a Student’s t-test. \(P < 0.05\) was used as a cutoff for statistical significance.

2.3 Birefringence analysis

To demonstrate the possibility of accessing RNFL birefringence, Long Evans rats were investigated. For this purpose, RNFL thickness maps and phase retardation maps were generated from 3D PS-OCT data sets consisting of \(400 \times 512\) A-scans. Every B-scan was repeated five times at the same location. The B-scans of three consecutive locations were then averaged. Hence, each A-scan was averaged over 15 scans using an approach described previously [30]. To segment the inner limiting membrane (ILM) and the border between the inner plexiform layer and the RNFL, we used a segmentation based on reflectivity.

Since the sample light beam passed the birefringent cornea on its way to and from the retina, phase retardation images had to be corrected for corneal birefringence [40]. For each A-scan in the PS-OCT data set, phase retardation and optic axis orientation values were extracted from the pixel with the highest intensity located in a ±2 pixel neighborhood of the segmented ILM. The resulting surface retardation and axis orientation maps were smoothed using a 10 (x) \times 8 (y) kernel and then used to construct the anterior segment Jones matrices for each A-scan location. These matrices were applied to all pixels beneath the retinal surface in the PS-OCT volume, yielding phase retardation images devoid of corneal birefringence impact [40]. Since the RNFL in rats is considerably thinner than in humans, birefringence could not reliably be assessed by determining the slope of the retardation increase within the RNFL [41–44]. Hence, the accumulated phase retardation projected onto posterior retinal layers was extracted in a 70 pixel deep slab located 20 pixels beneath the RNFL and, thereof, average RNFL phase retardation maps were computed [30,45]. For retardation calculation only pixels above an empirically chosen intensity threshold were used in order to reduce the influence of noise. RNFL birefringence maps were then generated by dividing the RNFL retardation maps by the respective RNFL thickness maps. The data processing for this analysis took ~15 min per volume. Circumpapillary RNFL thickness, phase retardation and birefringence were radially averaged in an annulus centered at the optic nerve head with inner and outer diameters of 660 \(\mu m\) and 890 \(\mu m\), respectively, and were displayed in TSNIT (temporal – superior – nasal – inferior – temporal) plots akin to those used in human RNFL analysis. The inner diameter was chosen such that it excludes the area of optic nerve head. The RNFL thickness is highest in the nerve head and decreases in the periphery. However, since in ONH the nerve fibers are bending downwards to form the optic nerve head, the signal from them is lower and also the observed phase retardation is decreasing. The reason for this is that the fibers are not perpendicular to the beam anymore, and both the backscattered signal
strength and the observed birefringence decrease. In part, the RNFL is also shadowed by vessels filled with blood. Therefore, we excluded this area from analysis and calculated circumferential distribution of the values around the optic nerve head.

2.4 Animals

Male non-pigmented Sprague Dawley rats (N = 4), pigmented Long Evans rats (N = 4), Brown Norway rats (N = 4) and C57BL/6 mice (N = 4) were purchased from the Medical University of Vienna breeding facility in Himberg, Austria (Sprague Dawley) and from Charles River, Germany (all other animals). The animals were kept under controlled lighting conditions (12 hours light, 12 hours dark). All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and under protocols approved by the ethics committee of the Medical University of Vienna and the Federal Ministry of Science, Research and Economy (protocols number GZ: BMWFU-66.009/0072-WF/V/3b/2015 and GZ: BMWFU-66.009/0318-WF/V/3b/2014). Animals were anesthetized using ketamine (Ketavet, rats 80 mg/kg body weight intraperitoneal, mice 100 mg/kg body weight, intraperitoneal) and xylazine (Rompun, rats 8 mg/kg body weight intraperitoneal, mice 5 mg/kg body weight intraperitoneal). The anesthetia was necessary to immobilize the animal and to avoid motion artifacts. Because animals are anesthetized during the measurements, the eyes had to be moisturized artificially. Eye drops (Oculotect, Novartis Pharma) were applied to the rodent cornea. Excess artificial tear fluid may act like a positive contact lens and hence decrease the image quality. Therefore, excess fluid on the eye was gently removed using a cotton pad. Tropicamide (Mydriaticum, topical) and phenylephrine (2.5%, topical) were used for pupil dilation. One animal for each strain (age 1 year) was used for OCT imaging (2D images) and histological imaging. Three animals from each strain were purchased later for statistical evaluation. Since pigmentation depends on age [46,47], we imaged animals of similar ages (age 4 months – rats, 5 months – mice).

2.5 Measurements

During the measurement, the animal was placed in a holder that allowed aligning the eye with respect to the measurement beam. In order to provide a comfortable stage, soft material cushioning was placed underneath the animal. The animal was kept warm using disposable heating pads during all measurements. Using the real time preview of the B-scans and OCT fundus projection images, the eye was placed in the desired position to acquire the 3D data set.

2.6 Histology

Histological imaging of retinas of several rats and mouse was performed after the PS-OCT measurements for qualitative comparison with OCT images. The eyes of one animal of each of the three rat strains, Sprague Dawley, Long Evans, Brown Norway rats and of a C57BL/6 mouse were fixed in 4% para-formaldehyde in 0.1 M phosphate buffered saline (pH 7.4) for 24 hours, cryoprotected in ascending concentrations of sucrose and infiltrated overnight in 20% sucrose in PBS (two parts) and Tissue-Tek O.C.T. compound (one part). Eyecups were flash-frozen in isopentane/liquid nitrogen and mounted to receive vertical frozen sections matching the plane of the OCT scans. Series of 20 μm thick frozen sections were collected onto Superfrost Plus slides, coverslipped in Aqua-Poly/Mount (Polysciences), and imaged on a Zeiss Axio Imager Z.2 using differential interference contrast.
3. Results

3.1 PS-OCT imaging

An OCT fundus view, averaged high resolution PS-OCT B-scans at different positions and enlargements of the scans of a Brown Norway (pigmented) rat are shown in Fig. 3. The field of view for Fig. 3(A) is 28° × 28°. In the reflectivity image, the retinal layers, choroid, sclera and retinal vessels can be observed. Even though the light penetration through pigmented tissues in general is low, the sclera is visible in this particular animal. It is noticeable in the phase retardation images, where the false-color gradient changed from blue to red and back to blue due to scleral birefringence. The depolarization effect of pigments is seen in the DOPU images, depicted in Fig. 4.

![Fig. 3. PS-OCT images of the retina of the pigmented Brown Norway rat (averaged over 10 B-scans). (A) OCT fundus image, field of view 28° × 28°. (B) Cross section images obtained at the position indicated by the blue line on the fundus image (reflectivity, fast axis orientation and phase retardation image accordingly). (C) Cross section images at the position indicated by the red line on the fundus image (reflectivity, fast axis orientation, phase retardation image accordingly). White arrows indicate the depolarizing effect of pigment. Blue arrows indicate the birefringent effect of the sclera. The red arrow denotes higher penetration at the location of choroid vessels. The zero delays are at the top of the images. (D) Enlargement of the red rectangle in (B).](image)

Here reflectivity and DOPU images of the eyes of a Sprague Dawley rat, a Brown Norway rat and a C57BL/6 mouse together with histological sections are shown. In the DOPU images (low DOPU values correspond to low polarization uniformity), the depolarizing effect of pigmented tissue is indicated by yellow to green false colors. In the pigmented animals, depolarization was also observed in the vicinity of the central retinal vessel and may be attributed to melanin present in the papillary region. In Sprague Dawley rats, which carry no pigment, penetration of the OCT beam was deeper, extending through the sclera to extraorbital tissue (Fig. 4). This extraorbital tissue showed sponge-like structure and exhibited depolarization in PS-OCT images.
Fig. 4. Imaging of pigmentation and depolarization by PS-OCT. Images are averaged over 10 B-scans of the retina of non-pigmented (Sprague Dawley) and pigmented (Brown Norway) rats. (A) Reflectivity and DOPU images of a non-pigmented Sprague Dawley rat, taken at the optic nerve head and in the peripheral retina. (B) Reflectivity and DOPU images of a pigmented Brown Norway rat taken at corresponding locations; blue arrows highlight higher signal beneath choroid vessels. (C) Reflectivity and DOPU images of a pigmented C57BL/6 mouse. In the DOPU images, polarization-preserving tissue exhibits values around 1 (red color), whereas depolarizing tissue exhibits lower values. The depolarizing effect of pigment is visible in the proximity of the blood vessel and in RPE and choroid. (D) Enlarged part of DOPU image of non-pigmented animal and pigmented animal, respectively, together with histological image from Brown Norway rat. On reflectivity images, red arrows show extraorbital tissue, on DOPU images white arrows mark the depolarization effect of the extraorbital tissue in the non-pigmented animal and in the proximity of the central vessels in pigmented animal. (E) Comparison of OCT reflectivity image, histological image (differential interference contrast) and DOPU image of non-pigmented rat, pigmented rat and pigmented mouse. White arrow points to choroid. In the histological images, melanin pigmentation is visible as brown color (present in RPE and choroid of pigmented animals, but absent in the RPE and choroid of non-pigmented animal).

3.2 Depolarization in retinal pigment epithelium and choroid

The depolarization characteristics of the RPE and the choroid were analyzed in the left and right eyes of 3 Brown Norway rats, 3 Long Evans rats, 3 Sprague Dawley rats, and 3 C57BL/6 mice. A comparison of reflectivity and DOPU images is shown in Fig. 5. In all pigmented strains, depolarization was observed in the RPE and even more pronounced in the...
The location of BM is indicated by the red line in the center reflectivity images in Fig. 5(B). Exemplary pigment epithelial and choroidal DOPU\textsubscript{mean} and DOPU\textsubscript{min} maps of a Long Evans rat are shown in Fig. 5(C), left panel. DOPU was rather high in the RPE maps (obscured only by shadowing artifacts from large retinal vessels shown as lower DOPU values under their locations). In contrast, the choroidal maps show a DOPU variation congruent with the locations of choroidal vessels. Average DOPU\textsubscript{min} and DOPU\textsubscript{mean} values are listed for RPE and choroid of the four strains in Table 1. Neither DOPU\textsubscript{min} nor DOPU\textsubscript{mean} was significantly different between left and right eyes (Fig. 5). However, for all rodent strains, there were significant differences between RPE and choroidal DOPU\textsubscript{min} as well as for DOPU\textsubscript{mean} in both left and right eyes. The respective p-values are listed in Table 2. DOPU\textsubscript{min} and DOPU\textsubscript{mean} data was also compared within the same tissue type among the different strains (e.g., choroidal DOPU\textsubscript{min} between Long Evans rats and C57BL/6 mice, et cetera). For all permutations, except one, the differences of the depolarization characteristics were significant, which is demonstrated by the p-values listed in Table 3.

### Table 1. Depolarization of RPE and choroid in different rodent strains/species. DOPU\textsubscript{mean} and DOPU\textsubscript{min} are given as average of each six eyes ± standard deviation.

| Strain            | DOPU\textsubscript{mean} | RPE | Choroid | DOPU\textsubscript{min} | RPE | Choroid |
|-------------------|---------------------------|-----|---------|--------------------------|-----|---------|
| Brown Norway      | 0.90 ± 0.01               | 0.73 ± 0.02 | 0.85 ± 0.02 | 0.45 ± 0.02               |     |         |
| Long Evans        | 0.87 ± 0.02               | 0.69 ± 0.01 | 0.80 ± 0.03 | 0.37 ± 0.03               |     |         |
| Sprague Dawley    | 0.95 ± 0.01               | 0.93 ± 0.01 | 0.93 ± 0.01 | 0.87 ± 0.01               |     |         |
| C57BL/6           | 0.71 ± 0.04               | 0.58 ± 0.03 | 0.58 ± 0.05 | 0.32 ± 0.03               |     |         |

### Table 2. P-values of comparison between depolarization of RPE and choroid in different rodent strains/species. OS - left eye, OD - right eye.

| Strain            | RPE vs. choroid (p-values) | OS | OD | OS | OD |
|-------------------|---------------------------|----|----|----|----|
| Brown Norway      |                           | 1.44 × 10^{-3} | 1.94 × 10^{-3} | 1.01 × 10^{-4} | 6.04 × 10^{-1} |
| Long Evans        |                           | 1.66 × 10^{-3} | 2.09 × 10^{-6} | 7.05 × 10^{-3} | 1.18 × 10^{-6} |
| Sprague Dawley    |                           | 4.17 × 10^{-3} | 9.01 × 10^{-3} | 5.09 × 10^{-4} | 1.14 × 10^{-3} |
| C57BL/6           |                           | 2.51 × 10^{-3} | 2.10 × 10^{-3} | 4.31 × 10^{-4} | 2.58 × 10^{-3} |

### Table 3. Bonferroni corrected p-values of comparison between depolarization within the same tissue type in different rodent strains/species. BN – Brown Norway, LE – Long Evans, SD – Sprague Dawley, B6 – C57BL/6

| Strain            | DOPU\textsubscript{mean} | DOPU\textsubscript{min} |
|-------------------|---------------------------|-------------------------|
| BN vs. LE         | 4.10 × 10^{-3}            | 3.46 × 10^{-2}          |
| BN vs. SD         | 1.38 × 10^{-3}            | 2.04 × 10^{-5}          |
| BN vs. B6         | 8.19 × 10^{-6}            | 1.13 × 10^{-6}          |
| LE vs. SD         | 4.13 × 10^{-12}           | 9.52 × 10^{-6}          |
| LE vs. B6         | 7.73 × 10^{-5}            | 9.30 × 10^{-6}          |
| SD vs. B6         | 6.43 × 10^{-10}           | 9.49 × 10^{-8}          |

### 3.3 Birefringence of the retinal nerve fiber layer

The birefringent properties of the RNFL were investigated in the left and right eyes of three Long Evans rats. Figure 6 provides exemplary images of a left rat eye. The semi-automatically segmented RNFL boundaries are shown in white in a reflectivity B-scan image. Beneath, the reflectivity fundus projection map, the RNFL thickness map, and the RNFL
retardation map of the same data set are shown. From the latter two maps, the RNFL birefringence map was computed in areas where RNFL thickness was more than 10 pixels (which corresponds to 26 µm in tissue). The highest thickness was observed along vessels and along radially distributed nerve fiber bundles around the optic nerve head. Retardation values were highest in the optic nerve head region and its close vicinity, while retardation at retinal vessel locations was close to zero. Birefringence was lowest at the location of retinal vessels and highest in the periphery of the optic nerve head. Circumpapillary RNFL thickness, phase retardation, and birefringence profiles were computed in an annulus around the optic nerve head for both left and right eyes (Fig. 6(B)). The average RNFL thickness in the annulus was 39.9 µm ± 5.6 µm and 37.8 µm ± 2.5 µm, and the average RNFL phase retardation was 6.6° ± 0.3° and 6.9° ± 0.1° for the left and right eyes, respectively. The average circumpapillary birefringence was 0.16°/µm ± 0.02°/µm and 0.17°/µm ± 0.01°/µm. By normalization with the central wavelength of 840 nm, these values can be expressed as dimensionless birefringence of 3.67 × 10⁻⁴ ± 0.36 × 10⁻⁴ and 3.9 × 10⁻⁴ ± 0.25 × 10⁻⁴. These values represent single pass birefringence and an average over three animals (mean ± standard deviation).

Fig. 5. DOPU analysis in RPE and choroid. (A) Reflectivity fundus projection image and exemplary B-scan with BM segmentation in a Long Evans rat. Depolarization was analyzed in slabs indicated in green (RPE) and orange (choroid). (B) Reflectivity and DOPU images in four different rodent strains. (C) The left panel shows DOPU en face projections for a Long Evans rat. In the right panel, DOPU mean and DOPU min are plotted for RPE and choroid in the four rodent strains (dark column represents left eye, light column represent right eye).
Fig. 6. Birefringence estimation of RNFL. (A) On top, a representative B-scan and the segmentation of the RNFL is shown. Reflectivity, retardation, thickness and birefringence maps are shown below. Pixels with a RNFL thickness below 10 pixels are displayed in grey. On the right, an enlargement of the peripapillary retardation plot depicting the evaluation area is shown. (B) Circumpapillary plots of RNFL thickness, retardation and birefringence in left and right eyes of Long Evans rats. Different colors represent three analyzed individuals. TSNIT stands for temporal–superior–nasal–inferior–temporal regions.
4. Discussion

Optical imaging methods provide rapid, noninvasive, structural and quantitative assessment of tissue structures. In this work, we used an OCT system for imaging pigmented and albino rodents using polarization sensitive detection and high axial resolution. OCT without polarization sensitive detection was used to study rodent eyes in the past [48,49]. For example, several groups imaged and studied healthy animals [50], animal models for photoreceptor degeneration and retinoblastoma in mice [48] and outer retinal degeneration in rats [51] using high axial resolution. Diabetes-induced changes in the retina were studied in mice [52] using label free angiography.

Polarization-sensitive imaging provides an additional contrast channel and enables quantitative measurements of polarization changes related to tissue microstructure. PS-OCT has proven useful in ophthalmic patients and considerably enhances diagnostic imaging in vision-threatening diseases such as AMD [29,53,54], diabetic retinopathy [55], macular telangiectasia [56], and glaucoma [45,57]. The improved visualization of pigmented structures, in particular the RPE, via depolarization and of birefringent structures such as the RNFL enabled quantitative measurements of pathology-related optical parameters and lesion extensions. Additional knowledge about light polarizing properties of tissues in health and disease can be obtained from preclinical research involving animals. Important information such as the conversion factor from birefringence to RNFL thickness via birefringence measurements in scanning laser polarimetry was obtained from animals and then translated to human imaging [58,59]. In a recent study, we used a PS-OCT system with lower resolution of 7.6 µm in air (5.6 µm in tissue) to study birefringence in the peripapillary rat sclera in vivo [33]. Very recently, we used the same system for quantifying depolarization in the posterior rat eye [60]. While that study did provide a correlation of in vivo PS-OCT images with ex vivo quantification of melanin density, the resolution was not sufficient to distinguish the RPE from the choroid. The high resolution PS-OCT system presented in this article allows to distinguish fine retinal layers and the RPE not only in reflectivity images, but also in PS-OCT images (Figs. 4 and 5). Unlike our previously reported system, the improved resolution now enables a better distinction of RPE and choroid. This should support the detection of clinically relevant features and lesions during in vivo studies concerning these layers (for instance in AMD models [27,35]).

In order to demonstrate the capabilities of high resolution PS-OCT, pigmented and albino animals were imaged. In pigmented animals, the beam penetration was less deep (restricted to choroid/sclera) than in non-pigmented ones (deep penetration down to sclera/extraorbital tissue). This can be explained by the lack of melanin pigmentation in the RPE and choroid in albino animals [15]. Due to reduced scattering and absorption of the beam in non-pigmented tissues, the beam travelled deeper into the eye wall. In the case of pigmented animals, beam penetration was deeper at the locations of choroidal vessels, as can be seen in Fig. 4. This could be because blood was absorbing and reflecting less than the densely pigmented surrounding tissue and thus the beam could penetrate deeper, sometimes down to the sclera. In the non-pigmented animals, penetration was deep enough to visualize the extraorbital tissue. As can be observed in Fig. 4, this tissue appears depolarizing as well.

Quantitative measurements of polarizing properties were performed in the melanin containing structures of the posterior eyes and in the RNFL of rodents. Melanin in RPE and choroid causes depolarization of backscattered light [15]. In our previous study on the depolarizing properties of these structures in rat eyes, our analysis was limited to measurements of the combined depolarizing effect of the RPE/choroid complex [60]. In this article, the improvement of axial resolution enabled us to use smaller DOPU evaluation kernels and, thus, to discriminate the depolarizing contributions of RPE and choroid. Fundus maps of DOPU in the RPE and choroid were extracted from the PS-OCT data sets and the depolarization properties of different structures were compared among four commonly used
rodent species (Fig. 5). The analysis revealed statistically significant differences between pigment epithelial and choroidal DOPU characteristics. In all species, the choroid exhibited stronger depolarization than the RPE, indicating that the choroid of the investigated rodents was more pigmented than their RPE (cf. Table 1 and Table 2). This effect was stronger in rats than in mice. The general trend showed the depolarization was more pronounced in the RPE and choroid of mice than in rats. Despite the different speckle size, there is a good agreement between DOPU values in choroid and choroid/RPE complex reported in our previous study. However, the RPE shows weaker depolarization than the choroid, which we were not able to analyze in our previous study due to the limited axial resolution [60]. Owing to the lack of melanin pigmentation, albino rats exhibited significantly higher DOPU_min and DOPU_mean than the pigmented rodents, both in RPE and choroid. Although this was never quantified in humans, qualitative comparison of albino and pigmented human eyes also showed reduced depolarization in the RPE of albino patients [15]. In contrast to rodents, the human choroid looked less depolarizing than the RPE [15,39,61]. However, since these data derive from a very small number of volunteers, larger study would be necessary to test this hypothesis.

RNFL thickness is an important parameter in glaucoma diagnostics [62–66]. Recently, RNFL thickness was also introduced as a potential parameter for the ocular assessment of neurological diseases such as multiple sclerosis, Parkinson’s disease, and Alzheimer’s disease where retinal pathology reflects neuropathology of the brain [67–69]. Animal models are a basic component of glaucoma research [26] (as well as of research in other diseases). Similar to ophthalmic diagnosis in humans, animal studies have benefitted from the noninvasive character of OCT [34,51,52,70,71]. In addition to RNFL thickness, RNFL birefringence represents another promising candidate for the assessment of RNFL integrity. RNFL birefringence is connected to the microstructure of axons [31,72] and has been hypothesized a parameter for glaucoma diagnostics even earlier and possibly more sensitive than RNFL thickness [73].

In this paper, we presented the first in vivo measurements of RNFL birefringence in rats. Owing to the low thickness of the RNFL in small animals, the high axial resolution of our system permits more accurate RNFL thickness measurements. It also enables a better estimate of the polarization state at the ILM, which is required for the compensation of corneal birefringence. Since the RNFL in rats is thin, the phase retardation values measured beneath the RNFL are quite low, rendering them more susceptible to noise. Therefore we performed averaging over 15 A-scans using Stokes vectors [30]. RNFL thickness, phase retardation, and birefringence maps were computed for Long Evans rats. Respective circumpapillary profiles were extracted from these maps. RNFL thickness values were found increased at the locations of retinal blood vessels while phase retardation (and, thus, also birefringence) was lower there. Thickness and phase retardation were highest in close vicinity of the optic nerve head, similar to human. In the circumpapillary annulus, RNFL birefringence amounted to 0.16°/µm ± 0.02°/µm and 0.17°/µm ± 0.01°/µm (3.67 × 10^−6 ± 0.36 × 10^−6 and 3.9 × 10^−6 ± 0.25 × 10^−6 dimensionless birefringence, single pass) for the left and right eyes, respectively. These values are higher than the birefringence values of 0.23 nm/µm ± 0.01 nm/µm (corresponds to 0.10 °/µm for 840 nm) reported for ex vivo measurements of rat RNFL [72]. However, post mortem measurements of polarization properties may be influenced by tissue shrinkage, fixation artifacts, as well as birefringence distortions induced by stress and strain during sample handling, which is not the case for in vivo imaging. For comparison, the conversion used in commercial scanning laser polarimetry retained from histopathologic correlation with ellipsometry data is 0.67 nm/µm (corresponding to a birefringence of 0.38°/µm at 632 nm or 0.29°/µm at 840 nm wavelengths) [58]. Birefringence measured in vivo with PS-OCT in human RNFL covered ranges of ~0.1 – 0.45°/µm (double pass, 830 nm) [42], 0.14°/µm (single pass, 840 nm) [43], 0.1 – 0.6 °/µm (double pass, 840 nm) [45], −0.07 – 0.15°/µm [44] (single pass, 840 nm) and 0.14°/µm ± 0.11°/µm [74] (double pass, 1040 nm). In macaque
RNFL, birefringence measured with in vivo PS-OCT ranged from 0.12°/µm to 0.2°/µm [34] (single pass, 1060 nm).

5. Conclusion

In this article, high resolution PS-OCT for ocular imaging in rodents is presented. The system acquires high resolution images which enables to study the morphology and polarization properties of single retinal layers. The performance of the system was demonstrated in pigmented and non-pigmented rats as well as in pigmented mice. The polarization properties of several ocular tissues were investigated. Melanin pigment related depolarization was assessed in RPE and choroid of four rodent strains and statistically analyzed. RNFL thickness, phase retardation, and birefringence were mapped and quantitatively measured in Long Evans rats. The results demonstrate that PS-OCT enables enhanced contrast and polarization-based tissue characterization and provides access to quantitative measurements of pigmentation, tissue organization, and microstructure. Hence, high resolution PS-OCT promises to be a powerful tool in preclinical studies of AMD, glaucoma, and other diseases of the eye.

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