Quantitative Analysis of Mouse Retinal Layers Using Automated Segmentation of Spectral Domain Optical Coherence Tomography Images

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Purpose: Quantification of retinal layers using automated segmentation of optical coherence tomography (OCT) images allows for longitudinal studies of retinal and neurological disorders in mice. The purpose of this study was to compare the performance of automated retinal layer segmentation algorithms with data from manual segmentation in mice using the Spectralis OCT.

Methods: Spectral domain OCT images from 55 mice from three different mouse strains were analyzed in total. The OCT scans from 22 C57Bl/6, 22 BALBc, and 11 C3A.Cg-Pde6b⁺Prph2^{Rd2}/J mice were automatically segmented using three commercially available automated retinal segmentation algorithms and compared to manual segmentation.

Results: Fully automated segmentation performed well in mice and showed coefficients of variation (CV) of below 5% for the total retinal volume. However, all three automated segmentation algorithms yielded much thicker total retinal thickness values compared to manual segmentation data ($P < 0.0001$) due to segmentation errors in the basement membrane.

Conclusions: Whereas the automated retinal segmentation algorithms performed well for the inner layers, the retinal pigmentation epithelium (RPE) was delineated within the sclera, leading to consistently thicker measurements of the photoreceptor layer and the total retina.

Translational Relevance: The introduction of spectral domain OCT allows for accurate imaging of the mouse retina. Exact quantification of retinal layer thicknesses in mice is important to study layers of interest under various pathological conditions.

Introduction

The optical properties of the eye make it possible to image a part of the central nervous system, the neuroretina, in vivo. Retinal imaging has been revolutionized by optical coherence tomography (OCT),¹ which, in the last 20 years, has been refined with ever increasing resolution and speed. Spectral domain (SD)-OCT offers high resolution ($<5 \mu\text{m}$)² and, in humans, is used for longitudinal monitoring of retinal diseases, such as age-related macular degeneration and diabetic retinopathy among others.³

However, because disease monitoring has relied largely on qualitative parameters, recent research has

focused on obtaining quantitative parameters from OCT by retinal layer segmentation. Manual OCT segmentation is labor-intensive, and biased by inter- and intrarater variability. Automated segmentation arguably allows for accurate and repeatable delineation of individual retinal layers, which then can be objectively and precisely quantified.^{4–7} This has been used recently for clinical brain research, particularly in patients with multiple sclerosis, where inner layers (nerve fiber, ganglion cell layer [GCL], inner plexiform layer [IPL], and inner nuclear layer [INL]) have been shown to undergo thinning.^{8–10}

Quantification of retinal layer changes in experimental eye research up to now has largely relied on

histology. However, histology is time-consuming and interferes with the ability to monitor individual animals over time. Additionally, histological samples are potentially associated with preparation artifacts leading to inaccurate thickness measurements of individual retinal layers.¹¹ OCT has been adapted to image the retina in small rodents, especially mice, with an increasing number of research reports published over the last decade.^{11–13} Furthermore, with the adaptation of SD-OCT for small animal research it only recently became possible to obtain retinal images that are similar to transverse histological sections of the retina. Because of the high speed of acquisition and averaging of scans, speckle noise in mouse imaging can be reduced significantly and allows automated segmentation to quantify individual layers of the mouse retina.¹⁴ As such, segmentation algorithms designed to delineate human OCT scans can now be used for small animal research. However, using such segmentation algorithms in OCT images of the rodent retina may violate model assumptions and lead to segmentation errors.

We recently have proposed an automated graph-based multisurface segmentation algorithm (AR-TORG Center Bern, Bern, Switzerland) that relaxes model assumptions by using soft constraints.¹⁵ This improves the accuracy of delineation and makes the segmentation of pathologies easier. The algorithm recently has been adapted for segmentation of murine retina as well. Using the Spectralis HRA OCT (Heidelberg Engineering, Heidelberg, Germany) for image acquisition in mice, we compared the performance of the inbuilt segmentation algorithm in the Heidelberg Explorer (Version 1.9.10.0) for the murine retina with our custom built segmentation algorithm and an alternative publicly available automated three-dimensional (3D) graph-theoretic retinal segmentation algorithm (Iowa Reference Algorithms; Iowa Institute for Biomedical Imaging, Iowa City, IA, USA, available in the public domain at <https://www.iibi.uiowa.edu>).

To use automated retinal layer segmentation in animal models, one must be aware of the limitations of such algorithms. Additionally, the segmentation results acquired with different segmentation algorithms must result in similar values to provide comparable data between different studies and centers. The current study compares the results from three different automated retinal layer segmentation algorithms with each other, as well as those from manual segmentation, and assesses the repeatability

of measurements in wild type mice and a mouse model of retinitis pigmentosa.

Methods

Mice

All procedures were performed following governmental approval according to the Federal Swiss Regulations on Animal Welfare and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Wild type inbred C57Bl/6 (pigmented) and BALB/c (nonpigmented), and C3A.Cg-*Pde6b*⁺*Prph*2^{Rd2}/J (congenic mutant strain, pigmented, C57Bl/6 background) mice were used for the experiments. All animals were housed under standard conditions (food and water ad libitum, 12-hour dark-light-cycle).

Animal Preparation

For mouse anesthesia, subcutaneous (s.c.) injection of 0.75 mg/kg medetomidine (Dormitor, 1 mg/mL; Orion Pharma, Provet, Switzerland) and 45 mg/kg ketamine (Ketalar 50 mg/mL; Parke-Davis, Pfizer, Switzerland) was used. At least half an hour after injection, anesthesia was reversed with 0.75 mg/kg atipamezole s.c. (Antisedan, 5 mg/mL; Pfizer) and 300 μ L of 0.9% NaCl s.c. was used to avoid dehydration. Pupils were dilated using tropicamide 0.5% and phenylephrine HCl 2.5% eye drops (ISPI, Bern, Switzerland). Methylcellulose (Methocel 2%; OmniVision, Neuhausen, Switzerland) diluted 1:1 with balanced salt solution (Alcon, Schaffhausen, Switzerland) was applied onto the cornea to prevent corneal desiccation.

OCT Image Acquisition

Optical coherence tomography images were acquired using the Heidelberg Retina Angiograph (HRA) Spectralis system (Heidelberg Engineering), and imaging was performed with best results according to previous reports.^{16,17} A 78 diopter lens (Volk Optical, Inc., Mentor, OH, USA) was added in front of the OCT camera and the murine eye was covered with a contact lens (9 mm diameter, base curve 7.2, power [F'] +4 diopters; Bausch & Lomb; OmniVision). An OCT volume scan, centered on the optic nerve head (ONH), was obtained from every eye (Fig. 1). Optical coherence tomographic scans were acquired in the automatic real-time mode (ART), averaging 18 frames per image. The scanned area

(512 × 496 pixels) covered the central 20° and consisted of 19 to 25 horizontal B-scan lines.

In five animals from each mouse strain, repeated OCT measurement was performed within a time interval of 1 month to assess repeatability. Additionally, monthly repeated OCT measurement over a time course of 6 months was acquired within these animals to assess variation of retinal thickness over time.

Histology

After the experiment, histological sections were prepared from all animals (Fig. 1). Eyes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) solution at 4°C for 12 hours. After gradual dehydration in an alcohol series, samples were embedded in paraffin, cut in 5-μm sections through the ONH and mount on glass slides. Staining of histological sections was performed using hematoxylin and eosin stain (H&E).

Image Analysis

Three OCT segmentation algorithms were used for automated segmentation of the entire OCT volume scans:

- The implemented Heidelberg Spectralis system software (Heidelberg Eye Explorer 1.9.10.0; Heidelberg Engineering)
- OCTExplorer 3.5, IOWA (The Iowa Reference Algorithms, Retinal image analysis laboratory, Iowa Institute for Biomedical Imaging), available in the public domain at <https://www.iibi.uiowa.edu/content/iowa-reference-algorithms-human-and-murine-oct-retinal-layer-analysis-and-display>^{18–20}
- OCT Segmentation App, developed by the AR-TORG Center (Ophthalmic Technology Group, University of Bern, Switzerland; Dufour PA, et al.¹⁵)

Additionally, an experienced annotator performed a manual segmentation using the Heidelberg system.

Thickness of individual retinal layers was quantified using a standard Early Treatment Diabetic Retinopathy Study (ETDRS) grid, which was centered manually on the ONH (Fig. 2C). Mean values of the inner ring (corresponding to approximated diameter of 600 to 1800 μm) were analyzed further. The central area with the ONH and peripheral areas with potential image disparity (ETDRS grid outer ring) were excluded from analysis. Note that measurements of lateral distances might not be accurate,

Figure 1. Histology and OCT of the retina of a C57Bl/6 mouse. Individual layers are marked besides (abbreviations see Table 1).

since the dimensions of the mouse eye differ significantly from a human eye, and a 78 diopter optical lens and a contact lens were used. However, axial OCT measurements seem to be accurate.²¹

Statistics

Prism software version 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Mean thickness of individual retinal layers was compared by the nonparametric Friedman test (1-way ANOVA) with posttest analysis. *P* values less than 0.05 were considered statistically significant. For assessment of repeatability, the Interclass Correlation Coefficient (ICC) was calculated using a 2-way mixed model with absolute agreement.

Automated Segmentation Software

Heidelberg Engineering Germany

The automated segmentation algorithm from the Heidelberg Eye Explorer segments 11 different retinal boundaries: the inner limiting membrane (ILM), the boundaries between the retinal nerve fiber layer (RNFL) and the GCL, between the GCL and the IPL, between the IPL and the INL, between the INL and the outer plexiform layer (OPL), between the OPL and the outer nuclear layer (ONL), the external limiting membrane (ELM), two photoreceptor layers (PR1/2), the RPE, and the basement membrane (BM) with the underlying choroid (Fig. 2B2, Heidelberg).

For every OCT scan, each segmented layer line can be individually shown (or hidden) and can be manually adjusted. From the layer segmentations, retinal thickness profiles were calculated for the complete retina, RNFL, GCL, IPL, INL, OPL,

Figure 2. Manual and automated retinal layer segmentation in an OCT of a C57Bl/6 mouse. (A) Native OCT with indicated layers used for thickness comparison. (B) Retinal layer segmentation using manual segmentation (B1) and three different programs: Heidelberg (Heidelberg Engineering, [B2]), IOWA (IOWA, USA, [B3]), and ARTORG (ARTORG center Bern, Switzerland, [B4]). All segmentation lines provided by the software are displayed. (C) Retinal thickness was analyzed within the inner ring of the ETDRS grid.

ONL, RPE, and PR. These can be displayed as color-coded thickness maps. An implemented ETDRS-grid then is used for mean thickness calculations of every segmented retinal layer within all grid areas.

IOWA USA

In the segmentation software from IOWA, segments 11 retinal boundaries using a 3D graph-theoretic approach with simultaneous segmentation of multiple surfaces:^{20,22} the ILM, boundaries between the RNFL and GCL, between the GCL and IPL, between the IPL and INL, between the INL and OPL, between the OPL and Henle fiber layer (HFL), the boundary of myoid and ellipsoid inner segments (BMEIS), the junction between the inner and outer segments (IS/OSJ), the inner boundary of the outer segment–RPE complex (IB_OPR), the inner boundary of the RPE (IB_RPE), and the outer boundary of the RPE (OB_RPE; Fig. 2B3, IOWA). The segmentation modality “macula” or the “ONH” can be chosen. Despite the central area of the ETDRS grid, which was placed on the ONH and which was not further analyzed in this study, the two segmentation modes macula and ONH delivered similar results for the retinal thickness of individual layers.

All segmented layer lines can be individually marked and be adjusted manually. Thickness maps from every individual retinal layer can be displayed as color-coded maps or intensity maps. An implemented ETDRS grid is used for calculation of mean thickness value for every segmented retinal layer within all grid areas. The segmentation process can be batch processed and the thickness measurements can be directly exported to an Excel file.

The IOWA segmentation algorithm also provides an additional tool for segmentation of OCTs from the murine retina, but this modality only supports Biotigen mouse OCTs.

ARTORG Center Bern

In the automated segmentation algorithm from the ARTORG Center Bern, an automated graph-based multisurface segmentation algorithm is implemented.¹⁵ This method builds a 3D mesh for the different layers, making sure they do not collide, yet still follow coherent shape patterns. The implemented option “segment mouse” was chosen. The software segments 6 different retinal boundaries: ILM, the boundaries between the RNFL and GCL, above the INL (GCL and IPL together), above the ONL (INL and OPL

Table 1. Abbreviations

BM	Basement membrane
BMEIS	Boundary of myoid and ellipsoid inner segments
ELM	External limiting membrane
GCL	Ganglion cell layer
HFL	Henle fiber layer
IB_OPR	Inner boundary of the outer segment-RPE complex
IB_RPE	Inner boundary of the RPE
ILM	Inner limiting membrane
INL	Inner nuclear layer
IPL	Inner plexiform layer
IS/OS (J)	Inner/outer segment (junction)
OB_RPE	Outer boundary of the RPE
OPL	Outer plexiform layer
PR1/2	Photoreceptor 1/2
RNFL	Retinal nerve fiber layer
RPE	Retinal pigment epithelium

Index of abbreviations of retinal layers used in text and figures.

together), the ELM, above the inner segments of the photoreceptor (IS), and the BM (Fig. 2B4, ARTORG).

All segmented layer lines can be individually marked and manually adjusted. Color-coded retinal thickness maps from the total retina, RNFL, GCL + IPL, INL + OPL, ONL, and PR can be displayed. An implemented ETDRS-grid is used to calculate the mean thickness values for every segmented retinal layer within all grid areas.

Manual Segmentation Using the Heidelberg Explorer Software

Manual OCT segmentation was based on the automated segmentation of the Heidelberg Explorer. Every single segmentation line for each retinal layer was individually verified and adjusted when necessary in case of erroneous delineation. Visible changes in reflectivity were used as visual cues for manual correction or insertion of individual retinal layers. Manually segmented lines were the ILM, RNFL–GCL/IPL boundary, IPL–INL boundary, INL–OPL boundary, OPL–ONL boundary, ELM, and RPE/BM (Fig. 2B1). The boundary between GCL and IPL was difficult to distinguish in OCT scans and, therefore, the sum of the thickness of these two layers was used for further analysis. In the automated OCT

segmentation, the PR layer between the ELM and BM was further divided in PR1, PR2, and RPE. In the thickness profile, thickness values for the whole PR layer, including the RPE and for the RPE alone were displayed. As the single cell line of the RPE was in some case challenging to discern, the PR and RPE were manually segmented as the same layer using the BM as the outer boundary of the RPE. All manual segmentations then were verified by an independent expert.

Results

In total, 55 SD-OCT images from 22 C57Bl/6, 22 BALBc, and 11 C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J mice were analyzed (one volume scan per animal). Figure 1 presents the labeling of the individual retinal layers in OCT and histological images (H&E staining, for Abbreviations see Table 1). All volume scans used for OCT segmentation were from high quality (mean $\pm$ SEM, 27.2 ± 0.813 decibel).

Retinal Layer Measurement of the Murine Retina Using Three Different Fully Automated Segmentation Algorithms in C57Bl/6 and BALBc Mice

Because the three segmentation programs used in this study segmented different layers (Fig. 2B) only the RNFL, GCL/IPL complex, INL/OPL complex, ONL, and PR/RPE complex were further analyzed for comparison purposes (Fig. 3).

Manual segmentation, Heidelberg, and IOWA automated segmentation showed small scatter range with coefficients of variation (CV) of 2.8 to 13.7% for the thickness of individual retinal layers (mean CV = 6.2%, 6.8%, and 8.1% for manual segmentation, Heidelberg, and IOWA, respectively; Table 2). Segmentation via ARTORG featured higher variability, especially for the RNFL, INL/OPL complex, and ONL (CV = 34%, 36%, and 19%, respectively; mean CV = 16.8%). For the RNFL, GCL/IPL, and INL/OPL, Heidelberg and IOWA segmentation displayed similar thickness measurements. The ARTORG segmentation measured highly significantly thicker RNFL and ONL, and thinner INL/OPL compared to the manual segmentation ($P < 0.0001$). The IOWA algorithm defines a roughly 20 μ m thicker ONL compared to the manual segmentation and the Heidelberg system.

For the outer retinal layers from the ELM to the BM, summarized as PR+RPE layer, the automated

Figure 3. Comparison of retinal layer thickness (RNFL, GCL+IPL, INL+OPL, ONL, PR+RPE) between the different segmentation modalities for C57Bl/6 mice ($n = 22$, [A]) and BALBc mice ($n = 22$, [B]): manual segmentation (m, ●/green), Heidelberg Engineering (H, ■/red), IOWA (I, ▼/blue), ARTORG (A, ▲/orange; abbreviations see Table 1).

segmentation of Heidelberg and the ARTORG segmentation defined significantly thicker values than the manual segmentation ($P < 0.0001$). This difference originates from an automated segmentation error of the basement membrane, which was placed within the choroid, below the RPE/BM line in the Heidelberg and ARTORG segmentation algorithms. For the Heidelberg program, this value was partly corrected by subtraction of the RPE thickness from the PR thickness value. In the IOWA segmentation, the thickness values for the PR and RPE were individually calculated.

By comparing segmentation results of C57Bl/6 and BALBc mice, thinner inner retinal layers (162.5 vs.

149.3 μm , $P = 0.0005$) with the biggest difference of 7.6 μm within the ONL were measured in the nonpigmented mouse strain. This finding might be explained by increased photo exposure in BALBc mice due to missing photo protection by melanin, resulting in a slight thinning of the inner retinal layers. Within the photoreceptor layer, there was no difference (59.8 vs. 59.85 μm , $P = 0.96$) between the two mouse strains in manual segmentation. However, the automated segmentations yielded thicker PR in BALBc than in C57Bl/6 mice (105 vs. 85.6 μm , $P < 0.0001$). Due to missing pigmentation of the RPE in BALBc mice, OCT might reach deeper structures and thereby present more choroidal structures. As the RPE/BM boundary automatically was segmented within the choroid, enhanced depth in BALBc mice, therefore, may result in thicker values of total retinal thickness.

Total Retinal Thickness Measurements in C57Bl/6 and BALBc Mice

Manual as well as automated segmentations showed small coefficients of variation within the groups (2.4%–4.7%). All three automated segmentation algorithms defined significantly thicker total retinal thickness values for both mouse strains compared to the manual segmentation data ($P < 0.0001$, Fig. 4A). In the Heidelberg and ARTORG software, this difference was found to originate from the automated alignment of the BM within the choroid instead of the hyporeflective OCT boundary between the PR/RPE and the choroid (Fig. 4B). In the Heidelberg segmentation and the IOWA software, this issue could partly be corrected for by subtraction of the RPE thickness from the total retinal thickness value. The differences between automated and manual segmentation are summarized in Table 3.

Comparison of Fully Automated Segmentation of Six Retinal Layers With Manual Segmentation Using the Heidelberg Explorer

For the RNFL, there was no difference between the manual and automated segmentation (20.94 vs. 20.42 μm , $P = 0.95$; Fig. 3). In the manual segmentation, the GCL/IPL was thicker (+5.2 μm , $P < 0.0001$), whereas in all other layers manual segmentation showed thinner values (INL, −1.4 μm , $P = 0.0005$; OPL, −0.2 μm , $P = 0.0037$; ONL, −2.5 μm , $P = 0.00283$; PR [minus RPE], −9.2 μm , $P < 0.0001$).

Table 2. Retinal Thickness Measurements With Different Segmentation Algorithms

	Manual Segmentation			Heidelberg Engineering			IOWA			ARTORG		
	Mean	SEM	CV	Mean	SEM	CV	Mean	SEM	CV	Mean	SEM	CV
C57Bl/6												
Automated: $n = 22$												
Manual: $n = 10$												
Total	222.30	2.11	3.00%	232.10	1.87	3.70%	247.90	1.55	2.93%	259.30	2.60	4.70%
RNFL	21.53	0.56	8.28%	21.81	0.33	7.08%	27.05	0.52	9.05%	44.98	3.22	33.60%
GCL+IPL	47.58	1.03	6.84%	44.36	0.60	6.30%	45.54	0.53	5.42%	42.88	1.08	11.81%
INL+OPL	42.73	0.61	4.54%	44.18	0.38	4.08%	36.26	0.40	5.12%	26.59	2.02	35.59%
ONL	53.65	1.26	7.44%	53.39	1.27	11.11%	73.02	1.54	9.92%	75.99	3.08	19.01%
PR	59.80	1.19	6.30%	68.63	0.68	4.55%	45.83	1.34	13.73%	68.85	2.05	13.95%
BALBc												
Automated: $n = 22$												
Manual: $n = 10$												
Total	209.20	1.88	2.84%	225.20	1.71	3.55%	243.30	1.70	3.29%	249.70	1.68	3.15%
RNFL	19.32	0.45	7.43%	20.06	0.54	12.64%	25.33	0.68	12.54%	30.70	1.47	22.48%
GCL+IPL	45.09	0.28	1.97%	41.29	0.42	4.73%	42.23	0.36	3.99%	39.62	1.06	12.59%
INL+OPL	41.92	0.52	3.92%	43.55	0.41	4.37%	35.99	0.49	6.40%	36.61	0.32	4.07%
ONL	46.09	1.24	8.52%	51.34	0.99	9.01%	70.97	1.08	7.14%	64.84	1.13	8.17%
PR	59.86	1.36	7.18%	69.49	0.54	3.65%	46.46	0.73	7.34%	77.95	1.15	6.93%
C3A.Cg-<i>Pde6b</i>⁺<i>Prph2</i>^{Rd2}/J												
Automated: $n = 11$												
Manual: $n = 5$												
Total	159.40	1.70	2.39%	176.80	1.37	2.57%	185.50	1.71	3.06%	195.50	2.15	3.65%
RNFL	19.45	0.83	9.59%	8.77	1.06	40.18%	27.17	0.53	6.45%	28.05	0.55	6.51%
GCL+IPL	57.50	1.42	5.53%	24.66	0.27	3.61%	45.32	2.20	16.07%	35.82	3.05	28.24%
INL+OPL	41.95	0.60	3.22%	39.93	1.08	8.93%	36.08	1.49	13.73%	21.14	2.84	44.47%
ONL	22.75	0.91	8.93%	39.86	1.05	8.74%	39.07	3.03	25.68%	63.89	0.89	4.63%
PR	19.50	1.32	15.14%	65.05	0.45	2.32%	17.27	0.92	17.64%	46.62	1.13	8.06%

Mean layer thickness ($\mu\text{m} \pm \text{SEM}$) and CV of retinal layers in C57Bl/6, BALBc, and C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J mice using manual segmentation, and the segmentation software from Heidelberg Engineering, ARTORG Center, and IOWA.

Performance of Automated Segmentation for Total Thickness Measurement in C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J Mice

Optical coherence tomography segmentation of the total retinal thickness in C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J mice showed consistent values for each segmentation modality with little variance within the groups ($\text{CV} < 4\%$, Fig. 5B). As in C57Bl/6 and BALBc mice, in the Heidelberg and ARTORG software, the BM layer was automatically segmented within the choroid and, therefore, was thicker than in the manual segmentation. The differences are summarized in Table 3.

Automated Segmentation Versus Manual Segmentation for Individual Layers in C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J Mice

The IOWA software showed adequate segmentation of all retinal layers with consistent thickness values within all OCTs analyzed (Figs. 5A, 5C). In the ARTORG program, the RNFL thickness was comparable to manual segmentation and the ELM was segmented correctly. However, the IPL–INL and the OPL–ONL boundaries were not adequate in the C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J mice due to a missing PR layer. The automated Heidelberg segmentation consistently defined the OPL as the ELM and conse-

quently placed the RNFL–GCL/IPL, IPL–INL, INL–OPL, and OPL–ONL boundary above the ELM (Fig. 5A). Therefore, none of the individually segmented layers from the automated Heidelberg segmentation was applicable for further analysis.

Repeatability of OCT Measurement and Segmentation Between Two Different Acquisition Dates

The comparison of total retinal thickness between segmentation of OCTs from two independent acquisition dates at least 1 month apart, revealed ICCs between 0.96 and 0.99. The lowest ICC was measured in C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J mice, most likely explained by the progressive retinal degeneration resulting in thinner thickness values (Supplementary Fig. 1). The mean ICC for thickness of individual retinal layers lay between 0.6 and 0.98 depending on the retinal layer and segmentation program.

Total Retinal Thickness Analysis of C57Bl/6, BALB/c, and C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J Mice Over Time Using Automated Segmentation of the Heidelberg Explorer

Total retinal thickness analysis of all three mouse strains over 6 months revealed constant thickness values for C57Bl/6 and BALBc mice (249 ± 1.3 and 243 ± 1.6 μm , respectively [mean $\pm$ SEM]). In C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J mice, at the first measurement at the mouse age of 1 month, thinner values were measured (208 ± 7 μm) compared to wild type strains ($P < 0.0001$). Over the course of 7 months the total retinal thickness decreased by 19% to 169 ± 7.2 μm (Supplementary Fig. 2).

Discussion

Until recently, quantification of individual retinal layers has largely relied on histology, which remains the gold standard for calculating the layer thickness and the number of individual cells within these layers. However, histology often is afflicted with visible artifacts, caused by postmortem or fixation artifacts that may lead to separation of the sensory retina from the RPE and tissue distortion. Furthermore, tangential section planes in histology may distort retinal layer quantification. With the recent introduction of high resolution SD-OCT and the adaptation of this technique for basic sciences, visualization of distinct retinal layers has become feasible in vivo and has been

shown to correlate well with histology.²³ This has numerous advantages; foremost, quantitative assessment of retinal layers over time facilitates longitudinal assessment of pathological processes within the same animal and may become the standard for the assessment of effects of novel therapeutic substances.

Whereas total retinal thickness measurements in the mouse retina is an established method,^{24–26} segmentation of individual retinal layers has only just emerged as a new tool in basic retinal research. Some graph-based methods for segmentation of retinal layers of SD-OCT using the Bioptigen (Bioptigen, Inc., Morrisville, NC, USA) scanner designed for OCT imaging in mice and rats have been published.^{22,27} Previous studies, including those of Antony BJ et al.²² and Pennesi ME et al.²⁸ have applied retinal layer segmentation in diabetic mice and in mouse models of retinal degeneration, respectively, using the Bioptigen OCT system. They reported good accuracy and reproducibility of their OCT segmentation algorithms suited to observe changes of retinal thickness over time.^{22,28} However, as major efforts are made to improve segmentation in OCT devices for human use the same equipment frequently is used for imaging mice in eye research.^{12,29} In the end, such commercially available SD-OCT may allow for in vivo structural data on mouse models of retinal disease. Furthermore, researchers must be aware of limitations using different segmentation algorithms and how they compare among each other.

We have shown that quantification of retinal layers in two commonly used mouse strains, C57Bl/6 and BALBc, can be performed using fully automated segmentation software intended for use in humans. Due to segmentation errors, there are limitations in the use of these in mice. An important finding is that the delineation of the retinal pigment epithelium was moved into the sclera, yielding consistently higher total retinal thickness values in both mouse strains in all three segmentation algorithms (Fig. 4). Possible sources of defective RPE/BM segmentation might be differences in the OCT reflection profile between the human and murine retina and/or anticipation of a minimal retinal thickness values defined for human retinas. Furthermore, the absence of pigmentation in the RPE of BALBc mice, may facilitate signal penetration beyond the RPE, thereby enhancing the visibility of the choroidea. As the RPE/BM boundary was automatically segmented within the choroid, this may have contributed to thicker values of total retinal thickness in BALBc mice. However, as these values are very consistent, automatically computed total

Figure 4. (A) Comparison of total retinal thickness of C57Bl/6 and BALBc mice with four different segmentation modalities: manual segmentation (*m*), Heidelberg Engineering (*H*), IOWA (*I*), and ARTORG (*A*) (C57Bl/6, black symbols, violet bars; BALBc, white symbols, blue bars). Correction of total retinal thickness values can partly be achieved by subtraction of the RPE thickness values (gray symbols). (B) Histology and OCT images of C57Bl/6 and BALBc mouse retina. Automated segmentation algorithms define the choroid-sclera boundary as RPE, resulting in higher total retinal thickness values (abbreviations see Table 1).

thickness measurements can be used to assess changes in relative thickness over time within the layer of interest. If accurate measurements of retinal layers or their thickness proportions are of interest, the automatically calculated thickness values can either

be corrected by manually adjusting the delineation for the retinal pigment epithelium or by subtracting a correction factor for total retinal thickness measurements, as described in Table 3. These findings also implicate that thickness measurements of the photo-

Table 3. Correction Factors for Automated Segmentation of Total Retinal Thickness in Mouse OCT

	C57Bl/6	BALBc	C3A.Cg-Pde6b +Prph2 ^{Rd2/J}
Heidelberg	−27	−52	−32
ARTORG	−37	−41	−36
IOWA	−36	−37	−29

Total retinal thickness values of automated segmentation can be corrected using following values (μm).

receptor layer yield thicker values than manually segmented data.

The thickness values for the inner retinal layers are very consistent within the individual mouse strains and between the segmentation programs from Heidelberg Engineering, IOWA, and the manual segmentation. Furthermore, they are largely similar between the BALBc and C57Bl/6 strains. Our experimental results demonstrated that automated segmentation algorithms are able to achieve high precision with low intraclass variability in the normal mouse retina when compared to manual segmentation. Whereas the Heidelberg Engineering software and the IOWA

Figure 5. The OCT segmentation in C3A.Cg-Pde6b⁺Prph2^{Rd2/J} mice. (A) Histology and OCT of the retina of C3A.Cg-Pde6b⁺Prph2^{Rd2/J} mice at the age of 6 months (upper row) with different segmentation modalities (lower row). The photoreceptor layer is not identifiable in the histology and corresponding OCT images. (B) Comparison of total retinal thickness between the four different segmentation algorithms ($n = 11$). (C) Comparison of individual retinal layers between the manual segmentation and the IOWA segmentation (●/green = manual, $n = 5$; ▼/blue = IOWA, $n = 11$; abbreviations see Table 1).

public accessible segmentation algorithm can delineate 10 retinal layers, our custom built software segments only five layers.

When analyzing the data in the C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J mouse, a mouse model of retinitis pigmentosa, we found an increase in segmentation errors with higher intraclass variability than the one observed in wild type mice. The most adequate segmentation results were obtained using the IOWA segmentation algorithm. The C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J carries a retinal degeneration slow (rds 2) mutation (*Prph2*^{Rd2}) and is characterized by slow degeneration of the ONL of the retina beginning at 5 weeks, loss of retinal rod and cone cells by 10 months, and loss of all retinal structures by 12 months of age. The main limitations of automated segmentation are caused by absence of individual layers, such as the OPL in C3A.Cg-*Pde6b*⁺*Prph2*^{Rd2}/J and subsequent erroneous delineation of neighboring layer boundaries. This is not surprising, as retinal layer delineation procedures have not yet reached a satisfactory level of performance in human macular disease. Therefore, segmentation algorithms themselves need to be further refined. Most automated segmentation algorithms for retinal layers identify the ILM and RPE/BM boundaries in the first step, as they are the two most prominent layer boundaries with the highest contrast in OCT images. Afterwards the residual retinal boundaries are used. This leads to inherent artifacts in retinal diseases, as retinal layers display intraretinal fluid or thinning and, thus, interfere with demarcation of the layer boundaries.

The analysis of repeated measurements within the same animals revealed very high intraclass correlations for total retinal thickness values. This indicates high reproducibility of data analyzed by automatic segmentation. The ICC for individual layers decreased, probably due to segmentation errors of individual layers.

Our findings have several implications. On the one hand it is important to know that especially the inner retinal layers are well delineated using the Heidelberg Engineering and IOWA software for both wild type mouse strains analyzed. This is particularly relevant for experimental brain research as this may ultimately allow to monitor RNFL in mouse models of multiple sclerosis, such as experimental autoimmune encephalomyelitis (EAE)³⁰ or Theiler's Murine Encephalitis Virus-Induced demyelinating disease.³¹ However, performance of automated segmentation of inner retinal layer pathologies remains to be evaluated. On the other hand, we were able to show that reliable

noninvasive quantification of retinal layers can be obtained using a clinical OCT system and commercially available OCT segmentation software. Low variability within individual segmentation algorithms allow for longitudinal measurement of retinal layer thickness. Thereby, it will facilitate research of various retinal disease models in small animals and should help to monitor putative therapeutic effects of novel interventional strategies.

Conclusion

The results presented in this study demonstrated that the image quality of murine retinal OCT images is sufficient to obtain reliable retinal layer segmentation. Comparing three automated segmentation algorithms, we found comparable volume data of the inner retinal layers in wild type mouse strains and a mouse model of retinal degeneration. However, the outer retinal layers were more difficult to segment due to errors in the localization of the basement membrane of the RPE, but are highly consistent and, thus, do not interfere with longitudinal analysis of murine retinal layers.

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