DEPTH-RESOLVED MULTI-CONTRAST RETINAL IMAGING
WITH HIGH AXIAL RESOLUTION

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

submitted by

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People are the sum of their experiences, and I want you to know that having you in my life has influenced the words in this thesis. No matter who you are, if we had never met, it would have been different somehow.

This one’s for you.
DECLARATION

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Retinal histological analysis was led by Martin Glösmann at the University of Veterinary Medicine, Vienna, with assistance from Tanja Himmel and Stefan Kummer. Cerebral histology was performed at the Institute of Neurology, General Hospital and Medical University of Vienna, by Johanna Gesperger, Martina Muck and Antonia Lichtenegger under the supervision of Adelheid Woehrer.

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Melanin phantoms were made at the Institute of Chemical Technologies and Analytics, TU Wien, by Danielle J. Harper, using melanin particles which were fabricated by Thomas Konegger.

The 840-nm optical coherence tomography system used in the experiment in Section 2.3 was originally developed by Stanislava Fialová and alterations were made by Conrad W. Merkle. The post-processing software for this system was developed by Marco Augustin.

The refocusing telescope within the white light optical coherence tomography system was designed by Carlos Reyes as part of his Masters thesis project.

In vivo imaging experiments were conducted and led by Danielle J. Harper, Marco Augustin and Bernhard Baumann, with assistance from Kornelia Schutzenberger, Pablo Eugui, Conrad W. Merkle and Antonia Lichtenegger. All animal experiments were performed at the Division of Biomedical Research, Medical University of Vi-
enna in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Directive 2010/63/EU. Ethics protocols were approved by the ethics committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Research, with numbers:

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The work described in this thesis is original work, and any work and/or words of others has been appropriately cited or quoted. The content of this thesis provides an accurate account of the experiments that were performed, and has not been and will not be used as a whole or in parts for submitting another thesis.
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ABSTRACT

Developed in the early 1990s, optical coherence tomography (OCT) is a non-invasive optical imaging technique which is capable of providing 3D morphological information of a sample at resolutions within the micrometer range. In order to achieve such resolutions, either the central wavelength must be sufficiently low, and/or the spectral bandwidth must be sufficiently high. However, there are also ways to infer information about the sample microstructure without requiring the resolution to visualize it directly. Additional sources of contrast, such as polarization state alterations, spectroscopic characteristics and motion can also be measured using extensions of conventional OCT. With this in mind, the aim of this work was to explore both high resolution imaging, and to exploit some native sources of contrast to gain a deeper insight into both the healthy and diseased rodent retina.

A white light polarization-sensitive (PS-) OCT system was developed, tailored specifically for multi-contrast imaging of the rodent retina. A supercontinuum laser was used as the light source, coupled with spectrometers which were designed to detect backscattered light across the whole visible light range. Owing to the low central wavelength and broad spectral bandwidth, sub-micron resolution images of the mouse eye were acquired, taking advantage of the inherent reflectivity- and polarization-based contrasts present within the retinal layers. Spectroscopic analysis was also performed in the mouse retina, sacrificing spatial resolution in favor of spectral resolution. Preliminary red-green-blue (RGB) OCT images successfully identified blood vessels in the retina, and showed color contrast in the retinal pigment epithelium (RPE) - a retinal layer which is known to contain the pigment melanin. To further investigate the spectroscopic capabilities of the white light OCT system, the concept of hyperspectral OCT was introduced. Images of rat eyes were acquired with the system, and a hyperspectral stack of 27 wavelengths (440-700 nm) was created in post-processing for each depth-resolved image. The RPE of the Brown Norway rat, in particular, contains melanin granules which are of the size, shape and concentration that they scatter light in the Mie regime upon visible light illumination. After proving this concept with numerical simulations and phantom measurements, the hyperspectral OCT image data was able to detect the presence of these melanin
granules. Hyperspectral OCT therefore offers a solution for melanin identification based on single granules, removing the requirement of high melanin concentration which is relied upon for conventional melanin-imaging techniques.

Running in parallel to the development of the white light OCT system, a longitudinal study was also being conducted on the retina of an APP/PS1 mouse model of Alzheimer’s disease (AD). The role of the retina in AD is one which is currently under debate in both patient studies and also in mouse models of the disease. Using optical methods, retinal imaging provides a unique opportunity to non-invasively visualize a part of the central nervous system. A study was therefore conducted on both retinas of 24 APP/PS1 transgenic mice and 15 of their wildtype littermates. For imaging, a multi-contrast OCT system with a superluminescent diode centered at 840 nm as a light source was used. This system was capable of providing data based on standard reflectivity, polarization sensitivity and motion (in the form of OCT angiograms). This triad of contrast mechanisms provided simultaneous information on the thickness and structure of the retinal layers, the presence or absence of hyper-reflective foci, abnormalities in phase retardation properties, and the network of retinal vasculature. To compare these in vivo retinal measurements to the cortical amyloid-beta (Aβ) plaque load, an advanced histology protocol was carried out in both retinal and cerebral tissue, allowing a thorough documentation of what is, and what is not, visible in the retina of these mice.

Having performed similar measurements with both the white light system and the 840 nm system, the advantages and disadvantages of both systems are discussed, and a short outlook to where the future of high resolution OCT may be going is provided.
ZUSAMMENFASSUNG

Die in den frühen 90er Jahren entwickelte Optische Kohärenztomographie (OCT) ist eine nicht-invasive optische Bildgebungstechnik, die in der Lage ist, morphologische 3D-Informationen einer Probe mit Auflösungen im Mikrometerbereich bereitzustellen. Um solche Auflösungen zu erreichen, muss entweder die zentrale Wellenlänge ausreichend niedrig und/oder die spektrale Bandbreite ausreichend hoch sein. Es gibt aber auch Möglichkeiten, Informationen über die Probenmikrostruktur zu erhalten, ohne dass die Auflösung benötigt wird, um sie direkt zu visualisieren. Zusätzliche Kontrastquellen wie Polarisationszustandsänderungen, spektroskopische Eigenschaften und Bewegung können ebenfalls mit Erweiterungen des konventionellen OCT gemessen werden. In diesem Sinne war das Ziel dieser Arbeit, sowohl die hochauflösende Bildgebung zu erforschen, als auch einige native Kontrastquellen zu nutzen, um einen tieferen Einblick in die gesunde und kranke Nagetier-Netzhaut zu erhalten.


Nachdem ähnliche Messungen sowohl mit dem Weißlichtsystem als auch mit dem 840 nm-System durchgeführt wurden, werden die Vor- und Nachteile beider Systeme diskutiert, und es wird ein kurzer Ausblick darauf gegeben, wohin die Zukunft des hochauflösenden OCT gehen könnte.
PUBLICATIONS ARISING FROM THIS THESIS

The references below list the peer-reviewed journal publications which have both arisen from, and are directly included in, this thesis. A full list of publications and first-author international conference contributions of the author Danielle J. Harper can be found in the curriculum vitae at the end of this thesis.


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<td>Aβ</td>
<td>amyloid beta</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
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<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
<td>BM</td>
<td>Bruch’s membrane</td>
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<tr>
<td>CH</td>
<td>choroid</td>
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<tr>
<td>DOP</td>
<td>degree of polarization</td>
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<tr>
<td>DOPU</td>
<td>degree of polarization uniformity</td>
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<tr>
<td>ELM</td>
<td>external limiting membrane</td>
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<tr>
<td>FD</td>
<td>Fourier domain</td>
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<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
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<td>GCL</td>
<td>ganglion cell layer</td>
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<td>HCM</td>
<td>hyperspectral confocal microscopy</td>
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<tr>
<td>HRF</td>
<td>hyper-reflective foci</td>
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<tr>
<td>INL</td>
<td>inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>inner plexiform layer</td>
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<tr>
<td>IS</td>
<td>inner segments</td>
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<tr>
<td>LED</td>
<td>light emitting diode</td>
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<tr>
<td>MPE</td>
<td>maximum permissible exposure</td>
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<tr>
<td>NIR</td>
<td>near-infrared</td>
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<td>OCT</td>
<td>optical coherence tomography</td>
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<td>OCTA</td>
<td>optical coherence tomography angiography</td>
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<tr>
<td>ONL</td>
<td>outer nuclear layer</td>
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<td>OPL</td>
<td>outer plexiform layer</td>
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<tr>
<td>OS</td>
<td>outer segments</td>
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<tr>
<td>PCF</td>
<td>photonic crystal fiber</td>
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<td>PS</td>
<td>polarization sensitive</td>
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<tr>
<td>PS1</td>
<td>presenilin 1</td>
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<td>RGB</td>
<td>red-green-blue</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RNFL</td>
<td>retinal nerve fiber layer</td>
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<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
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<tr>
<td>SC</td>
<td>sclera</td>
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<tr>
<td>SLD</td>
<td>superluminescent diode</td>
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<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
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<td>sOCT</td>
<td>spectroscopic optical coherence tomography</td>
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<tr>
<td>TD</td>
<td>time domain</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>VLDLR</td>
<td>very-low-density-lipoprotein-receptor</td>
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To Bernhard, my supervisor, who always knew exactly what to do. It’s been an honor to work for you. Thank you for all you taught me.

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And to Mum, Lauren, John, Tara and the rest of my family, who have loved and supported me forever. I will always appreciate that.
1. INTRODUCTION

The results of this thesis include three full manuscripts, each of which contains a detailed introduction including literature references specific to the respective investigation. I therefore provide here instead a general overview, the aim being to guide the reader swiftly on a journey through the science which ultimately culminates in the work conducted for this thesis. With that in mind, I would like to take the time to acknowledge the five textbooks which have shaped not only the optics section of this introduction, but also my understanding of the field as a whole. Textbooks by Griffiths [1] and Jackson [2] served as references for fundamental knowledge of electrodynamics. Born and Wolf’s Principles of Optics [3] allowed me to apply electrodynamics to the field of classical optics, and Tuchin’s Tissue Optics [4] taught me how to see tissues as optical components themselves. Last, but certainly not least, Drexler and Fujimoto’s Optical Coherence Tomography: Technology and Applications [5] provided me with the specifics necessary to understand the field of the imaging modality upon which this thesis is based.

1.1 Light

Life as we know it can only exist because of the fundamental physical phenomenon that is light. Without the energy transfer between the sun and our beloved planet Earth, the seas would be made of ice, the concept of weather would cease to exist, and the process known as photosynthesis, essential for the creation of food and oxygen for life on Earth, would simply stop. It is therefore not surprising that natural philosophers throughout the ages have chosen to focus their attention on this topic, each building upon what came before, resulting in the knowledge we have now.

In 300 BC, Euclid proposed the idea that light travelled in straight lines, and first documented the laws of reflection. In around 1000 AD, Islamic scholar Ibn al-Haytham made, arguably, the most important scientific breakthrough of ancient times. Contrary to Euclid’s work, he proposed that the eye itself does not emit any form of light beam, and rather vision is only possible due to the reflection of the sun’s rays into the eye. However, despite being present since (literally) the beginning of
time, it was only in the 17th century that our modern views of light began to take shape. The corpuscular theory of light, more commonly known today as “light as a particle”, was first documented in 1637 by René Descartes [6], and mathematically built upon by Isaac Newton. In Newton’s *Opticks* [7], he described his experiments with prisms, lenses, glass sheets and pigments, discussing the concepts of refraction, diffraction, absorption and polarization - some of the fundamental properties upon which this thesis is based.

The other half of the story, “light as a wave”, was published in 1690 by Christiaan Huygens in his *Treatise on Light* [8]. Despite fuelling the search for the luminiferous aether, the invisible yet infinite medium through which light could apparently travel, the wave theory of light gave a more complete description of properties such as interference, and perhaps a more intuitive picture of the ideas of amplitude and phase. Today, thanks to the scientific breakthroughs of Einstein and Planck, we understand that light exists as a superposition of states, more commonly known as the theory of “wave-particle duality” - one of the building blocks of quantum mechanics.

However, the foundations upon which this thesis was built was the work of Scottish physicist James Clerk Maxwell, who worked in the pre-quantum era. Maxwell unified the ideas of electricity and magnetism, mathematically describing the ability of a changing electric field to create a changing magnetic field, and vice versa. His work resulted in a definition of light: a fusion of orthogonal waves through a single field, travelling in a third orthogonal direction.

**Maxwell’s equations**

Maxwell elegantly summarized his findings into four equations, which are stated here in differential form:

\[ \nabla \cdot \mathbf{D} = \rho \]  \hspace{1cm} (1.1)

\[ \nabla \cdot \mathbf{B} = 0 \]  \hspace{1cm} (1.2)

\[ \nabla \times \mathbf{E} = -\frac{\partial \mathbf{B}}{\partial t} \]  \hspace{1cm} (1.3)

\[ \nabla \times \mathbf{H} = \mathbf{J} + \frac{\partial \mathbf{D}}{\partial t} \]  \hspace{1cm} (1.4)
The first equation (Eq. 1.1) is known as Gauss' Law, where \( \mathbf{D} \) represents the electric flux density, \( \rho \) is the electric charge density and \( \nabla \cdot \) is the divergence operator. Neglecting relativistic effects, the electric flux density is directly proportional to the electric field, \( \mathbf{E} \), related by the permittivity of the material, \( \epsilon \), within which the measurement is taken. In summary, Gauss' Law states that a non-zero divergence in electric flux density (and therefore electric field) exists if and only if there exists an electric charge or, in other words, the divergence of the electric flux density over the surface of any volume is equal to the net charge within it. We will discuss later the importance of Gauss' Law in the definition of polarization.

The equivalent equation for the magnetic flux density, \( \mathbf{B} \) is known as Gauss' Law for magnetism (Eq. 1.2). Like Gauss' Law (Eq. 1.1), the magnetic flux density is related to the magnetic field \( \mathbf{H} \) by \( \mathbf{B} = \mu \mathbf{H} \), where \( \mu \) represents the permeability of the material, again neglecting relativistic effects. This means that replacing the magnetic flux density with the magnetic field in Eq. 1.2 also holds true, as the constant on the right hand side of Eq. 1.2 is zero. Since this equation tells us that magnetic fields flow in a closed loop, the conclusion can be drawn that unlike for electric charges, magnetic monopoles do not exist anywhere in the universe. It should be noted here that the concept of a plane light wave seems to break this law, but actually the plane wave is defined as the limit where the radius of the closed loop tends towards infinity.

Maxwell's third equation (Eq. 1.3) is also known as Faraday's Law, which can be summarized by the conclusion that a changing magnetic field around a circuit induces current, i.e., an electric field. Coupled with the fourth of Maxwell's equations, Ampere's Law (Eq. 1.4), this describes the self-propagation of electromagnetic waves as Eq. 1.4 states that a changing electric field also produces a changing magnetic field, which in turn produces an electric field (Eq. 1.3), and so the cycle continues.

**From Maxwell to the wave equation**

Assuming a wave propagating in free space in the absence of external electric or magnetic fields (an assumption we will continue to use throughout the duration of this thesis), Eq. 1.4 can be simplified to

\[
\nabla \times \mathbf{B} = \mu_0 \epsilon_0 \frac{\partial \mathbf{E}}{\partial t}
\]

(1.5)
where \( \mu_0 \) and \( \epsilon_0 \) correspond to the permeability and permittivity of free space, respectively. Taking the curl of both sides of Eq. 1.3 and substituting in Eq. 1.5
\[
\nabla \times (\nabla \times \mathbf{E}) = -\mu_0\epsilon_0 \frac{\partial \mathbf{E}}{\partial t} \tag{1.6}
\]
then substituting the “curl of curl” vector identity
\[
\nabla \times (\nabla \times \mathbf{E}) = \nabla (\nabla \cdot \mathbf{E}) - \nabla^2 \mathbf{E} \tag{1.7}
\]
results in a loss of the \( \nabla (\nabla \cdot \mathbf{E}) \) term due to Gauss’ Law (Eq. 1.1) such that
\[
\nabla^2 \mathbf{E} = \mu_0\epsilon_0 \frac{\partial^2 \mathbf{E}}{\partial t^2}. \tag{1.8}
\]
Only a small rearrangement is then required to transform this into the 3D wave equation
\[
\nabla^2 \mathbf{E} - \mu_0\epsilon_0 \frac{\partial^2 \mathbf{E}}{\partial t^2} = 0 \tag{1.9}
\]
where
\[
\frac{1}{c^2} = \mu_0\epsilon_0 \tag{1.10}
\]
defines the speed of light in a vacuum, \( c \). For this derivation, we have started with the curl of both sides of Faraday’s Law (Eq. 1.3), although similar equation manipulation of Ampere’s Law (Eq. 1.4) would result in an equivalent expression for the magnetic field:
\[
\nabla^2 \mathbf{B} - \mu_0\epsilon_0 \frac{\partial^2 \mathbf{B}}{\partial t^2} = 0. \tag{1.11}
\]
The general solutions to these equations take the form of
\[
\mathbf{E}(\mathbf{r}, t) = f(\phi(\mathbf{r}, t)) = f(\omega t - \mathbf{k} \cdot \mathbf{r})
\]
\[
\mathbf{B}(\mathbf{r}, t) = f(\phi(\mathbf{r}, t)) = f(\omega t - \mathbf{k} \cdot \mathbf{r}) \tag{1.12}
\]
where \( \omega \) is the angular frequency and \( \mathbf{k} \) corresponds to the three-dimensional wave vector. As this is a linear system, the superposition principle can be applied, such that any number of solutions of this form can be combined to form a new solution.
1.1.1 Refractive index

From here, the concept of refractive index, $n$, can be introduced. The refractive index is a dimensionless quantity which relates the speed of light in a medium, $v$, to that of the speed of light in a vacuum, i.e.,

$$n = \frac{c}{v}.$$  \hspace{1cm} (1.13)

In the case of light in a vacuum, the refractive index is equal to 1 and, as light only slows down in a medium, the speed of light decreases with increasing refractive index. This refractive index can be calculated when the material’s relative permittivity, $\varepsilon_r$, and relative permeability, $\mu_r$, are known:

$$n = \sqrt{\varepsilon_r \mu_r}.$$ \hspace{1cm} (1.14)

The wave equation for a wave travelling through a material then becomes

$$\nabla^2 \mathbf{E} - \frac{n^2}{c^2} \frac{\partial^2 \mathbf{E}}{\partial t^2} = 0.$$ \hspace{1cm} (1.15)

1.1.2 Reflection and refraction

When a light beam is incident on a boundary between two materials of different refractive indices, both transmission and reflection of the light occur. This concept will now be shown through Maxwell’s equations.

The plane wave solution to the wave equation with frequency $\omega$ can be described as having electric and magnetic fields corresponding to

$$\mathbf{E}(\mathbf{r},t) = E_0 e^{i(\mathbf{k} \cdot \mathbf{r} - \omega t)}$$  \hspace{1cm} (1.16)

$$\mathbf{B}(\mathbf{r},t) = B_0 e^{i(\mathbf{k} \cdot \mathbf{r} - \omega t)}$$ \hspace{1cm} (1.17)

The wave vector, $\mathbf{k}$, is related to to the phase velocity, $v$, by

$$v = \frac{\omega}{k},$$ \hspace{1cm} (1.18)

where $k$ is the magnitude of the wave vector, $k = |\mathbf{k}|$. Since the direction of propagation of a transverse wave is orthogonal to the electric and the magnetic fields, $\mathbf{E}_0 \cdot \mathbf{k} = \mathbf{B}_0 \cdot \mathbf{k} = 0$. In combination with Eq. 1.3, $\mathbf{E}_0$ and $\mathbf{B}_0$ are related thus:
\[ \mathbf{B}_0 = \frac{\mathbf{k} \times \mathbf{E}_0}{v}, \]  

where \( \mathbf{k} \) describes the unit vector in the direction in which the wave is travelling.

Now we consider the situation where a light beam travelling in the \( z \)-direction is incident upon a boundary, the surface of which defines the \( x - y \) plane, as shown in Fig. 1.1. This surface \( x - y \) plane intersects the \( z \)-axis at \( z = 0 \), and the incident beam approaches the boundary from the \( -z \) direction, travelling through a material with refractive index \( n_1 \). The second material, located in the \( +z \) direction, has refractive index \( n_2 \). This defines the unit wave vectors as \( \mathbf{k} = +\hat{z} \) for both the incident and transmitted waves, and \( \mathbf{k} = -\hat{z} \) for the reflected wave.

\[ \begin{align*}
E(z, t) &= E_i e^{i(k_1 z - \omega t)} \hat{x}, \\
E(z, t) &= E_r e^{i(-k_1 z - \omega t)} \hat{x}, \end{align*} \]

Due to the principle of superposition, no loss of generality occurs if we assume that the electric field contains only an \( x \)-component (i.e., the light is polarized), and therefore for simplicity we will continue with this definition, defining the incident wave as

where \( \mathbf{B}_0 \) is the magnetic field, \( \mathbf{E}_0 \) is the electric field, \( v \) is the speed of light, \( \mathbf{k} \) is the wave vector, and \( i \) is the imaginary unit.
\[ \mathbf{E}(z, t) = E_t e^{i(kz-\omega t)} \hat{x}. \] (1.22)

At the boundary, the wave is continuous and all components (incident, transmitted and reflected) are parallel to one another, and so the boundary condition \( E_{\parallel 1} = E_{\parallel 2} \) can be applied such that

\[ E_i + E_r = E_t. \] (1.23)

Following similar notation to describe \( \mathbf{E}(z, t) \) and making use of Eq. 1.19,

\[ \frac{E_i - E_r}{v_1} = \frac{E_t}{v_2}, \] (1.24)

and since \( \frac{v_1}{v_2} = \frac{n_2}{n_1} \), this equation can be solved to find the amplitudes of the reflected and transmitted rays, respectively:

\[ E_r = \left( \frac{n_1 - n_2}{n_1 + n_2} \right) E_i \] (1.25)

\[ E_t = \left( \frac{2n_1}{n_1 + n_2} \right) E_i. \] (1.26)

From these equations some conclusions can be drawn. The first is that if the refractive indices of the two materials are equal, i.e., \( n_1 = n_2 \), then the amplitude of the reflected wave falls to zero and the transmitted wave has the same amplitude as the incident wave. This makes sense, as if the two materials had the same refractive index, there would be no boundary at all. When the refractive indices are different, however, some component of reflection will always occur. This has implications for optical design, as it makes 100% transmission through an optical component, such as a lens, fundamentally impossible.

To convert these amplitudes into coefficients of reflection, \( R \), and transmission, \( T \), the ratio of the intensities of the waves must be considered rather than the amplitudes. Since the intensity, \( I \), is directly proportional to the square of the amplitude, \( E \), we can define these coefficients as

\[ R = \frac{I_r}{I_i} = \left( \frac{E_r}{E_i} \right)^2 \] (1.27)

for reflection, and
\[ T = \frac{I_t}{I_i} = \frac{v_1}{v_2} \left( \frac{E_t}{E_i} \right)^2 = \frac{n_2}{n_1} \left( \frac{E_t}{E_i} \right)^2 \]  

(1.28)

for transmission. By substituting in the relations in Eq. 1.25 and Eq. 1.26, the coefficients of reflection and transmission can be defined purely by the relationship of the refractive indices

\[ R = \left( \frac{n_1 - n_2}{n_1 + n_2} \right)^2 \quad \text{(1.29)} \]

\[ T = \frac{n_2}{n_1} \left( \frac{2n_1}{n_1 + n_2} \right)^2 \quad \text{(1.30)} \]

resulting in the intuitive equation

\[ R + T = 1. \quad \text{(1.31)} \]

If the coefficients did not sum to 1, this would indicate some energy loss at the boundary. This relationship between the reflected and transmitted beams must also hold when the incident light ray is at an angle to the normal line, as shown in Fig. 1.2.

Fig. 1.2. A pictorial representation of Snell’s law.

By following a similar derivation to that described for the case in Fig. 1.1, we arrive at Snell’s law,

\[ \frac{\sin \theta_i}{\sin \theta_t} = \frac{v_t}{v_i} = \frac{n_1}{n_2}, \quad \text{(1.32)} \]
which states the equivalence of the ratio of the sines of the incident light angle, $\theta_i$, and the transmitted light angle, $\theta_t$, to the phase velocities in the respective media ($v_i$ and $v_t$). Furthermore, this ratio can also be calculated from the reciprocal of the ratio of the refractive indices within the media. Equation 1.32 also demonstrates that the incident light angle, $\theta_i$, and the reflected light angle, $\theta_r$, are equal. As the refractive index $n_1$ does not change between the incident and reflected rays, the velocity term only changes direction, but not magnitude. In OCT, we measure the directly backscattered light from different layers within the sample, along the $z$-axis. It is therefore not necessary for this work to repeat the previous derivation including an angular component, other than for the recognition that the energy of light at a boundary is conserved.

1.1.3 Scattering

In an optical context, scattering occurs when a light beam is redirected following an interaction with matter. Matter contains atoms and molecules, which themselves contain electric charges. When a light beam is incident upon a scatterer, the field oscillations excite these charges, causing them to radiate. These radiations are the scattered waves, and a superposition of the incident wave and the scattered wave is what can then be detected. This process involves an energy transfer. In the event that the emitted wavelength is different to the incident wavelength, this is known as inelastic scattering, to which Raman scattering and fluorescence belong. However we will limit our discussions here to elastic scattering only, i.e., the incident wavelength and the scattered wavelength are equal.

Elastic scattering takes one of three forms depending on the ratio of the particle size to the wavelength of the incident light. A graphical representation of these scattering profiles and the corresponding backscattering cross-sectional areas can be found in Fig. 1.3, and a description of each regime can be found below.

Optical scattering

If the particle radius is much larger than the wavelength of the incident beam (more than a factor of around 10), we find ourselves in the optical, or geometric scattering regime. Here, forward scattering dominates, and the backscattering cross-sectional area is largely wavelength-independent. In the large-particle limit, the light scattering
is governed by standard geometrical optics (Snell’s law, etc.) and therefore complex mathematical simulations are not required to predict the behavior of the light as it interacts with the particle.

**Rayleigh scattering**

On the other end of the scale, where the particle radius is much smaller than the wavelength of the incident light beam, Rayleigh scattering dominates. In this case, the scattering is inversely proportional to $\lambda^4$. When light is incident upon a particle with a small radius, its electric field acts upon the charges within the particle, resulting in particle oscillation. These oscillations occur elastically, i.e., at the same frequency, and the radiation has no preference for either the forward or backward direction.

In nature, Rayleigh scattering is responsible for the blue color of the sky. White sunlight travels down to the Earth through air molecules, which are very small compared to the wavelength of the sunlight. As the scattering is so inversely wavelength dependent, the shorter wavelengths (i.e., blue light) scatters much more than the longer wavelengths. As such, the light which we see from the sky (scattered towards the direction of our eyes from air molecules) shows a preference for blue light.
Mie scattering

In between the limits of Rayleigh and optical scattering sits the Mie regime, where the particle radius is of a comparable size to the wavelength of the incident light. The Mie solution to Maxwell’s equations was first described by Gustav Mie in 1908 [9], and converges to optical scattering in the large particle limit, and Rayleigh scattering in the small particle limit. However in between these two extremes, an interesting phenomenon occurs where there is a strong angular dependency of the scattered intensity. A detailed mathematical derivation of the theory exists in [3] (p. 759 - 789), and therefore only a brief description is provided here.

Mie theory describes the scattering behavior of a single sphere, the center of which forms the origin of a spherical coordinate system. An incident plane electromagnetic wave is also expanded into vector spherical functions, as is the field within the particle itself. After setting up the problem, the Poynting vector (describing the magnitude and direction of energy flow of the wave, $\frac{1}{\mu_0} E \times B$) is integrated with respect to both angle and space, resulting in the determination of the coefficients of scattering, $Q_s$, and attenuation, $Q_t$. These coefficients take the form

$$Q_s = \frac{2}{x^2} \sum_{j=1}^{\infty} (2j + 1) \left( |a_j|^2 + |b_j|^2 \right)$$

(1.33)

$$Q_t = \frac{2}{x^2} \sum_{j=1}^{\infty} (2j + 1) \text{Re} \{a_j + b_j\}$$

(1.34)

where $x$ is the diffraction parameter (relationship of the particle circumference to the wavelength). $a_j$ and $b_j$ describe the Mie coefficients in the form of Riccatti-Bessel functions, giving the backscattering cross-sectional area observed in Fig. 1.3 its characteristic modulations. Since Mie theory incorporates scattering of spherical particles of any size, it has found many applications in both simulation and measurement of biological tissue [10]. Although traditional Mie theory assumes spherical particles, extensions to the theory have also been demonstrated to accurately predict light scattering from particles of other shapes [11–13].
1.1.4 Attenuation

When considering only reflection and refraction, it is sufficient to define the refractive index, $n$, as a real number. Geometric optical calculations using only the real refractive index assume lossless propagation of the electric field. However in the case where losses exist, the complex refractive index, $n$, must be considered,

$$n = n + i\kappa,$$  \hspace{1cm} (1.35)

where $\kappa$ corresponds to the extinction coefficient. Inserting this expression into the plane wave solution (Eq. 1.16) yields

$$E(z, t) = e^{-2\pi k z/\lambda} \text{Re} \left[ E_0 e^{i(kz-\omega t)} \right]$$  \hspace{1cm} (1.36)

where $\lambda$ here is the vacuum wavelength. This equation defines an exponential attenuation term characterized by an attenuation coefficient, $\mu_t$, which an electric field experiences when traversing a material,

$$\mu_t = \frac{-2\pi \kappa}{\lambda}. \hspace{1cm} (1.37)$$

Attenuation of an electric field consists of two components, scattering and absorption, with their respective coefficients $\mu_s$ and $\mu_a$ related thus:

$$\mu_t = \mu_s + \mu_a. \hspace{1cm} (1.38)$$

Consideration of the complex refractive index and its associated attenuation is necessary for the application of Mie scattering theory, and for consideration of the physical propagation of light through anything other than a vacuum.

1.1.5 Polarization

The polarization of light is a property which defines the geometric orientation of the electric field of a light wave. Most common light sources (the sun, incandescent bulbs, flames) are unpolarized, i.e., there is no preference for a single orientation, and therefore a random distribution of all polarization states can be observed. Light from such sources can be manipulated to be polarized, usually by introducing a polarizer into the path of the beam in order to only allow light of one particular polarization.
state to pass through. Returning to the wave equation (Eq. 1.9) and the principle of superposition, the vector $\mathbf{E}$ of a simple plane wave can be described by two orthogonal field components $E_x$ and $E_y$ travelling along the $z$-direction, i.e.,

$$\mathbf{E}(x, y, z, t) = E_x \hat{x} + E_y \hat{y} \quad (1.39)$$

where the field components take the form

$$E_x(z, t) = E_{\text{max},x} e^{i(kz - \omega t + \alpha)} \quad (1.40)$$
$$E_y(z, t) = E_{\text{max},y} e^{i(kz - \omega t + \gamma)}. \quad (1.41)$$

$E_{\text{max},x}$ and $E_{\text{max},y}$ are the maximum amplitudes of the electric field of the $x$- and $y$-components, respectively, and $\alpha$ and $\gamma$ correspond to the phases of each component. The relationship of $E_y$ to $E_x$ then defines the state of polarization of a light wave.

**Jones vector representation**

The Jones vector representation of polarization combines $E_x$ and $E_y$ into a single vector which describes the state of polarization:

$$\begin{pmatrix} E_x \\ E_y \end{pmatrix}. \quad (1.42)$$

encoding both amplitude and phase information, although the amplitude is often normalized to 1. If the relative phases differ by $0^\circ$ or $180^\circ$, this is known as a linear polarization state. If the amplitude is contained solely within either $E_x$ or $E_y$, this corresponds to a horizontal or vertical polarization state. Equal amplitudes with these phase differences would correspond to linear polarization at $\pm 45^\circ$, and when the amplitude is equal but the phase difference is $\pm 90^\circ$, a circular state occurs.

Jones vectors, describing the polarization state of a light wave, can be operated upon by Jones matrices, $\mathbf{J}$, which define a change of polarization state caused by an optical element such as a mirror, a retarder, or a lens.

$$\mathbf{E}_{\text{out}} = \mathbf{J} \mathbf{E}_{\text{in}} \quad (1.43)$$

Using Eq. 1.43, the electric field components of an output light beam can be calculated if the input state and the properties of the optical component are known.
Although full phase information is retained with the Jones formulation, it cannot represent partially polarized or unpolarized light.

Stokes vector representation

Stokes vectors define a polarization state of light in terms of intensities, sacrificing absolute phase information of the waves (maintaining only the relative phase difference) in favor of analysis of the degree of polarization. A Stokes vector comprises four real-valued parameters, calculated as follows:

\[
\vec{S} = \begin{pmatrix}
I \\
Q \\
U \\
V \\
\end{pmatrix} = \begin{pmatrix}
|E_x|^2 + |E_y|^2 \\
|E_x|^2 - |E_y|^2 \\
2 \text{Re}(E_xE_y^*) \\
-2 \text{Im}(E_xE_y^*) \\
\end{pmatrix}
\]

(1.44)

where \(I\) is directly proportional to the intensity of the electric field, and the remaining three parameters describe the polarization. For fully polarized light, the relation

\[
I = \sqrt{Q^2 + U^2 + V^2}
\]

(1.45)

holds true. When conducting an analysis of polarization states, it is often the case that the magnitude of the intensity is not of interest, but a degree of polarization (DOP) is. The DOP can be calculated by

\[
\text{DOP} = \frac{\sqrt{Q^2 + U^2 + V^2}}{I}
\]

(1.46)

which is equal to 1 for fully polarized light, and 0 for unpolarized light. For a visual representation of the polarization state, the Poincaré sphere can be utilized, as shown in Fig. 1.4. Each Stokes parameter is first normalized to the intensity, \(I\), and the remaining three parameters, \(Q\), \(U\) and \(V\) are assigned an axis in Cartesian coordinates. In this representation, a polarization state can be plotted on the sphere. By connecting this point to the origin, the resulting vector describes the polarization where the radius corresponds to the DOP, and the angular components describe the polarization state itself.

Similar to the Jones matrix for Jones vectors, Stokes vectors can be operated upon by Mueller matrices:
Fig. 1.4. The Poincaré sphere. Excluding the first Stokes parameter which is related to the intensity, the remaining three are assigned an axis in Cartesian coordinates. Polarization states highlighted on the axes of the sphere correspond to special cases of linear or circular polarization states, although any arbitrary elliptical state can be described by its position on the sphere.

\[ \vec{S}_{\text{out}} = M \vec{S}_{\text{in}} \quad (1.47) \]

A Mueller matrix is square matrix of order 4, and is a generalization of the Jones matrix. Any Jones matrix, \( \mathbf{J} \), can be converted into its respective Mueller matrix, \( \mathbf{M} \), by the following equation:

\[ \mathbf{M} = \mathbf{G} (\mathbf{J} \otimes \mathbf{J}^*) \mathbf{G}^{-1} \quad (1.48) \]

where \( \otimes \) is the Kronecker product, and \( \mathbf{G} \) represents the following transformation matrix:

\[ \mathbf{G} = \begin{pmatrix}
1 & 0 & 0 & 1 \\
1 & 0 & 0 & -1 \\
0 & 1 & 1 & 0 \\
0 & -i & i & 0
\end{pmatrix}. \quad (1.49) \]
However, as the absolute phase information is lost in this transformation it is not possible to convert a Mueller matrix directly into its corresponding Jones matrix as there are an infinite number of solutions.

**Polarization-altering material**

Materials which alter the polarization state of light incident upon them can be described using either Jones or Mueller matrices, depending on how they interact with the light. Non-isotropic materials such as uniaxial crystals or fibrous tissues, for example, present with differing refractive indices depending on the polarization of the incident light beam. A material with this property is known as birefringent, and is defined by the difference in refractive index, $\Delta n$, experienced by the electric field components:

$$\Delta n = n_{\text{high}} - n_{\text{low}}.$$ (1.50)

Excluding any non-linear effects such as the Kerr effect [14] and dealing with only linear birefringence, this change in refractive index induces a phase retardation between the orthogonal polarization states, $\delta$, which is directly proportional to the distance travelled through the material along the direction of the optical axis:

$$\delta = \Delta nz.$$ (1.51)

The phase retardation values in this case are predictable, indicating the presence of a defined axis orientation. When this is not the case, i.e., the phase retardation becomes random after passing through a material, that material is said to be depolarizing. Such depolarization can be attributed to many mechanisms, including scattering from non-spherical particles such as melanin granules, or by multiple scattering [15, 16].

The third property is known as diattenuation, which refers to a polarization-dependent transmittance of light through a material. While success has been had in using diattenuation measurements to highlight certain regions within the rat brain [17], it is generally accepted that the contributions of diattenuation to the polarization properties of tissues are small and can often be considered negligible [18, 19].

Birefringence and diattenuation can be described using either the Stokes or the Jones formalism, as the DOP is equal to 1. When dealing with depolarization, how-
ever, this must be tackled using the Stokes formalism, as the Jones matrices do not consider partially polarized or unpolarized light.

**Polarization-preserving material**

A material which is not birefringent, depolarizing nor diattenuating can be considered a polarization-preserving material, i.e., the polarization state of light does not change following interaction with it. Mathematically speaking, this can be described simply by either Jones or Stokes formalism; both the Jones matrix and the Mueller matrix will correspond to the identity matrix of the respective order.

1.1.6 Coherence and interference

The concept of coherence is one of the most important in optics, and is one of the fundamental properties which allows interference to occur. Coherence describes a fixed relationship of the phase of a light wave between one point and another, in either the spatial or temporal dimension. For the purposes of this work, it is very important to differentiate between spatial coherence and temporal coherence, and to discuss the physical limitations and implications for interferometry.

If a light source exhibits spatial coherence, there is a strong correlation between the light waves at different points in space, transverse to the direction of wave propagation. In other words, it tells us how regular the pattern of the wavefront appears. A light source is likely spatially coherent if it demonstrates directionality. Temporal coherence, on the other hand, occurs when there is a strong phase correlation between a light wave and the same light wave delayed by a period of time. A highly temporally coherent light source is generally monochromatic; propagating as sinusoidal oscillation of only one frequency [20].

Interference can be considered the mathematical addition of the fields of light waves, and a fixed phase relationship, i.e., spatial and temporal coherence, must be present in order for interference to occur. If two waves have no phase difference, they are said to be “in phase”, and constructive interference occurs. In this case, the amplitude of the interfered wave is equal to the sum of the amplitudes of the original waves. If the waves are out of phase by exactly 180°, destructive interference occurs, and the amplitude of the resultant wave falls to zero (assuming the amplitudes of the
two waves were equal). Any other phase difference results in a superposition of the waves such that the resultant amplitude falls between these two limiting cases.

Although both spatial and temporal coherence are required for interference to occur, the idea of partial coherence also exists. A truly monochromatic light source would be infinitely temporally coherent, a theoretical phenomenon which does not exist in practise. The distance over which a light source remains temporally coherent is defined by the length over which the fringe visibility reduces by a factor of 3 dB. This is known as coherence length, \( L_c \), and is defined as

\[
L_c = \frac{4 \ln 2 \lambda_0^2}{\pi n \Delta \lambda}
\]

(1.52)

for a Gaussian spectrum in \( k \)-space, where \( \lambda_0 \) corresponds to the center wavelength, \( \Delta \lambda \) to the spectral bandwidth, and \( n \) to the refractive index of the medium. The concept of coherence length is also important for interferometric imaging techniques.

As a note, light waves of orthogonal polarization states are incapable of interfering with one another.

1.1.7 Light sources

A broadband light-emitting diode (LED) exhibits low temporal and spatial coherence, demonstrated by its broad emission spectrum and its lack of a single propagation direction. A typical narrow-band laser source, on the other hand, is both highly temporally and spatially coherent, which can be demonstrated even with a simple laser pointer. Its high monochromaticity speaks to its high temporal coherence properties, and the fact that its output remains highly directional even over long distances (i.e., the beam still has a defined shape) demonstrates high spatial coherence [21]. However, the light sources used in this work exhibit properties of both standard LEDs and lasers, maintaining a high spatial coherence but with low temporal coherence.

Superluminescent diodes

The superluminescent diode (SLD) was first described in 1971 [22]. Like typical LEDs, SLDs are based on the recombination of electron-hole pairs over a junction between positive-type and negative-type semiconductor-based materials (p-n junction). However in the case of the SLD, the p-n junction is interrupted by an angled waveguide,
and the whole diode is encased in mirrors which allow amplification of the light. Spontaneous electron-hole recombination across the p-n junction can occur across a range of energy gaps, corresponding to the emission of many optical frequencies of light. The directional output caused by amplification across the waveguide gives rise to a high degree of spatial coherence, making them highly suitable for interferometry [23], which is not the case for an LED.

**The supercontinuum laser**

Like the SLD, the supercontinuum emits a continuous, broad-bandwidth optical spectrum, i.e., its temporal coherence is low [24]. However unlike for the SLD, the source of this spectrum originates from a highly coherent laser source. As first described in 1970 [25], the wide spectrum is achieved by sending a short, highly amplified optical pulse through a bulk medium such as glass, exploiting its nonlinearity. As this medium acts as an optical waveguide, the high spatial coherence of the pump laser is preserved [24].

A wide range of solids, liquids, gases and waveguides have been used as the nonlinear element in supercontinuum generation [26]. However in recent years, there has been an increasing interest in supercontinuum generation using photonic crystal fibers (PCFs) [26–29], a diagram of which can be found in Fig. 1.5. PCF chromatic dispersion characteristics allow strong nonlinear interactions to occur over long lengths of fiber. This fact coupled with the inherent nonlinearity of an optical fiber under high-power conditions means that broad spectra can be achieved with relatively low pulse energy when compared to that required in a bulk medium. This has simplified the process of building a supercontinuum laser from large, complex systems to something which can be pumped by common commercially-available femtosecond sources [26].

Owing to the nonlinearity, the underlying physics behind supercontinuum generation differs depending upon both the fiber and the incident light pulse. The properties of self-phase modulation, Raman scattering, soliton fission and four-wave mixing can all be responsible for the creation of continuous spectra [30]. When the pump laser emits pulses on the order of femtoseconds, for example, it is usually the self-phase modulation effect which dominates [31]. However the successful development of reproducible supercontinuum spectra requires a complex understanding of how all four of these nonlinear effects interplay with one another. It is also important to con-
Consider how each of these effects contributes to the noise of the source, particularly when considering imaging applications. Modelling the noise characteristics of a supercontinuum is not an easy task. The nonlinear interactions between the solitons are highly amplitude- and phase-dependent, and fluctuations occur from one pulse to the next as both the central wavelength and the phase vary [32]. This causes unavoidable interference between solitons which overlap in k-space; the only way in which a smooth spectrum can be obtained is by integrating over several pulses. For imaging applications, this means either increasing the repetition rate of the laser, or reducing the temporal spectral acquisition rate, both of which result in averaging over more pulses [33]. Herein lies the disadvantage of the supercontinuum laser as a light source. There lies a fundamental trade-off between acquisition speed and signal-to-noise ratio in the images, much more so than for other light sources such as SLDs.

1.1.8 Interferometry

Interferometry refers to a measurement technique built upon extracting quantitative data from the interference of light. In principle, an input beam is split into two, and one of the resulting beams is delayed with respect to the other. Upon recombination of the two beams within the coherence length, interference occurs. By measuring the
power of the interfered beam, data can be recovered regarding the delay which was introduced between the two.

When a single beamsplitter is used for both beam separation and reunification, this interferometer is known as a Michelson interferometer, made known by the famous Michelson and Morley experiment of 1881 [34]. This interferometer is the basis of all OCT performed during the course of this work, and from now on, the discussions regarding interferometry will mainly focus on this technique.

1.2 Optical coherence tomography

The predecessor to today’s ocular imaging using OCT utilized low coherence interferometry to perform length measurements of the whole eye with only a handful of measurement points, with the results detected on photographic film [35,36]. Today, optical coherence tomography [37] has developed into an imaging technique capable of providing 3D morphological information of tissue with up to micron-scale resolution [38], at A-scan line rates of tens, hundreds or even thousands of kHz [39–41].

A 3D image is reconstructed step-by-step in OCT. While some 3D image reconstruction methods scan preferentially in the en-face ($x$–$y$) plane [42–45], this discussion will focus on the construction of a 3D image from an optical A-scan. In this case, light incident upon the sample is only considered to travel along the $z$-axis, meaning that information is only acquired in the depth-dimension. To turn this into a 3D image, the laser beam must then be scanned across the $x$–$y$ plane. Most commonly, galvanometric scanners are used for this purpose, and they undertake a raster scanning pattern. One scan across the fast axis ($x$) of the raster creates a two-dimensional B-scan, and then B-scans can be acquired at different $y$-positions, building up a 3D image. An example of the construction of a 3D image of the mouse retina can be found in Fig. 1.6.

We will now discuss the method of obtaining the A-scan in the first place. In traditional Michelson interferometry, the beams are redirected to the beamsplitter by two mirrors. In OCT, one of these mirrors is replaced with a sample, defining a so-called “sample arm”. The remaining beam path still employs the use of a mirror, and is known as the reference arm. Interference then occurs between the sample and reference arm, where the data returning from the sample is unknown.
Fig. 1.6. Demonstration of how 3D images of the mouse retina are created with optical coherence tomography. Each point acquisition results in a depth profile after post-processing. Using a galvo scanner to scan the incident laser beam in x-direction generates a 2D B-scan. Scanning also in the y-direction allows 3D images to be reconstructed. Images are averaged to increase SNR.

Time domain OCT

Original implementations of OCT operated in the time domain (TD). The interfered light beam was sent to a photodiode for detection, and the reference arm mirror was longitudinally translated in order to measure the interference pattern corresponding to depth position [37]. The measured intensity in time domain OCT, $I(z)$, takes the form [3]

$$I(z) = I_r + I_s + \left(2\sqrt{I_r I_s} \cdot |\gamma(\Delta z)| \cdot \cos[2k_0\Delta z]\right). \quad (1.53)$$

$I_r$ and $I_s$ correspond to the intensities measured from the reference and sample arm beams, respectively. The remaining final term includes contributions from these two intensities, as well as the complex degree of coherence, $|\gamma(\Delta z)|$. The complex degree of coherence has a value between zero (for incoherent light) and 1 (for perfectly coherent light), and carries information about the interference envelope and optical carrier frequency for a Gaussian spectrum. The peak of the Gaussian envelope encodes the information about the depth position of the sample, its amplitude representing
the reflectivity at that depth position. The width of the coherence function defines the axial resolution of the OCT system.

While the acquisition rates in TD-OCT increased rapidly in the early years [46], another OCT image acquisition technique which did not require the slow physical translation of the reference arm mirror was being developed in parallel.

**Fourier domain OCT**

The first demonstration of Fourier domain OCT (FD-OCT) was reported in 1995 with the application of corneal thickness measurements [47]. FD-OCT involves the simultaneous measurement of all light echos present by measuring an interference spectrum on a detector array. The full A-scan is acquired in a single shot, and the detected interference occurs in the Fourier domain. An inverse Fourier transform is then required to reconstruct the depth profile; the location of a particular signal in depth is encoded into a modulation frequency over the investigated spectral range. A simplified sketch of a spectral domain OCT system can be found in Fig. 1.7.

Similarly to what has been described for intensity detection in TD-OCT (Eq. 1.53), an equivalent expression exists for the recorded interference spectrum, $S$, in FD-OCT, this time as a function of both $\Delta z$ and $k$:

$$S(k, \Delta z) = S_r(k) + S_s(k) + \left(2\sqrt{S_r(k)S_s(k)} \cdot \cos[2k\Delta z]\right)$$

where $S_r(k)$ and $S_s(k)$ correspond to the k-dependent spectral densities returning from the reference and sample arms, respectively, and the final term encodes the interference and depth information.

The move towards FD-OCT also improved the sensitivity of detection, resulting in shorter acquisition times required to gain the same image SNR [48]. For this reason, FD-OCT technology overtook TD-OCT technology very quickly [49]. FD-OCT can be further divided into two sub-categories, dependent upon the type of light source, and therefore also detection unit [5]. For a light source based on a SLD or a supercontinuum laser, as is relevant to this work, the detection unit typically takes the form of a spectrometer. Spectrometer-based detection measures all frequencies of light simultaneously. However, FD-OCT can also be performed using a photodiode as the detector. Rather than separating the spectral frequencies in space, as in a spectrometer, they are rather separated in time by the light source itself. Such a
Fig. 1.7. Simple sketch of a Fourier domain optical coherence tomography system. Light from a broadband laser source is incident upon a beamsplitter, where the light is split between a sample arm and a reference arm. In the reference arm, the light is reflected back to the beamsplitter by a mirror, and the light has travelled a distance \( z \). In the sample arm, the light travels \( z + \Delta z \), with the \( \Delta z \) changing as the light is reflected from different depth positions. The light which returns from the sample arm is interfered with the reference arm beam, leading to an encoding of \( \Delta z \) in the modulations of the spectrum. By passing this interfered beam through a diffraction grating (or a prism), the beam is chromatically dispersed for detection. Post-processing software including an inverse Fourier transform then allows the depth profile to be reconstructed.

source is known as a swept source laser, rapidly sweeping through the frequencies of its spectral bandwidth.

1.2.1 Towards high axial resolution

Unlike in many other imaging modalities, the axial and lateral resolutions remain largely decoupled in OCT. The axial resolution is directly proportional to square of the central wavelength of the light source, \( \lambda_0 \), and inversely proportional to the full width at half maximum (FWHM) of the total detected spectral bandwidth, \( \Delta \lambda \). For a Gaussian spectral profile, the axial resolution is equal to half of the coherence length defined in Eq. 1.52, while the lateral resolution, \( \Delta x \), is
\[ \Delta x = \frac{4\lambda_0}{\pi} \left( \frac{f}{d} \right) \]  

where \( f \) is the focal length and \( d \) is the diameter of the final objective lens in the system. This definition of lateral resolution also relies on a spatially Gaussian laser beam profile.

In order to achieve as high an axial resolution as possible, the central wavelength should be reduced while the spectral bandwidth is increased [50]. A reduction in the central wavelength also improves the lateral resolution, but another way to increase the lateral resolution is to use an objective lens with a higher numerical aperture, i.e., it can accept light from a wider range of angles. This can be realized physically by using a lens with a shorter focal length for the same diameter. Unfortunately for retinal imaging, the objective lens in the system is the eye itself, and therefore further improvements in lateral resolution are not possible with current measurement techniques.

### 1.2.2 Additional sources of contrast

Traditional OCT utilizes the intrinsic contrast based on backscattered light intensity in order to generate images [5,37]. However it is also possible to generate images based on other sources of contrast such as polarization properties, motion or spectroscopy.

**Polarization sensitive OCT**

First introduced in 1992 by Hee et al. [51], polarization sensitive (PS)-OCT can be used to infer properties of the tissue under investigation based on an analysis of polarization state of the light beam. In a commonly used spectral domain PS-OCT configuration, a basic diagram of which can be found in Fig. 1.8 [52], light from a partially coherent light source is polarized by a linear polarizer before passing through a beamsplitter. In the sample arm, the beam passes through a quarter-wave plate at 45° to the polarizer orientation, illuminating the sample with circularly polarized light. Following a second traversal of the quarter-wave plate at 45°, a beam which has not had its polarization state altered by the sample will return to a linear state, otherwise it will have an elliptical state. In the meantime, light returning from the reference arm has travelled through a quarter-wave plate at 22.5° twice, creating a
linear polarization state where horizontal and vertical polarization state intensities are equal. Before detection, the interfered signal is split by a polarizing beam splitter into the horizontal and vertical components, and these polarization states are detected separately but simultaneously on two identical spectrometers.

![Diagram of a polarization-sensitive spectral domain optical coherence tomography system](image)

Fig. 1.8. A simplified diagram of a polarization-sensitive spectral domain optical coherence tomography system [52] based on the method of Hee et al. [51].

Light sources for OCT are usually polarized, with the exception of supercontinuum lasers [53]. However as polarization states in the reference and sample arms must be matched in order for interference to occur, the incident light beam to a supercontinuum-based OCT system also traverses a polarizer. Without PS detection, any orthogonal field components to this incident polarization state will be lost, which will occur if the sample is not polarization-preserving. In PS-OCT, this data is not lost, but detected by the second spectrometer. The full backscattered intensity, $I$, can therefore be recovered, regardless of the depolarization/birefringence of the sample. This intensity information, known as reflectivity, is directly proportional to the sum of the squares of the amplitudes, $E$, detected on each camera:

$$I(z) \propto E_x^2(z) + E_y^2(z).$$  (1.56)
Again using this amplitude information, the phase retardation between the electric field components, \( \delta \), can also be recovered in PS-OCT [51, 52]:

\[
\delta = \arctan \left( \frac{E_y}{E_x} \right),
\]

When analyzing the phase retardation data, one of three cases is possible:

- The phase retardation values are largely uniform. In this case it can be assumed that the sample is polarization-preserving within the region of interest.

- The phase retardation values scale linearly with increasing z-position (although due to the ambiguity of the arctangent, and therefore a “banded” appearance may occur). This indicates that the sample is birefringent.

- A random assortment of phase retardation values are observed. In this case, the sample is said to be depolarizing.

If the sample is birefringent, it makes sense to calculate the fast axis orientation to gain direct directional structural information of the tissue. This makes use of the phase difference, \( \Delta \phi \) between the two channels after Fourier transform [54]:

\[
\theta = \frac{\pi - \Delta \phi}{2}
\]

To test for depolarization, it would be of interest to calculate the DOP values for each pixel. However due to the coherence of the light used in OCT, the DOP will always equal 1. The more depolarizing the sample is, the more the Stokes vectors will tend towards being random between one speckle and the next. This is a fact which can be used to extract information regarding the depolarization of the sample, calculating a metric known as degree of polarization uniformity (DOPU) [55]. DOPU calculation finds the normalized length of the average Stokes vector within a sliding window containing several speckles:

\[
DOPU = \sqrt{Q^2 + U^2 + V^2}
\]

Similar to the DOP, depolarizing tissue will have a DOPU value close to zero, while polarization-preserving (rather than “polarized” as in DOP) tissue will have values close to one. A typical example of a reflectivity, phase retardation, and DOPU image of the optic nerve head region of the mouse retina can be found in Fig. 1.9.
Fig. 1.9. Examples of PS-OCT B-scans in the region of the optic nerve head (ONH) of the mouse retina. While the reflectivity B-scan gives morphological information of the layer structure, the phase retardation image highlights regions where the polarization state has been altered by the sample. The low DOPU values in the RPE, choroid and ONH regions show the depolarization caused by melanin granules.

**OCT Angiography**

First attempts to visualize and quantify motion-based contrast in OCT utilized Doppler OCT [56, 57]. By performing differential phase analysis between successive A-scans (or B-scans), images can be created which highlight the moving red blood cells against a background of the static surrounding tissue. While Doppler OCT has the advantage that it can perform quantitative blood flow measurements, a major drawback lies within the fact that measurements can only be performed along the axis of the probing beam (i.e., the z-direction), and therefore Doppler OCT is insensitive to motion which occurs at 90° to the incidence angle. The more recent OCT angiography (OCTA) methods [58] remove this restriction. Since OCT images are acquired in a pixel-by-pixel manner, speckle patterns which vary as a function of time manifest themselves in the OCT images as changing spatial speckle frequencies between repeated B-scans. Speckle variance images can then be created in post-processing, highlighting the locations of motion-based contrast using only the intensity (or amplitude) information, and not the phase. However since the phase information is provided “for free” in FD-OCT, phase-variance-based OCTA [59, 60] and complex OCTA [61, 62] are also viable options, providing that the phase of the light source is stable enough, and that bulk sample motion is either negligible or has been previously corrected for [63].
An example of complex OCTA images of two layers within the mouse retina, compared to the regular reflectivity images, can be found in Fig. 1.10. The angiogram highlights the blood vessels which contain moving blood cells.

Fig. 1.10. Comparison of intensity-based contrast (left) and motion-based contrast (right) OCT images. The blue and orange arrows in the B-scan image indicate the position of the en-face superficial and deep vascular plexuses, respectively. The green line in the superficial vascular plexus en face images shows the location of the respective B-scan. Scale bar = 100 µm and applies in all images.
Spectroscopic OCT

As the bandwidth of light sources used for OCT has become broader, it has become possible to use post-processing-based software methods to split the detected spectrum up into discrete spectral bands, sacrificing axial resolution in favor of spectral contrast. There are several chromophores in the human body which have a wavelength-dependent backscattering coefficient in the spectral range covered by OCT sources. Spectroscopic OCT (sOCT) has already been used to visualize and/or quantify chromophores such as melanin [64] and bilirubin [65], but the most frequently used application for sOCT today is retinal oximetry [66–70]. Situated in the middle of the visible light range, wavelengths corresponding to the absorption peaks of oxy- and deoxy-hemoglobin can be spectrally filtered and compared to models of light attenuation, resulting in a quantitative measurement of blood oxygen saturation.

As the spectral bandwidths for OCT become broader still, either the axial resolution of the spectroscopic contrast or the number of spectral sampling points can be increased. The current problem with in vivo spectroscopic OCT, though, is the lack of a ground truth comparison. The wavelength dependence on features including the sensitivity roll-off, absorption (particularly through the anterior eye when considering retinal imaging) and backscattered intensity itself make it difficult to reliably quantify the spectroscopic properties of the tissue in question. To help to answer these questions, calibrations and comparisons may be performed with another imaging modality.

1.3 Hyperspectral imaging

Hyperspectral imaging in its simplest form can be realised by scanning a traditional spectrometer over 2D space, developing a spectral profile for each pixel in an image. Originally developed for remote sensing applications [71], hyperspectral imaging is quickly gaining traction in the medical field, having shown positive results for surgical guidance and non-invasive disease diagnosis amongst other applications [72]. However, like many other spectroscopic techniques, traditional hyperspectral imaging lacks depth information.
1.3.1 Hyperspectral confocal microscopy

The hyperspectral confocal microscope was first introduced in 2006 [73]. Confocal microscopy makes use of a high numerical aperture combined with a pinhole to localize the region of the investigated sample in the depth dimension ($z$). Light from a source, often a supercontinuum laser, is tightly focused onto a single spot within the sample, and by scanning the beam laterally a 2D image is reconstructed. By scanning the sample also in the z-dimension, a 3D image can also be generated. The hyperspectral aspect introduces a fourth dimension to confocal microscopy: wavelength. The 3D scans are acquired repeatedly, each time with a different wavelength for the incident light beam, forming a 4D hypercube. Each pixel can then be defined in terms of its lateral position ($x$ and $y$), its depth position ($z$), and its wavelength ($\lambda$). A big advantage of hyperspectral confocal microscopy (HCM) is the ease of calibration. With visible light HCM systems which are commercially available today, it is possible to select a desired wavelength range from 470 - 670 nm, and to choose in how many spectral bins to acquire the hyperspectral image stack. The desired acquisition protocol can then be used to acquire hyperspectral images of color filters before applying the same protocol to the actual sample of interest.

Unfortunately, HCM images are slow to acquire (on the order of hours for a $512 \times 512 \times 100$ cubic pixels over 21 wavelengths) making this technique largely unsuitable for in vivo imaging. However, it is possible to perform HCM on ex vivo samples, a step which could be hugely important for ex vivo validation of in vivo spectroscopic measurements, including sOCT.

1.4 The retina

On a macroscopic level, the eye consists of the cornea and lens to focus light, and the retina to detect it. As a natural optical system itself, it is not surprising that OCT has found its most successful application in the field of ophthalmology [74], making use of the natural focusing properties of the eye to act as the final objective lens of the system.

The human retina is a thin, laminar structure which coats the inner posterior surface of the eye cup until its termination at the serrated *ora serrata*. The retina consists of epithelial, neural and glial cells, oriented in such a fashion that allows us to conveniently divide the retina into 10 layers, which are clearly visible with OCT due
to differences in reflectivity from one layer to another. Perhaps counterintuatively, the direction of neural activation lies in the opposite direction to the direction of the incoming light. Thus, the visual pathway begins with the rods and cones in the posterior retina (see Fig. 1.11).

Fig. 1.11. Connections of neurons in the retina. The direction of neuronal activation is in the opposite direction to that of the incident light.

Synapses then occur between a series of four neurons, three of which lie within the retina (rods/cones, bipolar cells and ganglion cells). The axons of the ganglion cells then combine to form the fibers of the optic nerve, cross at the optic chiasma, and synapse with the final neurons of the visual pathway in the lateral geniculate body of the diencephalon. The axons of this final neuron then transfer the information to the visual cortex. Directly connected to the brain, the retina is the only part of the central nervous system which can be directly visualized non-invasively.
1.4.1 Human vs. rodent

The rodent eye and the human eye share many common features, and therefore the rodent eye is often used as a model of the human during retinal studies. A macroscopic comparison of the human and rodent (mouse/rat) eye can be found in Fig. 1.12. Compared to the human eye, the mouse and rat eye are very similar, and therefore the mouse eye will be considered here.

![Macroscopic comparison of human and rodent eyes](image)

Fig. 1.12. A macroscopic comparison between the human eye (left) and the rodent eye (right). The rodent eye is smaller and does not contain a fovea.

Apart from the obvious discrepancy in size, the biggest difference between the human and the mouse eye is that the latter does not contain a foveal pit or a macula, i.e., no center of sharp vision. The mouse eye cannot accommodate either. When viewing the structure of the eye macroscopically, the mouse lens takes up a much greater percentage of the total eye volume than in the case of the human. As the cornea/lens focuses the light onto the retina in a much shorter distance in the mouse, the numerical aperture is greater for a fully dilated pupil (0.49 in the mouse [75] vs. 0.23 in the human [76]). The transmission of light through the eye is intuitive, because the same optical properties of the eye which allow us to perform imaging are those which allow us to see in the first place. Therefore, the higher numerical aperture in the mouse eye should allow a higher lateral resolution when imaging, not taking aberrations into account [77].

Although there are differences between the human eye and the mouse eye, there are many similarities too. Even considering the large difference between the size of the eyes, the same is not true for the retina. Retinal thickness between humans and mice are relatively similar, falling in the low hundreds-of-microns region [78, 79].
retinal layer structure is also very similar between the two. A vertical histological slice of the mouse retina can be seen in Fig. 1.13, where the retinal layers are labelled. These layers occur in the same order in the human retina, making OCT images of the two comparable.

Fig. 1.13. Vertical histological slice of the mouse retina in the region of the optic nerve head. The layer structure is similar between mice and humans. **RNFL/GCL** Retinal nerve fiber layer/ganglion cell layer. **IPL** Inner plexiform layer. **INL** Inner nuclear layer. **OPL** Outer plexiform layer. **ONL** Outer nuclear layer. **ELM** External limiting membrane. **IS** Inner photoreceptor segments. **OS** Outer photoreceptor segments. **RPE** Retinal pigment epithelium. **BM** Bruch’s membrane. **CH** Choroid. **SC** Sclera.

In general, the mouse is often used as a model of the human as an average of 85% of the mouse protein-encoding genome overlaps with that of the human [80]. Additionally, their small size makes them cheap and easy to breed, and their comparably short lifespan allows a lifetime of observation in a realistic time frame. This is particularly true when considering mouse models of human diseases, where the disease pathology can be reproduced and monitored.

### 1.5 Alzheimer’s disease

As the most common form of dementia worldwide, Alzheimer’s disease (AD) affected 4.7 million people over the age of 65 in the United States alone in 2010. By 2025, this number is expected to reach 7.1 million and then to approximately double again in the following 25 years, leaving 13.8 million people over the age of 65 in the US suffering
from the disease. In the absence of a development or medical breakthrough capable of preventing or curing AD, this figure will only continue to increase [81]. A key hurdle in the development of treatment is the lack of definitive diagnosis. A time delay of up to 20 years between the start of the disease and the presentation of symptoms means that the disease is already at an advanced stage before the patient may first consult their physician [82]. Patients suffering with AD typically present with symptoms of cognitive impairment. This is caused by the formation of amyloid beta (Aβ) proteins, and intracellular neurofibrillary tangles caused by hyperphosphorylation of tau proteins [83]. These processes then lead to a degeneration of neurons in the brain resulting in memory loss and lack of motor control, amongst other symptoms. Current diagnostic tests, including the mini-mental state examination score, can only diagnose dementia as a symptom but not AD as its cause. To rule out other causes of the symptoms, clinicians may also perform laboratory analyses such as blood tests and urine tests. Current areas of research into early AD diagnosis have included imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). Although there have been promising studies with all three [84–87], it remains true that in standard clinical practice AD can only be confirmed by pathological autopsy of brain tissue. This has been the case since the 1907 documentation of tau tangles by Alois Alzheimer, describing for the first time the disease which would go on to bear his name [88].

Today, post mortem diagnosis of AD is realized by the positive, simultaneous histological identification of Aβ plaques and tau tangles in the brain [89, 90]. Both the plaques and tangles are small (only up to tens of microns in diameter [91, 92]), and are largely invisible even when observed under high magnification [93]. For this reason, routine histopathological procedures typically employ two out of a number of recognised staining techniques: one for Aβ and another for tau. Such stainings are robust, and provide a clear, unambiguous post mortem AD diagnosis. However a huge hurdle in the development of a treatment of AD lies in the fact that the disease is poorly understood. Guided largely by post mortem studies of late stage AD brains, it has not yet been possible to document with certainty the development of Aβ plaques or tau tangles, as longitudinal histopathology studies are not possible. There is therefore a great need to be able to monitor the plaque/tangle formation as a function of time - a task which proposes clear logistical difficulties in the brain.
1.5.1 The eye as a “window to the brain”

The retina is also a part of the central nervous system; the possibility that it could also be affected by AD has been the subject of many recent studies. However which changes may occur is currently a highly debated topic in the field. An elegant summary of the current status of research into ocular biomarkers in AD was recently published by Ong et al. [94]. This analysis concludes the following:

- The presence of Aβ plaques and neurofibrillary tau tangles in the human retina has not yet been definitively proven.
- Structural changes (including retinal layer thinning and general degeneration) do occur in AD patients, but it is difficult to distinguish these changes from those observed in, for example, glaucoma.
- In vivo retinal measurements must be correlated to findings in the brain and the cerebrospinal fluid, for patients at all stages of AD.

As an optically transparent material, it would be of particular interest to be able to diagnose AD through the eye. By using OCT, it has been long proven that it is possible to clearly distinguish retinal features noninvasively and with high resolution [95–98]. However as the retinal plaques are small (only a few µm in diameter [99]), in order to be able to visualize something on this scale, a high resolution would be required. In addition to the high resolution, another promising technique for the identification of retinal Aβ plaques is the extension of PS-OCT. Since birefringent cerebral Aβ plaques have been shown to be visible with PS-OCT [100], it is possible that retinal plaques could be distinguished from the retinal layer structure by the use of this functional extension too. However the question still remains if these plaques are indeed present in the retina at all.

1.5.2 Mouse models of AD

Mouse models of AD often belong to one of three groups [101]. The first is based on a transgenic expression of a mutation of human amyloid precursor protein (APP), known to be a cause of familial AD [102]. APP mouse models generally develop Aβ plaques in the brain and develop cognitive impairment as they age. As a result, many AD mouse models are built upon this base. In order to form Aβ, APP must be
cleaved by two enzymes: beta secretase and gamma secretase. The sub-component of gamma secretase responsible for the APP cleaving is known as presenilin, the addition of which can accelerate the Aβ production process in transgenic mice. The most common presenilin mutation used for this purpose is presenilin 1 (PS1), leading to the doubly-transgenic APP/PS1 mouse model. It is worth noting here that mice with a single PS1 mutation do exist, but as they do not increase the expression of Aβ40 with respect to Aβ42, they do not develop cognitive deficits or model typical AD pathology [104]. This lack of phenotype is likely due to the fact that mouse APP and human APP differ by 3 amino acids within the Aβ region alone (and 17 overall) [105], and therefore the human PS1 cannot cleave mouse APP.

The third type of mouse model is that which emulates the role of human tau protein. While the presence of neurofibrillary tangles in the brain is a key pathology of AD, tau mutations alone do not cause the disease. In fact, mouse models to date have been unable to replicate the complex relationship between extracellular Aβ and tau tangles. In humans, tau pathology appears later than that of Aβ, while in the mouse model both are overexpressed simultaneously [101, 106].

The APP/PS1 mouse

In this work, we have focused on analysis of a particular APP/PS1 mouse model (APPswe, PSEN1dE9 MMRRC stock number 34829-JAX) [107–109]. The APP/PS1 mouse replicates human Aβ pathology in the brain [110, 111]. An example of an APP/PS1 mouse brain which has undergone immunohistological staining against Aβ can be found in Fig. 1.14, alongside a wildtype littermate control. Small brown plaques are visible in the Alzheimer’s disease-affected mouse brain, but not in the control. Similarly to what has been reported in the human, changes observed in the retina of this mouse model so far are disputed and inconclusive [99, 112–114]. Potential changes include, but are not limited to, retinal layer thickness changes, Aβ plaque formation and vascular changes. A thorough investigation of this mouse model with high resolution, multi-contrast OCT hopefully serves to answer some of these open questions, and therefore forms part of this work.
Fig. 1.14. Histological slices of the APP/PS1 mouse brain and a corresponding wildtype littermate, after immunohistochemical staining against amyloid-beta (Aβ). Stained Aβ plaques appear as brown structures in the Alzheimer’s disease (AD)-affected mouse brain only.

1.6 Aims of this thesis

The aims of this thesis can be broken down into three discrete points:

1. Design and construct a high resolution PS-OCT system based on a white light supercontinuum laser source, tailored for the imaging of the mouse retina.

2. Investigate the spectroscopic properties of melanin granules in the rodent retina in the visible light range.

3. Conduct a full characterization of the retina of an APP/PS1 mouse model of Alzheimer’s disease using high resolution OCT and additional sources of contrast, including PS-OCT and OCTA.
2. WHITE LIGHT POLARIZATION SENSITIVE OPTICAL COHERENCE TOMOGRAPHY

With the goal of increasing the axial resolution of OCT, a PS-OCT system was constructed with a lower central wavelength and larger spectral bandwidth than that which is typical for OCT systems. The light source for this system was a supercontinuum laser, offering partially coherent light all the way down to the blue light range: a recent development in the field. By illuminating the sample with the whole visible light range, an axial resolution of 1 µm in air (corresponding to 0.73 µm in retinal tissue) was achieved. The use of such a spectral range in OCT allowed not only for high resolution PS-OCT images to be acquired, but also for additional spectroscopic analysis to be performed, investigating the dependence of the reflectivity of light upon wavelength. This high resolution, three-fold contrast was used to image the retinas of both healthy mice and the very-low-density-lipoprotein receptor (VLDLR) mouse model. VLDLR mice are known to form neovascularizations similar to those found in patients with retinal angiomatous proliferation, a form of neovascular age-related macular degeneration (AMD) [115]. These neovascularizations have already been proven to be observable with OCT [116]; however the increase in axial resolution may provide a better insight into their growth and development.

The system design and characterization, and three-fold contrast images of healthy and VLDLR murine retinas were published in Biomedical Optics Express with the title “White light polarization sensitive optical coherence tomography for sub-micron axial resolution and spectroscopic contrast in the murine retina” [38]. The PDF file of the manuscript can be found on the following pages.
White light polarization sensitive optical coherence tomography for sub-micron axial resolution and spectroscopic contrast in the murine retina

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Abstract: A white light polarization sensitive optical coherence tomography system has been developed, using a supercontinuum laser as the light source. By detecting backscattered light from 400 – 700 nm, an axial resolution of 1.0 µm in air was achieved. The system consists of a free-space interferometer and two homemade spectrometers that detect orthogonal polarization states. Following system specifications, images of a healthy murine retina as acquired by this non-contact system are presented, showing high resolution reflectivity images as well as spectroscopic and polarization sensitive contrast. Additional images of the very-low-density-lipoprotein-receptor (VLDLR) knockout mouse model were acquired. The high resolution allows the detection of small lesions in the retina.

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References and links
1. Introduction

The axial resolution of an optical coherence tomography (OCT) system is directly proportional to the square of the central wavelength of the light source, and inversely proportional to the full width at half maximum (FWHM) of the source's spectral bandwidth [1]. The development of supercontinuum lasers has therefore opened up a fresh wave of spectral domain (SD-) OCT. Higher repetition rates and lower relative intensity noise fluctuations have now made it possible to perform OCT with broader spectral bandwidths [2, 3] and lower central wavelengths [4–7], thus resulting in a better axial resolution.

OCT has proven itself time and time again as being an important technique in retinal imaging, both in the clinic in human subjects [8] and in preclinical imaging of healthy animals as well as in models of diseases [9]. Despite the lack of a fovea, the murine retina shows many similarities to that of a human retina [10], particularly in its layer structure [11]. The mouse is therefore often used as an animal model, as they are cheap and easy to look after and can be bred quickly. Their short lifespan means that if their genome is manipulated, the pathologies associated with this progress more quickly than they would in other animals with longer lifespans, thus resulting in shorter experiment times if a longitudinal study of the disease progression is to be performed. To be able to characterize the progression of a disease (in humans or animal models) with imaging techniques, it is important that the resolution is as high as it possibly can be in order to detect more subtle changes.

However it is not only higher resolution which plays a role in better image interpretation. In conventional OCT reflectivity images of the retina, it can often be difficult to distinguish pathological features if their particle distribution and backscattering properties are similar to that of the surrounding tissue. In this case, functional extensions of OCT are often applied to highlight contrasts which are not visible in the reflectivity images. Doppler OCT [12, 13], for example, is often used to quantify blood flow in the retina by performing differential phase analysis between successive A-scans (or B-scans). This method detects motion along the axis of the probing beam, resulting in contrast between the moving red blood cells and their static surrounding tissue [14–16]. OCT angiography (OCTA) can also be used to visualize blood flow, but this time by looking at either phase or intensity variations to detect decorrelation [17, 18]. In the retina, this results in a detailed map of where the blood flow occurs down to the capillary level [19].

To further quantify blood vessels in terms of blood oxygen saturation, a spectroscopic analysis can be performed on visible light OCT data, visualizing the behavior of different wavelengths corresponding to the absorption peaks of oxyhemoglobin and deoxyhemoglobin [20–24]. While this can also be done at infrared wavelengths, the effect is much more pronounced in the visible light range [25], and since the central wavelengths are lower a better axial resolution is maintained [7]. Although these studies have been performed in blood vessels, the method is not dependent upon the movement of blood cells and can be performed in other tissues. If the wavelength range is extended to the full visible light spectrum, it is also possible to reconstruct "true color" images if the wavelengths chosen fall at red, green and blue, providing a different contrast in static tissue capable of chromophore detection [26]. It has already been shown that scattering properties of retinal layers can change with wavelength [27] and therefore visible light OCT could play a role in a spectroscopic investigation of these layers.

Also enhancing the contrast in static tissue, polarization sensitive OCT (PS-OCT) [28, 29] highlights the intrinsic contrast caused by the fact that some tissues change the polarization state of light incident upon them. In the healthy retina of both humans and mice, for example, the retinal pigment epithelium (RPE) depolarizes light due to the melanin granules which are present...
in this layer [30, 31]. Each granule scatters the light and alters the polarization state, and the cumulative effect of the interaction of light with many granules is a random polarization state, given that the pigment density is sufficient. This is known as depolarization [32, 33]. It therefore follows that if there are any subtle irregularities in the shape of the RPE, this can also be detected by PS-OCT.

Previous studies have shown the use of PS-OCT in human retinal imaging for both healthy volunteers [34–39], and in patients with diseases such as age-related macular degeneration (AMD) [40–45], glaucoma [35, 39, 46] and choroidal nevus [42]. Recent PS-OCT studies in our group have focused mainly on rodent models of such diseases. Since intraocular pressure is an important parameter in glaucoma [47], this was studied in the rat eye using a high resolution PS-OCT system (axial resolution = 3.8 µm in retinal tissue) described in [48]. It was shown that scleral birefringence, a property defined by the axis orientation of collagen fibers, could be correlated to the intraocular pressure [49]. The system used to perform this study was further used to image the very-low-density-lipoprotein-receptor (VLDLR) mouse model which forms type 3 neovascularizations similar to those found in patients with retinal angiomatosus proliferation, a form of neovascular AMD [50]. In this case, the melanin displacement was visualized with PS-OCT over time in a longitudinal study [51].

In this work, we present a new PS-OCT system operating across the whole visible light range with an axial resolution which is approximately five times higher than in our previous system. As the system was specifically designed for mouse retinal imaging, we discuss its design, measure its specifications and finally we demonstrate reflectivity, phase retardation and spectroscopic retinal images of both healthy mice and the VLDLR knockout mouse model as acquired by the system.

2. Materials and methods

2.1. System

![Diagram of the white light PS-OCT system. Polarization optics are drawn in red. FC Fiber Collimator. DM Dichroic Mirror. BPF Bandpass Filter. WC Water Cuvette. ND Neutral Density Filter. POL Polarizer. BS Beam Splitter. QWP Quarter Wave Plate. DC1 Dispersion Compensation Water Cuvette. DC2-4 Dispersion Compensation Prisms. RM Reference Mirror. L Lens. PBS Polarizing Beam Splitter. DG Diffraction Grating. CMOS Line Scan Camera. C Computer. (b) Wavelength range detected by the two spectrometers (reference spectra).](image)

A white light PS-OCT system was developed as shown in Fig. 1(a). A supercontinuum laser
was chosen as the light source, with the desired spectrum (400 – 700 nm) selected using a homemade filter box (indicated in the green dashed rectangle) consisting of a dichroic mirror (DMSP805, Thorlabs), a bandpass filter (FESH0750, Thorlabs), a water cuvette (lightpath = 10 mm) and a neutral density (ND) filter (optical density = 1.8). As the dichroic mirror and the bandpass filter break down at longer wavelengths (>1500 nm), the water cuvette was added to absorb the remaining infrared part of the spectrum. Heat sinks were attached to the water cuvette for efficient heat dissipation. The neutral density filter was then added to attenuate the remaining beam power in the visible light range before passing the beam through a photonic crystal fiber (SuperK FD7, NKT Photonics) to the interferometer. The photonic crystal fiber was necessary to maintain single mode transmission over the whole wavelength range.

The incident white light beam was first linearly polarized by a Glan-Thompson polarizer (5524, Newport Spectra-Physics) and then separated into a sample and reference arm by a 50/50 beam splitter. In the sample arm, a variable telescope (AC127-030-A, focal length = 30 mm and AC127-025-A, focal length = 25 mm, Thorlabs) was used to correct for poor focus of each individual mouse eye during the measurement. A set of X-Y galvanometric scanners (GVS002, Thorlabs) were then used in combination with a final telescope (AC508-075-A, focal length = 75 mm and AC254-030-A, focal length = 30 mm, Thorlabs) to scan a beam with a 1/e² diameter of 0.3 mm through the anterior eye. The system then relied on the natural optics of the mouse eye to focus the light onto the retina. Such a setup does not require any physical contact with the mouse eye. In the reference arm, glass prisms of various glass types (similar to those in the lenses in the sample arm) were added to compensate for the phase dispersion introduced by the two telescopes. A water-filled cuvette (type 96 X-rite colorimeter cell, lightpath = 2.5 mm, FireflySci) was also added to loosely compensate for the dispersion introduced by the eye itself.

Polarization optics were also added to the interferometer. In the sample arm, the beam passed through a quarter wave plate (QWP) (10RP44-1, Newport Spectra-Physics) angled at 45° to illuminate the sample with circularly polarized light. A change in polarization state caused by the sample generally results in an elliptical state returning to the beam splitter, which interferes with the beam returning from the reference arm (which itself has traversed a QWP at 22.5° twice). A polarizing beam splitter then splits the resultant beam by polarization state, allowing a polarization sensitive detection. Each beam passes through a photonic crystal fiber (SuperK FD7, NKT Photonics) to a homemade spectrometer, comprising a diffraction grating (1800 lines/mm), a custom designed lens and a CMOS line scan camera (ELiiXA+ 16k, e2v) with four rows of 16384 pixels, each measuring 5 µm × 5 µm. However as the diameter of the diffraction limited spot size of the beam lies between 6 – 9 µm (depending on the wavelength), the pixels were binned to 8192 pixels of 10 µm × 10 µm, resulting in a spectrometer resolution of 0.044 nm/pixel. The wavelength spectrum as acquired by the spectrometers is shown in Fig. 1(b).

Data from the CMOS line scan cameras were sent to the computer via a frame grabber (Komodo, Kaya Instruments) over a CoaXPress [52] link. The acquisition and the X-Y galvanometric scanners were synchronized and controlled by LabVIEW (Version 15.0f2, 64-bit, National Instruments). For 3D data acquisition, the scanners were set to execute a smoothed raster scan pattern with 512 × 400 data points at an A-scan rate of 25 kHz. Although the maximum line rate of the cameras was 40 kHz (for acquisition with 12 bit resolution), the A-scan frequency was sacrificed in favor of the exposure time and therefore the signal-to-noise ratio (SNR).

2.2. Data acquisition and post processing

2.2.1. Reflectivity: backscattering based imaging

Following acquisition, each data set was processed using a combination of MATLAB (R2015b, MathWorks) and Fiji (ImageJ 1.51p) [53]. To obtain standard reflectivity OCT images based on backscattering, the background is first removed by subtracting the average spectrum of the whole B-scan and then the spectral data is resampled to be linear in k-space. Prior to processing of the
second channel, the data is first passed through an equation of the form $y = mx + c$ in order to align the spectrum to that of the first channel (previously calibrated using color filters), and a normalization is performed to correct for the intensity differences between channels at each wavelength. Numerical dispersion compensation is then applied using a variation on the method described by Wojtkowski et al. [54] (see section 2.2.2), and the Fourier transform is computed. In some cases, multiple frames are averaged for speckle reduction. The reflectivity images are then calculated by summing the squares of the signals from each channel [55].

2.2.2. Dispersion compensation for broad bandwidth

A method for dispersion compensation involving the addition of a phase correction term to the complex analytic representation of the spectral fringe pattern has previously been described [54]. In this method, the coefficients $a_2$ and $a_3$ are adjusted to balance the second- and third-order dispersion terms, respectively. However, due to the broad spectral bandwidth in the white light OCT system, the numerical constants $a_2$ and $a_3$ are themselves wavelength dependent and can differ by up to an order of magnitude across the whole wavelength range. The correction terms were therefore calculated for three separate wavelength ranges, $(\lambda_1, \lambda_2, \lambda_3)$, independently, and the phase correction curves for these wavelengths were then concatenated and smoothed to ensure no discontinuities. In doing this, a first order dispersion term is introduced which must again be corrected for. This technique is a generalization of that shown in Eq. 1, where the phase correction term in k-space, $\Phi$, is dependent not only on the central wavenumber $k_0$ and the wavenumber $k$, but also on wavelength dependent constants. The generalization of this case to three wavelength ranges means that the exact relationship between the numerical constants and the wavelength range need not be known and a trial and error approach can be taken for each data set, finding the phase correction constants by judging the quality of the images by eye. In this case, the $a_1$ term is also required to ensure the image appears at the same position in depth for each wavelength range, correcting for first order dispersion.

$$\Phi(k, \lambda, i = 1, 2, 3) = a_1(\lambda_i) \times (k - k_0) - a_2(\lambda_i) \times (k - k_0)^2 - a_3(\lambda_i) \times (k - k_0)^3 \quad (1)$$

As this process is rather lengthy, it was not performed for all data sets. For the remainder, dispersion compensation was performed exactly as outlined in [54].

2.2.3. Polarization sensitive image processing

After all spectrum alignment and normalization (see section 2.2.1), the phase retardation for each pixel, $\delta$, can be calculated [28,55]:

$$\delta = \arctan\left(\frac{A_V}{A_H}\right) \quad (2)$$

In this equation, $A_V$ and $A_H$ are the amplitudes of the vertically and horizontally polarized channels, respectively. Prior to calculating the retardation, an intensity threshold was set in the corresponding reflectivity image ($\approx 3 - 5$ dB above the mean noise level), below which the retardation was not calculated. This ensured that the retardation image consisted only of retinal signal, removing the background noise.

2.2.4. Spectroscopic OCT

The original spectra were filtered by Gaussian windows centered at blue (460 nm), green (550 nm) and red (640 nm) wavelengths to gain an additional spectroscopic contrast. The filtering was performed after resampling of the spectra to k-space to ensure a constant axial resolution ($\approx 4$ $\mu$m in tissue) for each wavelength channel. The resulting images were first normalized by histogram shape, and then combined to form an RGB image - a "true color" representation of the
OCT data [26]. As different wavelengths produce different speckle patterns in the OCT images, each wavelength channel B-scan was smoothed by a mean filter (rectangular kernel, $3 \times 2$) before the RGB image was created.

### 2.3. Mice

Both healthy adult control mice (from a BL/6 background) and very-low-density-lipoprotein-receptor (VLDLR) knockout mice (8-10 months old) were measured using the system. The mice were anesthetized using an isoflurane/oxygen mixture and pupils were dilated using tropicamide and phenylephrine, topically administered mydriatic agents. The eyes were kept moist during the experiments using artificial tear drops. A stage was designed and built to position the mouse and to hold the anesthesia apparatus to the nose for the duration of the experiments. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and under a protocol approved by the ethics committee of the Medical University of Vienna and the Austrian Federal Ministry of Science, Research and Economy (BMWF-66.009/0360-WF/V/3b/2016).

### 3. Results

#### 3.1. System characterization

#### 3.1.1. Specifications

![Fig. 2](image-url)

**Fig. 2.** (a-b) Axial resolution measurement in (a) the horizontally polarized channel and (b) the vertically polarized channel. Red lines indicate the full width at half maximum (FWHM) of the signal intensity which is equal to 1.0 $\mu$m in air for both channels. (c-d) Sensitivity roll-off as a function of depth (in air) for (c) the horizontally polarized channel (roll-off decay $\approx 14$ dB/mm) and (d) the vertically polarized channel (roll-off decay $\approx 15$ dB/mm).

The axial resolution of the system was measured by placing a mirror in the sample arm and measuring the FWHM of the resultant point spread function after Fourier transformation. Spectral phase correction as described in detail by Choi et al. [56] was performed to remove dispersive effects. An axial resolution of 1.0 $\mu$m in air was measured in both channels, which corresponds to 0.73 $\mu$m in rodent retinal tissue where the refractive index is assumed to be 1.37 [57].
2(a) shows the axial resolution in the horizontally polarized channel, while Fig. 2(b) displays the same in the vertically polarized channel.

The sensitivity and corresponding roll-off of the system were also measured. This was done by placing a ND filter of known optical density in the sample arm in front of a mirror, and calculating the maximum SNR which could be achieved at different depth positions. A maximum sensitivity of 96 dB in both channels was calculated (with an incident power of 1 mW) by adding the SNR to the attenuation factor introduced by the ND filter. Figure 2(c-d) shows the result of the sensitivity roll-off as a function of depth for both the horizontally (c) and vertically (d) polarized channels. The corresponding sensitivity roll-off was 14 dB/mm in the horizontally polarized channel and 15 dB/mm in the vertically polarized channel. The total imaging depth range was measured to be 2.2 mm, corresponding to ≈ 1.6 mm in retinal tissue. However since the thickness of the murine retina is < 300 µm, only the first 700 µm of the depth range was used for imaging.

3.1.2. Polarization measurement validation

Fig. 3. (a) Phase retardation at different depth positions. (b) Phase retardation at different waveplate orientations. The retardation stays within ± 4° for a 180° rotation of the waveplate.
(c) A wavelength dependence on retardation is observed with a smaller phase retardation at longer wavelengths. The error associated with the measurement is greater at the edges of the spectrum. All graphs display mean retardation ± propagated standard deviation.

In order to verify the capability of the system to resolve polarization properties of a sample, a phantom was constructed consisting of a QWP designed for 1300 nm and a mirror, as described in [55]. The retardation of this phantom was then measured at different distances from the zero delay and also for different orientations of the QWP. Figure 3(a) shows the retardation values as a
function of distance from the zero delay. Within the depth range used for murine retinal imaging (the first 700 µm) the measured retardation value stays approximately constant and does not go outwith ± 2°. For the retardation as a function of set waveplate orientation (Fig. 3(b)), the value is also relatively constant. The slight periodic shift (± 4°) is most likely due to a misalignment of the waveplate with respect to the beam. This would also explain the discrepancy between the measurements at 0° and 180°, which should theoretically be identical.

The acquired spectra were then filtered by Gaussian windows to sample the phase retardation at different central wavelengths ranging from 400 - 700 nm. The retardation shows a strong wavelength dependence, particularly in the region of 400 - 500 nm. The graph in Fig. 3(c) demonstrates the measurements for one particular depth position and waveplate axis orientation, but the trend is reproducible at different depths and orientations.

In this section all measurements were repeated 512 times without scanning the mirror, and the mean and standard deviation of the intensity values of each channel were calculated. The standard deviation of each channel was propagated through the retardation equation (Eq. 2) [58], and the mean retardation at each point is plotted with error bars indicating the associated error values.

3.2. Imaging the healthy murine retina

The white light PS-OCT system was used to image the retina of healthy adult mice. An example of a 30× averaged reflectivity B-scan image can be found in Fig. 4(a). The corresponding phase retardation image is seen in Fig. 4(b). Most of the mouse retina is polarization-preserving and therefore appears blue in the image. However when zooming in to the region including the RPE (reflectivity: Fig. 4(c), retardation: Fig. 4(d)), the addition of PS contrast immediately highlights the boundary of the depolarizing RPE, which is difficult to distinguish from the surrounding layers in the standard reflectivity images. An example of an en-face projection over the whole depth of the retina, including the optic nerve head, can be found in Fig. 4(e). The adjustable focusing telescope in the sample arm allows a tighter beam focus on the retina and therefore the vessels appear well-defined in the image. The horizontal stripes in the image are caused by breathing artifacts. In order to highlight the axial resolution, Fig. 4(f) shows a labeled section of the retina as measured with white light OCT plotted against depth position, and a histological sample of a similar region of the healthy mouse retina. The high resolution allows for clear retinal layer definition. Since the external limiting membrane (ELM) is a reflective boundary between two surfaces, its apparent thickness in OCT images can be considered as a measured in vivo axial resolution. In single frames, this corresponds to 0.91 - 0.97 µm. The discrepancy between this value and the theoretical value of 0.73 µm can be explained by imperfect dispersion compensation and attenuation of the edges of the wavelength spectrum with depth in the retina.

3.3. Spectroscopic OCT

Spectroscopic analysis, as represented in Fig. 5(a), was performed on the OCT images; sacrificing axial resolution to gain a spectroscopic contrast. The Gaussian windows were selected to give an axial resolution of 4 µm in retinal tissue at each wavelength. Figure 5(b-f) shows the results of such an analysis applied to the same image as in Fig. 4(a). The acquired spectra were filtered using Gaussian windows centered at red (Fig. 5(b)), green (Fig. 5(c)) and blue (Fig. 5(d)) wavelengths. From these images it is clear that the penetration depth is greatest in the red channel, and decreases with decreasing wavelength. By combining the three channels to form a "true color" RGB image (Fig. 5(e)), blood vessels are immediately highlighted as there is a greater component of backscattered light at red wavelengths compared to the others.

The spectroscopic analysis was also performed on 3D datasets and en-face projection maps were made across the whole depth of the retina (Fig. 5(f)). Again the vessels are immediately highlighted. This could prove a useful method for identifying blood without relying on the cellular motion to provide contrast.
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3.4. VLDLR knockout mouse model

The white light PS-OCT system was also used to image the retina of the VLDLR knockout mouse model which is known to develop retinal lesions [59]. The tomogram in Fig. 6(a) shows the standard reflectivity image in which a lesion (inset) can be found. When zooming into the
Fig. 5. (a) A cartoon representation of spectral filtering in wavelength space. The original spectrum is multiplied by three Gaussian windows centered at blue ($\lambda_1 = 460$ nm), green ($\lambda_2 = 550$ nm) and red ($\lambda_3 = 640$ nm) wavelengths. (b-d) Red (b), green (c) and blue (d) B-scan images after spectral filtering. (e) “True color” RGB image produced by the normalized and smoothed addition of the red, green and blue channels. White arrow indicates blood vessel. (f) Spectroscopic en-face projection over the whole retina including the optic nerve head. Vessels are immediately highlighted by color contrast. All scale bars correspond to 50 $\mu$m.

abnormal area (indicated by the orange box, 100 $\mu$m high), the lesion clearly has some structure which indicates RPE disruption. Figure 6(b) shows the phase retardation image of the B-scan. The inset shows that while the RPE is disrupted somewhat, the depolarizing melanin pigments have not yet been displaced into the photoreceptor layer. Figure 6(c) displays the true color spectroscopic representation of the lesion. It would appear that there is some color contrast within the lesion, but a more comprehensive analysis would be required to confirm the source of this.

While an en-face projection over the whole depth of the retina (Fig. 6(d)) does not highlight any immediate irregularities, manual segmentation of the RPE layer alone shows the location of lesion sections. Figure 6(e) shows an en-face projection of all signal from the top surface of the RPE and deeper retinal layers in grayscale, and a heat map of height above the RPE layer (also manually segmented) where additional high-intensity signal (indicating presence of a lesion) is observed. Due to the high axial resolution of the system, the lesions can be detected and monitored using white light OCT with a much more precise level of detail when compared to infrared light. For comparison, Fig. 6(f) shows an example of a histology image of the retina of the VLDLR knockout mouse model. The yellow arrow indicates a miniscule and eosinophilic thickening between the RPE and choroid, the growth of which could be studied in more detail in
Fig. 6. (a) Reflectivity B-scan of the VLDLR knockout mouse model as acquired by white light OCT (4x average of consecutive B-scans). The orange box indicates the presence of a retinal lesion. (b) The corresponding phase retardation image. The PS contrast indicates the position of the lesion, but in this case there is no indication of depolarizing melanin in the photoreceptor layer. (c) The true color spectroscopic RGB image. There appears to be some color contrast within the lesion. (d) En-face projection across an area of the retina superior to the optic nerve head. There is no indication of retinal lesions. (e) En-face projection from the top of the RPE (grayscale) with a heat map of height above the RPE layer where additional abnormal signal is seen. This highlights the lesion area. (f) Representative histology image of the retina of the VLDLR knockout mouse model. Yellow arrow indicates a miniscule and eosinophilic thickening between the RPE and choroid, which potentially features a lesion. High resolution imaging would be required to study the progression of this in vivo. Green asterisks indicate artefactual detachment. (g) A larger lesion (indicated by yellow arrow) which shows melanin has been displaced to the outer nuclear layer, and the whole layer structure is disrupted. All scale bars correspond to 50 µm. Insets in (a-c) show a depth of 100 µm.

In vivo with the white light system. While preparing the histology images, artefactual detachments can occur during paraffin embedding of the eye cup. Examples of this are highlighted by the green asterisks in Fig. 6(f). Figure 6(g) shows a very large lesion which causes disruption of all deeper retinal layers. Since these mice are used as a model of age-related macular degeneration, a more precise mapping of the growth of these lesions could help to gain a better understanding of the disease.
4. Discussion

As the OCT system presented in this work makes use of backscattered light from 400 - 700 nm, the high axial resolution allows a 3D reconstruction of the mouse retina to be visualized in great detail. The additional contrast channels of polarization sensitivity and spectroscopic information open up new possibilities to visualize properties of the mouse retina for this wavelength range. As the eye is designed for visible light, "true color" images can be reconstructed, which may give a more intuitive idea of how the retina behaves since the wavelength spectrum used for imaging overlaps the absorption spectra of the photoreceptors in the retina [60,61].

However the extension of OCT to the whole visible light range does not come without its challenges. In order to achieve the highest possible axial resolution, the dispersion must be corrected for to a high degree of accuracy. In this work, both hardware and software methods are used, and there is still a small amount of dispersion present in the images. The $a_2$ and $a_3$ parameters in Eq. 1 change from mouse to mouse, and therefore fully-automated post-processing has not yet been implemented.

The broad bandwidth also poses new challenges in the acquisition of the polarization sensitive data. As the detectors of the CMOS cameras in the spectrometers are 8.2 cm long, it was not possible to align both of them truly identically. The result of this was that an additional calibration step was required before Fourier transformation, aligning the spectra of the second channel to those of the first. Also, the polarization optics do not perform ideally across the whole wavelength spectrum which could lead to a slight offset in the retardation measurements. However it is not only the polarization optics which are being used at their limits. Every lens present in the system was modeled using Zemax (OpticStudio 15.0, Zemax LLC) to find a combination with minimal chromatic aberrations, and the lenses for the spectrometers were custom made. To extend the bandwidth of PS-OCT any further would require optical components which are not yet available off-the-shelf.

White light OCT also has its limitations, such as the limited penetration depth. As the melanin in the RPE is highly absorbing in the visible light range, there is very little light penetration to the choroid. While this system was designed specifically for the mouse eye, there is also the question about the transfer of white light OCT to humans [4]. As the human eye is very sensitive to shorter wavelengths, care must be taken to keep the power as low as possible, which in turn reduces the power incident upon the sample and therefore lowers the system sensitivity [4].

When performing the spectroscopic analysis, it is clear that the penetration depth decreases with decreasing wavelength and therefore the pure addition of the three channels results in a color gradient from white at the anterior surface of the retina to only red in the choroid (Fig. 5(e)). In order to correct for the effects of the spectral attenuation and the spectral sensitivity roll-off in the images, it would be possible to calculate the sensitivity roll-off at each wavelength and use this as a correction factor. However as the blue light signal intensity is low at the level of the RPE, this method significantly amplifies the noise and it is no longer possible to be confident of the spectroscopic data. For this reason, the three color channels were simply combined, with the idea that the spectroscopic contrast could be local to the surrounding tissue rather than global to the whole image. Although the signal is low in the blue channel in the posterior retinal layers, the anterior retinal layers allow a true color image to be reconstructed. The spectroscopic analysis indicates that there do appear to be structures in the RNFL/GCL which scatter only at specific wavelengths, but this needs further investigation. What is immediately clear is the vessel contrast due to the hemoglobin present in blood. If it is possible to detect blood in this manner, this could provide an interesting alternative to OCT angiography. In addition to highlighting the vessels, the spectroscopic analysis could also be used for leakage detection in the retina, or also in ex-vivo work, as the flow of blood is not necessary for contrast.

In addition to blood detection, the spectroscopic approach could also be used to detect other chromophores, such as melanin. As melanin pigment is brown, it would be reasonable to assume
that it could also be identified by spectroscopic analysis, leading to a brownish appearance of the RPE and the remnant of the hyaloid canal which are both known to contain melanin [31, 62]. While this brownish hue can be somewhat seen in Fig. 5(e) (RPE) and (f) (hyaloid canal, top of ONH region), more analysis must be performed to confirm that melanin is the source of this. This could perhaps be explored by measuring the melanin concentration with spectroscopic OCT. In terms of PS contrast, this study focused only on phase retardation measurements which shows a clear definition of the RPE due to the high axial resolution. Additional future studies include the extension of PS-OCT to degree-of-polarization uniformity (DOPU) measurements in the retina, and axis orientation measurements in the RNFL specifically. The additional ability to perform a spectroscopic analysis of such polarization properties in the visible light range could provide an in vivo, non-invasive insight into the micro-structure of the murine retina.

5. Conclusion

A white light polarization sensitive optical coherence tomography system has been developed for sub-micron resolution imaging in the murine retina. Both healthy mice and VLDLR knockout mice have been imaged in vivo, demonstrating high resolution reflectivity images, phase retardation images and a spectroscopic contrast. A system such as this could prove a candidate for high resolution imaging in longitudinal studies of mouse models of retinal diseases.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.
3. AN INTERLUDE ON PHASE RETARDATION MEASUREMENT UNCERTAINTY

The phase retardation, \( \delta \), is defined as the inverse tangent of the ratio of the measured amplitudes of each channel as shown in Eq. 1.57 [51,52]. While previous studies have considered the effect of a systematic error on the accuracy of phase retardation measurements as a function of SNR [19,117], the uncertainty associated with the measurement result itself has not yet been discussed. A brief interlude is therefore included here to discuss the fundamental unknown error in phase retardation measurements which cannot be corrected for.

In the case of the waveplate measurements (Fig. 3 in Chapter 2), each amplitude is measured several times and therefore a mean amplitude, \( E \), and associated standard deviation, \( \sigma \) can be calculated for each channel. To propagate these standard deviations (under the assumption that standard deviation \( \gg \) instrument error), the uncertainty in the ratio of the amplitudes is defined as per Eq. 3.1 [118], where \( \sigma_V \) and \( \sigma_H \) are the standard deviations of the vertical and horizontal amplitudes, respectively, and \( \sigma_{VH} \) is the covariance of the two measurements. However since the two amplitudes are uncorrelated (assuming the two spectrometers to be independent entities), the covariance, \( \sigma_{VH} \), is zero and therefore the equation is simplified to that in Eq. 3.2.

\[
\Delta \left( \frac{E_y}{E_x} \right) = \left| \frac{E_y}{E_x} \right| \sqrt{ \left( \frac{\sigma_V}{E_y} \right)^2 + \left( \frac{\sigma_H}{E_x} \right)^2 - 2 \left( \frac{\sigma_{VH}}{E_y E_x} \right)^2 } \tag{3.1}
\]

\[
\Delta \left( \frac{E_y}{E_x} \right) = \left| \frac{E_y}{E_x} \right| \sqrt{ \left( \frac{\sigma_V}{E_y} \right)^2 + \left( \frac{\sigma_H}{E_x} \right)^2 } \tag{3.2}
\]

Once the amplitude ratio has been determined, the remainder of the retardation calculation takes the form of a simple inverse tangent (Eq. 3.3). The overall uncertainty in the retardation measurements can therefore be calculated using the partial derivative method (Eq. 3.4) and substituting the \( x \) term for the amplitude ratio (Eq. 3.5).
\[ f(x) = \arctan(x) \quad (3.3) \]

\[ \frac{df}{dx} = \frac{1}{1 + x^2} \quad (3.4) \]

\[ \Delta \delta \approx \Delta x \cdot \frac{1}{1 + x^2} = \frac{\Delta \left( \frac{E_y}{E_x} \right)}{1 + \left( \frac{E_y}{E_x} \right)^2} \quad (3.5) \]

After following all of the above steps, the total uncertainty in the retardation measurements, \(\Delta \delta\), can be calculated as follows:

\[ \Delta \delta \approx \left| \frac{E_y}{E_x} \right| \sqrt{\left( \frac{\sigma_V}{E_y} \right)^2 + \left( \frac{\sigma_H}{E_x} \right)^2} \frac{1}{1 + \left( \frac{E_y}{E_x} \right)^2} \quad (3.6) \]

To go further, this equation can be rearranged to:

\[ \Delta \delta \approx \frac{\left| \frac{E_y}{E_x} \right|}{1 + \left( \frac{E_y}{E_x} \right)^2} \sqrt{\left( \frac{\sigma_V}{E_y} \right)^2 + \left( \frac{\sigma_H}{E_x} \right)^2} \quad (3.7) \]

Using the trigonometric identity \(\frac{2 \tan a}{1 + \tan^2 a} = \sin 2a\) and defining the signal-to-noise ratio of the vertical and horizontal channel as \(SNR_V = \left( \frac{E_y}{\sigma_V} \right)^2\) and \(SNR_H = \left( \frac{E_x}{\sigma_H} \right)^2\) respectively, we can simplify the equation to

\[ \Delta \delta \approx \frac{1}{2} |\sin 2\delta| \sqrt{SNR_V^{-1} + SNR_H^{-1}} \quad (3.8) \]

This equation indicates that in the event that the standard deviation of the retardation measurements is significantly higher than any systematic errors in the system, the expected value for the uncertainty in the retardation is dependent upon two terms. The first is the periodic dependence upon the total retardation, \(\delta\). For constant and equal noise terms, the uncertainty in the retardation approaches a minimum as the retardation approaches 0° and 90°. The second term, \((SNR_V^{-1} + SNR_H^{-1})^{1/2}\), dic-
tates the dependence of $\Delta \delta$ on the SNR contributions of each polarization channel. This is similar in form to the relation for the precision of phase variance measurements as they are required for Doppler OCT [119, 120], and also to the trend curves in the comprehensive noise analyses for phase retardation measurements which have previously been conducted [19, 117].

If the noise level and total signal intensity (reflectivity) remain constant Fig. 3.1A, the maximum uncertainty in retardation measurement occurs at a retardation value of 45°. In the case of unequal noise levels between channels, this second term shifts the maximum retardation error away from 45°. An example of this is shown in Fig. 3.1B, where the intensity of the noise in the horizontal channel is double that of the vertical channel. For a retardation value of 45°, Fig. 3.1C shows a plot of the uncertainty in retardation as a function of the total SNR, defined as:

$$SNR[dB] = 10 \log_{10} \left( \frac{E_x^2 + E_y^2}{\sigma_H^2 + \sigma_V^2} \right)$$  \hspace{1cm} (3.9)$$

Fig. 3.1. A-B) Uncertainty in retardation, $\Delta \delta$, plotted as a function of retardation, $\delta$, for different SNR values. A) Equal noise variance in both polarization channels. B) The horizontal channel noise variance is double that of the vertical channel. C) Error as a function of the total SNR for a retardation value of 45° where the SNR in both channels is equal.

where in this case, $\sigma_H = \sigma_V = \sigma$. The curve of this graph confirms our recent experimental data (see Fig. S1 in [100]). In addition, the plots in Fig. 3.1 confirm that the averaging of multiple images reduces the error in the retardation, $\Delta \delta$, for all retardation values, due to the increase in the SNR [121].
4. HYPERSONSPECTRAL OPTICAL COHERENCE TOMOGRAPHY

In Chapter 2, a 3D spectroscopic contrast was achieved by filtering the acquired spectra by Gaussian windows centered at 460 nm (blue), 550 nm (green) and 640 nm (red), and assigning each sub-image to its respective RGB channel for image visualization. As a next step, the filtration windows were increased in number and reduced in bandwidth, sacrificing the axial resolution in favor of increasing the spectral resolution. An axial resolution of around 6 µm in tissue was, however, maintained in the images. A total of 27 Gaussian windows were applied in post-processing to the spectral data of both the rat and mouse retina, and the deviations in the intensity of the data as a function of wavelength were used as a metric to source melanin granules within the RPE. The idea of using sOCT to detect particles based on their Mie backscattering coefficients is one which had been previously demonstrated [122, 123], however its in vivo application to melanin granules remained unexplored.

To prove the feasibility of the concept, theoretical Mie theory simulations were performed and phantoms consisting of melanin granules in silicone were imaged using the hyperspectral OCT system, a slight modification of the system presented in Chapter 2. The results of the simulations and measurements were then published together with in vivo results in the Journal of Biophotonics with the title “Hyperspectral optical coherence tomography for in vivo visualization of melanin in the retinal pigment epithelium” [124]. This study demonstrated the use of hyperspectral OCT for the successful detection of melanin in low-melanin-concentration biological systems. The PDF file of the manuscript can be found on the following pages.
Hyperspectral optical coherence tomography for in vivo visualization of melanin in the retinal pigment epithelium

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Abstract
Previous studies for melanin visualization in the retinal pigment epithelium (RPE) have exploited either its absorption properties (using photoacoustic tomography or photothermal optical coherence tomography [OCT]) or its depolarization properties (using polarization sensitive OCT). However, these methods are only suitable when the melanin concentration is sufficiently high. In this work, we present the concept of hyperspectral OCT for melanin visualization in the RPE when the concentration is low. Based on white light OCT, a hyperspectral stack of 27 wavelengths (440-700 nm) was created in post-processing for each depth-resolved image. Owing to the size and shape of the melanin granules in the RPE, the variations in backscattering coefficient as a function of wavelength could be identified—a result which is to be expected from Mie theory. This effect was successfully identified both in eumelanin-containing phantoms and in vivo in the low-concentration Brown Norway rat RPE.

KEYWORDS
hyperspectral imaging, melanin, optical coherence tomography, optical spectroscopy, retinal pigment epithelium, white light

1 | INTRODUCTION
The protective effects of melanin on ocular cells and tissues make it an interesting heteropolymer. By a combination of physical and biochemical mechanisms, the pigment simultaneously acts as a photo-screen and an antioxidant [1]. The pigmented middle layer of the eye, known as the uvea, consists of the iris and ciliary body in the anterior eye, and the
choroid in the posterior eye. In this layer, the melanin acts as an absorber for any stray light either reflected within the eye or transmitted through the sclera, improving the contrast of the final retinal image. Also located in the posterior eye is the retinal pigment epithelium (RPE). Alongside its absorption properties, the melanin in this layer is thought to play a role in many additional processes. Here, the melanin assists in the maintenance of visual function and serves as a site for sub-cellular molecular interactions [2, 3]. Owing to the close functional relationship of the RPE to the photoreceptors, it has been suggested that a change in RPE melanin content may be associated with diseases such as age-related macular degeneration [4].

As the RPE is separated from the also melanin-rich choroid by only Bruch’s membrane (a 2- to 4-μm-thick layer in humans, [5] and even thinner in the rodent [6, 7]), in vivo analysis of the melanin in the RPE alone has proven challenging. Chemical methods have been employed to study melanin in the choroid/RPE complex as a whole [8] and also in the RPE alone [9]; however, such approaches are only applicable post mortem. These methods, and also those based on electron spin resonance spectroscopy [9, 10], have shown that the melanin content within RPE cells can drop down to a fifth of its initial value by the age of 80, increasing the possibility of disease [4]. However, even in young subjects, the melanin concentration is not uniform across the retina [9, 11]. There is therefore a need for an in vivo method for visualization of the RPE melanin, independent from that of the choroid, and optical techniques have recently been employed for this purpose [12].

Photoacoustic microscopy has been proposed as a method for visualization and/or quantification of melanin in the ex vivo human retina [13], and also in vivo in the retina of both the chick embryo [14] and the rat [15]. Photothermal (PT) optical coherence tomography (OCT) has also recently demonstrated its potential as a functional technique to image melanin distribution in the RPE of the tyrosinase-mosaic zebrafish [16]. Another recent study compared the reflectivity contrast between visible light OCT and near-infrared OCT in the RPE, indicating that the difference in melanin absorption between these two wavelength bands may lead to an intrinsic contrast, detected by the intensity ratio of the OCT images [17]. All of these techniques make use of the absorption properties of melanin as the source of image contrast and therefore the melanin concentration must be sufficiently high to achieve good image contrast. Near-infrared autofluorescence imaging (NIR-AF) also detects the presence of ocular melanin and can be linked to its concentration [18]; however, this is only a two-dimensional imaging modality and therefore the choroidal melanin also contributes to the observed signal. Polarization sensitive (PS) OCT, exploiting the depolarizing property of the melanin granules, has also been applied in the retina [19, 20]. PS-OCT was utilized to show a relationship between the optical pigment density in the RPE/choroid complex and the degree of polarization uniformity of the light which is backscattered from these layers [21, 22]. PS-OCT was also combined with scanning laser polarimetry [23, 24] and NIR-AF [24, 25] for in vivo analysis of polarization properties of the RPE. However in these studies, it was not possible to directly correlate the polarization properties to the melanin concentration, most likely due to the scattering properties of the melanin granules themselves.

In order to investigate scattering properties, direct measurement of wavelength-dependent scattering and backscattering coefficients has been demonstrated in the visible light range using low coherence spectroscopy measurements of microspheres [26]. The results were in good agreement with Mie theory simulations. Since the melanin granules in the RPE are on the order of magnitude of the wavelength of visible light, it may be expected that the granules exhibit Mie regime-like scattering behavior upon visible light illumination. In conventional OCT, only the light which is directly backscattered by the sample is detected, resulting in an imaging modality in which the intensity of the signal is dependent upon the coefficient of backscattering, \( \mu_b \), across the entire bandwidth of the spectrum. To investigate the dependence of the backscattered signal upon wavelength, spectroscopic OCT (sOCT) is required. sOCT has been used to visualize and/or quantify chromophores such as melanin [27], bilirubin [28] and hemoglobin [29–31], mostly based on the absorption properties of the chromophores in the visible light range [32]. However, sOCT has also been used to quantify the backscattering coefficient of small particles, given that the relationship of the particle size to the central wavelength is such that the modulations can be adequately sampled by the detection apparatus [33–35]. The results of such studies were again consistent with Mie theory.

In this work, we propose the use of visible light sOCT to create a hyperspectral image stack to visualize melanin in the rodent RPE based on the wavelength-dependent backscattering coefficient. To validate the results, phantoms consisting of various concentrations of melanin in silicone were created and imaged with the same system. We present both phantom and in vivo hyperspectral OCT images, and discuss the feasibility and limitations of this technique for in vivo melanin concentration measurements.

## 2 | MIE THEORY

To determine the suitability of hyperspectral OCT for detection of Mie-regime backscattering, simulations based on code by Mätzler [36] were performed. Both melanin
phantoms (melanin in silicone) and the RPE (melanin in retinal tissue) were simulated, using plane wave illumination across the whole visible light range. Although the experiments use Gaussian beam illumination rather than plane wave illumination, this results only in a slight shift in spectra but the modulations remain at the same frequency [34].

Since melanin is a strong absorber in the visible light range, the wavelength-dependent complex refractive index, \( n(\lambda) \), must be considered [37]:

\[
 n(\lambda) = n_r(\lambda) + i \kappa(\lambda),
\]

where \( n_r(\lambda) \) and \( \kappa(\lambda) \) are the real part and extinction coefficient of the refractive index, respectively.

The real part of the refractive index of melanin, \( n_{mel}(\lambda) \), is given as

\[
 n_{mel}(\lambda) = 1.6840 - 1.8723 \times 10^4 \lambda^{-2} + 1.0964 \times 10^4 \lambda^{-4} - 8.6484 \times 10^3 \lambda^{-6},
\]

across the wavelengths, \( \lambda \), in the visible light range [38]. The extinction coefficient, \( \kappa_{mel} \), was calculated from the absorption coefficient (\( \mu_a \)) of mouse RPE melanin taken from the superior retina of wild type mice [39] by

\[
 \kappa_{mel}(\lambda) = \frac{\mu_a(\lambda) \lambda}{4\pi},
\]

[40] and therefore an equation can be fitted to \( \kappa_{mel} \)

\[
 \kappa_{mel}(\lambda) = 9.452 \times 10^{-13} \lambda^4 - 2.452 \times 10^{-9} \lambda^3 + 2.447 \times 10^{-6} \lambda^2 - 1.133 \times 10^{-3} \lambda + 0.2124,
\]

across the visible light range.

The refractive index of the surrounding media was also required for the simulations. From data extracted from Figure 3 of Meichner et al. [41], the real part of the refractive index of the silicone elastomer in the visible light range, \( n_{sil} \), can be approximated as:

\[
 n_{sil}(\lambda) = 1.527 \times 10^{-7} \lambda^2 - 2.260 \times 10^{-4} \lambda + 1.494,
\]

and since the elastomer is considered transparent at visible wavelengths, the imaginary part of the refractive index was set to zero [41, 42].

For the in vivo (RPE) case, the real part of the refractive index of the surrounding medium was considered to be an average value of 1.368 ± 0.004 [43] and the imaginary part of the refractive index was also set to zero. While the non-

\[
 \mu_b \text{(\(\mu_m\))},
\]

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\[
 \mu_b \text{(\(\mu_m\))},
\]
As seen in Figure 2B, revealing a peak particle diameter of 2.4 μm.

To create the ball lens rodent eye phantoms as sketched in Figure 2C, melanin particles were first mixed with a small volume of silicone (SYLGARD 184; Sigma-Aldrich) and ground with a mortar and pestle to remove large agglomerates. This mixture, along with additional silicone to reach the desired volume, was then mixed for 2 minutes at 2000 rev/minute using a planetary mixer (ARE-250; Thinky Corporation). Following this, hardener was added at a 1:10 hardener:silicone ratio and the mixing process was repeated. To remove air bubbles, an additional mixing step (1500 rev/minute for 4 minutes) was performed. The mixture was then divided between three wells, and N-BK7 ball lenses of 3 and 6 mm diameter (43-711 and 32-746; Edmund Optics) were suspended in two of these wells before being left to dry for 24 hours. This process was performed six times for melanin concentrations ranging from 0 to 30 mg/mL, up to double the melanin concentration which was recently reported in RPE tissue [13].

To investigate the suitability of the N-BK7 ball lenses for focusing, the propagation of visible light through the ball lenses was modeled using CODE V (2017, Synopsis, Inc.). As the system is spherically symmetric, only the longitudinal chromatic focal shift was investigated. By choosing the reference wavelength to be 500 nm, the shift in focus varies by ±24 μm across the whole wavelength range as shown in Figure 2D. The simulations also showed that the spot size for each wavelength is diffraction limited, and therefore the Airy radius was used for the following calculations.

The confocal parameter, $b$, was calculated for each wavelength from 400 to 700 nm:

$$b = \frac{2\pi(w_0)^2}{\lambda},$$

where $w_0$ is the beam waist, or half of the $1/e^2$ diameter of the beam at its tightest focus. Figure 2E shows a plot of the confocal parameter at each wavelength (in 50 nm intervals), indicated by the length of the colored bars, while the focal points are
indicated by white lines. As highlighted in the shaded gray area, there is a region of approx. 65 μm where all wavelengths can be considered in focus, that is, their confocal parameters overlap. The focus was therefore set in this area during the experiments.

The data shown in Figure 2D,E represents the 3 mm ball lens. For the 6 mm lens, the shift in focus is greater (±45 μm), however as the confocal parameter at each wavelength is longer, the region of focus overlap in depth increases. A photograph of the final phantoms can be found in Figure 2F.

### 3.2 Hyperspectral OCT

Ball lens phantoms and the retinas of mice and rats were imaged using a modified version of the white light OCT system described in detail elsewhere [47]. A diagram of the system, as it was used for this study, can be found in Figure 3. A magenta color filter was added to the system to suppress the green portion of the spectrum, allowing a higher relative intensity at the edges of the spectrum (Figure 4A). The total incident power on the cornea was 0.9 mW. The system operated at an A-scan rate of 25 kHz, acquiring three-dimensional images (512 × 400 A-scans) with an en-face area up to 1 mm². To enhance the SNR in the rodent eye images, an additional two-dimensional scanning protocol of 30 repeated B-scans was introduced to allow averaging, and these data were used for the in vivo melanin visualization. The refocusing telescope was manually adjusted before each acquisition to ensure the tightest focus.

The acquired spectra firstly underwent background removal and reference intensity normalization before being remapped to be linear in wavenumber as shown in Figure 4B. The remapped data were then sub-sampled into 27 wavelength bands (central wavelength = 440 to 700 nm, steps of 10 nm, bandwidth constant in wavenumber to ensure a constant axial resolution in tissue [6 microns], see Figure 4C). Second- and third-order dispersion was compensated numerically for each wavelength independently [48] and each sub-band was subsequently Fourier transformed. This resulted in a hyperspectral OCT stack of 27 images per B-scan position, which were then manually axially registered (correcting for first-order dispersion). For illustration purposes, Figure 4D-F shows a representation of how the Mie backscattering coefficient is detected. As an example, the wavelength-dependent backscattering coefficient taken from Figure 1B for the 0.7 μm melanin particle diameter in the rodent RPE is displayed in Figure 4D. By multiplication of Gaussian windows at the selected wavelengths by the Mie backscattering coefficient (Figure 4E), it is expected that the modulations introduced by the melanin particles can be observed using hyperspectral OCT (Figure 4F).

### 3.3 Animals

Both eyes of male non-pigmented Oncins France Strain A (OFA) rats (N = 3, age = 13 weeks), pigmented Brown Norway (BN) rats (N = 3, age = 17 weeks) and mice from a C57BL/6 background (N = 3, age = 66 weeks) were imaged...
in this study. The animals were cared for by the staff at the animal facility of the Center for Biomedical Research, Medical University of Vienna. A 12-hour photoperiod was observed, with food and water ad libitum. For the experiment, the animals were anesthetized using an isoflurane/oxygen mixture (4% isoflurane for the first 4 minutes to induce anesthesia and 2% thereafter, flow rate of 2 L/minute) and pupils were dilated using topically administered tropicamide. The eyes were kept moist during the experiments using artificial tear drops. During measurement, the animals were placed in a homemade translational/rotational stage that enabled alignment of the eye with respect to the measurement beam. The duration of anesthesia was less than 45 minutes in total for each animal resulting in a fast wake-up process, with full mobility regained within approximately 1 minute. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Directive 2010/63/EU. Ethics protocols were approved by the ethics committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Research [BMBWF-66.009/0216-V/3b/2018 (mice); BMBWF-66.009/0183-V/3b/2018 (rats)].

3.4 | Image analysis

For hyperspectral image analysis, the maximum intensity projection (MIP) was first taken across all 27 wavelength images. A watershed transformation [49] was then performed on the MIP image to define catchment basins on the order of single speckles. To avoid oversegmentation, a minimum basin area of 8 μm² (corresponding to just below the image resolution) was defined. The catchment basins were used to segment the original 27 wavelength images and the mean intensity values within each basin were calculated for each wavelength. These steps were performed to remove the effect of the wavelength-dependent speckle patterns on the hyperspectral analysis.

To test for Mie behavior, the mean intensity value across all 27 wavelengths was calculated and then subtracted from each wavelength individually. If the backscattering of the sample was largely independent of the incident wavelength, that is, in the optically scattering region where the particle diameter is much larger than the wavelength, the intensity values should cluster around this zero line. However in the case of Mie backscattering, the intensity of the catchment basin varies as a function of wavelength, resulting in larger deviations from the mean (DFM) values. As a method of visualization, the numerical integral of the absolute value of the deviation intensity, $I_{dev}$, with respect to wavelength, $\lambda$, was calculated,

$$DFM = \int_{\lambda_1}^{\lambda_2} |I_{dev}(\lambda)|d\lambda,$$

with larger numbers corresponding to greater deviations. These numbers were mapped back to the watershed.
catchment basins from which they originated, in a DFM image. All calculations were performed on images with a linear intensity scale. A diagram of the image analysis pipeline can be seen in Figure 5.

3.5 | Electron microscopy

For ultrastructural analysis, eyes from pigmented mice (C57BL/6j; Jackson Laboratory, Bar Harbor, Maine) and rats (Brown Norway; Charles River Laboratories, Wilmington, Massachussetts) were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in Epon. Series of ultrathin sections (70-90 nm) were collected on copper grids, contrasted with lead citrate and uranyl acetate and viewed with an electron microscope (EM900; Zeiss). Images were acquired using a 1024 × 1024 pixels frame transfer CCD camera (Tröndle TRS, Moorweiss, Germany) and Image SP Software (SYS-PROG, Minsk, Republic of Belarus), stitched using the Distortion Correction Plugin [50] in ImageJ and adjusted for brightness and contrast with Adobe Photoshop CS6.

4 | RESULTS

4.1 | Phantom measurements

All melanin phantoms were imaged by the white light OCT system, and the hyperspectral analysis was performed on these images. A MIP across all 27 wavelengths can be seen in Figure 6A (10 mg/mL, 6 mm ball lens). It was immediately clear that the MIP contained many more particles than any individual wavelength. To demonstrate this visually, an RGB image was created (Figure 6B) consisting of three consecutive wavelength channels encoded into the three colors: 540 nm in blue, 550 nm in green and 560 nm in red. The lens-silicone surface appears more white than the melanin particles. This suggests that the backscattering of this surface is less wavelength-dependent than the particles, which would be consistent with Mie theory.

For further analysis, the watershed transformation was applied (Figure 6C) and the DFM image, shown in Figure 6D, was calculated. This again shows the low variation in backscattering coefficient at the lens-silicone interface, while highlighting the differences in the melanin granules. The difference in the relative OCT signal intensity as a function of wavelength between the surface line and the melanin granules can be seen in Figure 6E. Also displayed is one of the melanin granules at a selection of the individual wavelengths. Not only does the intensity change, but the apparent location and shape of the granule shifts too. This is due to the scattering profile of the individual granules, demonstrating the need for the watershed method.

After proving that it was possible to visualize the melanin granules, the possibility of determining the absolute melanin concentration using this method was investigated. Figure 7A-F shows MIP B-scans across all wavelengths for known concentration values ranging from 0 to 30 mg/mL. The corresponding DFM plots can be seen in
Figure 7G-L. For all concentrations, the same trend as in Figure 6 was observed whereby the surface line is less visible in the DFM images. For both datasets, the surface line was excluded and the percentage of pixels above 3× the standard deviation of the noise was calculated over 50 μm in depth (smaller than the confocal parameter of the mouse eye phantom). Figure 7M-R shows the thresholded DFM images, with pixels above the threshold in green. Figure 7S shows a map of the observed percentage of high intensity pixels based on both the MIP intensity data and the DFM plots. The data plotted is the mean and standard deviation over 400 B-scans. When considering the intensity alone, the observed concentration linearly increases as a function of actual concentration across the investigated range. However this is not the case for the DFM measurements. The observed concentration follows a linear trend (with a small offset) until around 10 mg/mL, but then starts to reach a plateau. This effect can also be seen qualitatively from the images.

4.2 In-vivo RPE melanin visualization

To test if the same effect could be observed in vivo, eyes of three pigmented BN rats (example can be found in Figure 8A-C), three non-pigmented OFA rats (example in Figure 8D-F) and three pigmented mice from a C57BL/6 background (example in Figure 8G-I) were also imaged with the white light OCT system. Figure 8A shows an example of a MIP across all wavelengths for the BN rat, while Figure 8B shows its corresponding DFM image. In the DFM image, the melanin-containing RPE is highlighted as a layer which shows deviation unlike the rest of the retina. Some regions of the choroid also appear to show increased DFM. These effects are also highlighted in the thresholded binary DFM image in Figure 8C.
The six OFA albino rat retinas were imaged to test if this effect was specific to melanin (example intensity B-scan shown in Figure 8D, DFM image in Figure 8E) and thresholded DFM in Figure 8F). Displayed on the same colorscale, the DFM image did not highlight the RPE in any of the images, which can be observed by the thresholded DFM image (Figure 8F). In Figure 8J, examples of the intensity as a function of wavelength for single watershed-created pixels can be seen. For the non-pigmented case (indicated by the red box in Figure 8D,E), the deviations are small and fluctuate around the mean intensity. However for the BN rat (indicated by the green box in Figure 8A,B) the deviations are much further from the mean intensity values.

Imaging was also performed on three pigmented mice from a C57BL/6 background (six eyes imaged, one eye excluded due to motion artifacts) to see if the melanin in the RPE could also be observed here (examples in Figure 8G-I). However in images of all five mouse retinas, the DFM signal in the RPE was very similar to that of the albino rat (example shown in Figure 8J with a pixel indicated by the white box in Figure 8G,H), indicating that it was not possible to detect the melanin with this technique. To investigate this

**FIGURE 7** A-F, Maximum optical coherence tomography (OCT) intensity projections across all wavelengths. (G-L) Deviation from mean (DFM) B-scans of melanin ball lens phantoms as acquired by hyperspectral OCT. The percentage (by area) of the image which contained pixels above 3× the standard deviation of the noise was calculated in both intensity and DFM. A binary representation of pixels above the DFM threshold can be seen in (M-R). S, Melanin concentration calibration graph. Error bars indicate standard deviation across 400 B-scans. While the intensity information shows a linear increase in concentration across the measured range, the DFM plot levels off at higher concentrations. Scale bars = 50 μm and apply in (A-R)
further, ultrastructural analysis of both the BN rat retina and the C57BL/6 mouse was performed. From the transmission electron microscopy images in Figure 9, it can be seen that the concentration of melanin granules in the mouse RPE is much greater than in the BN rat. When considering the laser spot size on the retina during OCT imaging (the lateral resolution is estimated to be approx. 2–3 μm), the beam would likely only hit one or two melanin granules per A-scan in the BN rat retina. This would not be the case in the mouse, as the laser beam would hit several granules of different sizes, each of which contributes to the observed modulations.

FIGURE 8 In vivo results of melanin visualization by hyperspectral optical coherence tomography (OCT). A, Intensity; B, Deviation from mean (DFM) and C, Thresholded DFM images in the Brown Norway (BN) rat retina. In the DFM plots, high signal is only observed in the retinal pigment epithelium (RPE) and the choroid, both of which are melanin-containing. D, Intensity; E, DFM and F, Thresholded DFM in the albino OFA rat retina. The DFM image in (E) does not contain many pixels above the threshold (F). G, Intensity; H, DFM and I, Thresholded DFM in the retina of a mouse from a C57BL/6 background (B6). The signal in the RPE is much less pronounced than in the case of the Brown Norway rat. J, Examples of relative intensity as a function of wavelength from within single watershed boundaries for the pigmented Brown Norway rat (from the region indicated with the green box in [A] and [B]), the albino OFA rat (from the region indicated with the red box in [D] and [E]) and the the pigmented mouse (from the region indicated with the white box in [G] and [H]). Scale bars = 100 μm

FIGURE 9 Transmission electron microscopy images of the Brown Norway rat retina (A) and the C57BL/6 mouse (B). Colors indicate layers: Purple Rod outer segments; Green Retinal pigment epithelium (RPE); Red Bruch's membrane; Blue Choroid. Numbers indicate features: 1 Red blood cell. 2 RPE cell nucleus. 3 Melanin granules. 4 Endothelial cell. 5 Mitochondria. 6 Apical microvilli. 7 Basal microvilli
Eumelanin is highly absorbing and therefore provides a strong contrast in photoacoustic tomography and PT OCT. Previous simulations have therefore focused mainly on the absorption characteristics, treating the whole RPE as one homogeneous layer [51]. However, this only applies in the limit of a high melanin concentration. Since eumelanin is a heterogeneous macromolecule comprising numerous cross-linked polymers (5,6-dihydroxyindole [DHI] and its 2-carboxylated form 5,6-dihydroxyindole-2-carboxylic acid) [52], it forms pigmented granules in the RPE which vary in melanin content. For low melanin concentrations and small probing beam diameters, the overall size and shape of the pigment is the dominating factor for changes in the backscattering properties. It is therefore important to consider methods for melanin visualization, also when the concentration is low.

Hyperspectral OCT has successfully demonstrated its ability to detect varying backscattering coefficients as a function of wavelength both in melanin phantoms and in vivo. The phantoms used in this experiment contained melanin particles which were similar in shape to those found in the rodent RPE, albeit a little larger on average. Nevertheless, they fell into the detection limits of the hyperspectral OCT system. To mimic the rodent eye, a ball lens was used to provide the focus. For the OCT system used in this study, the fact that the focus always falls behind the back surface of the ball lens was not a problem as the system contained an additional manually adaptable refocusing telescope. However for a system without this capability, a more sophisticated lens configuration would be required to achieve a tight focus.

A linear relationship between the pixels above the threshold in the DFM plots and the actual concentration of the sample indicates that with calibration, the concentration of melanin granules in the phantoms could be measured. In the phantom measurements, the concentration could also be calculated directly from the conventional OCT images, as the number of hyperscattering pixels also scales linearly with the concentration. At the lens-silicone interface, the DFM values are generally low indicating that the backscattering coefficient is largely independent of the incident wavelength. High DFM values at the interface are mostly attributed to saturation of the signal in the intensity images, although there will also be some melanin granules exactly at the boundary too. However, as the dominant cause of the high DFM values is the saturation, the interface line was excluded from all concentration measurements. In the in vivo case, the melanin is not the only scatterer present, demonstrating the need for the DFM method. The enhanced contrast given in the DFM images is assumed to be specific to the melanin, as there is no increased signal in the albino rat RPE. However the phantom measurements show that the DFM method reaches a saturation point—the values become generally lower at high concentrations. This indicates that the DFM images, and therefore also the ability to detect Mie scattering by hyperspectral OCT, is only applicable at low concentrations with the current apparatus. Using this method, it could also be possible to estimate the size of the particles by correlating the spectral shape back to the Mie theory simulations. It would, however, be challenging to correlate this directly as the size and shape varies from one particle to another, and it cannot be ruled out that the observed signal does not come from only one melanin granule. This could cause some aliasing artifacts. However for qualitative visualization purposes, as the modulations are still observed in the spectrum, the presence of melanin can be identified without knowing the exact dimensions of each individual granule. In order to make quantitative statements regarding the size and shape of the particles, a more sophisticated particle backscattering model would be required which includes all possible sizes and shapes of melanin granules in the RPE, for example by the MONTCAIRL software [53, 54].

In the in vivo rodent retinas, differences between albino and pigmented rodent RPE were identified, but only in the animals where the melanin concentration is low as is the case in the BN rat. Owing to motion artifacts, image averaging becomes more challenging and therefore the modulation signal is generally less pronounced when compared to the phantoms, but the effect can still be observed. The example spectra shown in Figure 8J show a higher intensity of modulation for the BN rat than for the non-pigmented rat or the mouse. The spectrum in this example shows an increased intensity after 640 nm, perhaps corresponding to a melanin granule similar in size/shape to the 1 μm spherical granule shown in Figure 1B. Each individual granule, however, displays a different spectral signature, corresponding to the different sizes and shapes of the granules.

As the entire spectral information is acquired simultaneously in spectral domain OCT, only a single measurement is required to obtain each hyperspectral depth profile. No further spectral image registration is required other than that caused by dispersion. This results in a hyperspectral imaging stack of all retinal layers—not only the RPE. Consequently, the spectral data could be used for detection of other chromophores, such as oxy- and deoxy-hemoglobin in the blood vessels. Red blood cells themselves are too large to produce a pronounced Mie effect in this wavelength range and the absorption of single pixels within the vessels is not strong enough to increase the DFM above the threshold. However since the spectral information is already present, a more traditional spectroscopic analysis could be applied to the data for this purpose [29–31].
There is also some DFM signal from the choroid in the BN rat, but not in the other rodents. This is most likely due to a combination of the increased density of the melanin in the choroid as compared to the RPE (see Figure 9), the change in size of melanin particles between the regions (in the mouse) and the increase in light penetration to the choroid in the BN rat due to the lower RPE melanin concentration. In order to improve the resolution of the images to see this effect in more pigmented RPEs (such as in the mouse), or in the choroid, higher axial, lateral and spectral resolution would be required. A higher spectral resolution would increase the range of particle diameters which could be observed, which may also be useful in other pigment-containing tissues. However there is an inherent trade-off between axial and spectral resolution in OCT. With the broad bandwidth used in this study, filtration by 27 Gaussian windows was sufficient to observe the modulations while maintaining a high enough axial resolution (6 μm) to resolve the RPE. Methods to overcome this limitation have also been proposed [55–57] and could also be applied in similar studies. Since the lateral resolution is dependent upon the numerical aperture of the imaging lens, i.e. the rodent eye itself, it will differ slightly from one animal to another. In this system, it is estimated to be 2–3 μm, however this will also vary as a function of wavelength and therefore contribute to changes in the speckle pattern throughout the hyper-stack. Integrating adaptive optics into the system could help to solve this [58], although this would add further complexity to the system.

More generally, this study raises further questions for OCT imaging. If the acquired OCT spectra consist of irregular sample intensity across the wavelength range, this would reduce the axial resolution and perhaps cause side lobes to be present in the images. The speckle shape is also different for each wavelength, and the point spread function is modulated in depth [35], making traditional hyperspectral image analysis techniques more challenging in hyperspectral OCT. Pixel-based approaches are not suitable as the intensity data per single pixel does not give an accurate representation of the total backscattered intensity. For this study, the watershed segmentation method was utilized to overcome this limitation. Although a well-established technique in image segmentation [59], the application of the watershed transformation to OCT data is relatively new [60, 61]. This method is, however, sensitive to speckle. The speckle patterns were suppressed in this study by using the MIPs over the 27 independent wavelengths. Application of the watershed transformation to standard OCT images would require another means of speckle reduction, for example the Bayesian non local means filter as used by Girish et al. [60].

Owing to the spectrometer configuration, the recorded data was not measured linearly in wavenumber. This resulted in a wavelength-dependent sensitivity and roll-off which also had to be corrected for. This could be improved by designing a spectrometer based on a grating-prism setup [62–64]. Choosing to sample the spectra to be linear in wavelength for the hyperspectral images also resulted in a lower system sensitivity to higher frequency Mie modulations at shorter wavelengths, as the wavenumber sampling density increases with increasing wavelength. The non-linear wavenumber sampling is, however, also an advantage. In the case of aliasing, some modulation is still very likely to be observed and while this would not be suitable for particle size estimation, qualitative identification of the particles is still possible. While the image analysis method was designed to remove signal contributions due to laser source noise and spectrometer noise, the DFM method is sensitive to the wavelength-dependent sensitivity roll-off which occurs due to the unequal wavenumber sampling. To minimize such effects in vivo, the images are acquired using enhanced depth imaging [65], that is, the RPE is closest to the zero delay, with the inner retina further away.

When considering RPE melanin concentration alone, the mouse may be a better model of the human than the BN rat as the concentration is higher [3]. For visualization of melanin in these high concentrations, imaging methods based on melanin absorption and/or depolarization may be more appropriate than those based on backscattering. Nevertheless, there is not yet a consensus on the absorption, scattering and depolarizing properties of melanin in the RPE, and the average melanin concentration of the human RPE quoted in literature varies by up to three orders of magnitude [44, 51, 66]. In order to develop a greater understanding of the complex relationship between the melanin in the RPE and visual function, a detailed optical analysis consisting of absorption, backscattering and polarization analysis is required, and this study is a step towards that goal.

6 | CONCLUSIONS

It has been shown that with some prior knowledge of the granule size range and at low concentrations, it is possible to detect the presence of melanin using hyperspectral OCT. This was demonstrated both in phantoms and also in vivo in the BN rat retina. For the CL57BL/6 mouse, the melanin granules are too densely packed for the resolution of the system, but in that case it is likely that the absorption/depolarization properties dominate over the backscattering properties and therefore methods such as photoacoustic tomography may be more suitable for concentration measurements. Nevertheless, in order to fully understand the signals which come back from in vivo retinal imaging systems, a complete model consisting of absorption, scattering and polarization properties is required.
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CONFLICT OF INTEREST

The authors have no potential conflict of interest to disclose.

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5. AN INTERLUDE ON HYPERSPECTRAL CONFOCAL MICROSCOPY

The work presented in this chapter is only a pilot study, and therefore at the time of writing, the results have not been submitted for publication in a peer-reviewed journal. A conference abstract, presented at the ARVO Imaging in the Eye 2019 conference in Vancouver, is already freely available [125].

Introduction

Hyperspectral imaging of the retina has mostly been limited to 2D imaging techniques based on reflectometry [126] or fundus photography [127]. The recent emergence of supercontinuum lasers in the visible light range has now made it possible to construct sOCT systems which can spectroscopically sample the retina in 3D, while maintaining a sufficiently high axial resolution [50]. Although sOCT allows depth-resolved, wavelength-dependent reflectivity images to be reconstructed, it is subject to errors caused by a combination of wavelength dependent sensitivity roll-off, absorption/scattering properties of the layers above the layer of interest, and the efficiency of the OCT detection spectrometer at each individual wavelength. In this study, we reveal the spectroscopic properties of individual retinal layers of the C57BL/6 mouse measured both in vivo with white light hyperspectral OCT, and ex vivo by hyperspectral confocal microscopy (HCM). Furthermore, using HCM, we compare the spectral reflectivity of the ex vivo retinal layers of the heavily pigmented C57BL/6 mouse to those of the albino.

Materials and methods

In vivo hyperspectral optical coherence tomography

Both eyes of mice from a C57BL/6 background (N = 3, age = 66 weeks) were imaged using a white light optical coherence tomography system with an additional hyperspectral post-processing step [38,124]. Following image acquisition, the spectral data were filtered in 10 nm intervals by Gaussian windows with center wavelengths of 470
– 670 nm, sacrificing axial resolution in favor of spectral information. The bandwidth of each window was such that an axial resolution of 6 µm in retinal tissue was maintained. After anesthetizing the animals using a mixture of isoflurane and oxygen, tropicamide was topically administered for pupil dilation. Artificial tear drops were then applied to keep the eyes moist during the experiment. All experiments abided by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Directive 2010/63/EU. The ethics protocol (BMBWF-66.009/0216-V/3b/2018) was approved by the ethics committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Research.

**Ex vivo hyperspectral confocal microscopy**

The cornea and lens of C57BL/6 and albino (SWISS LTK, line 1033) mice were removed from the eye and radial cuts were made to flatten the eyecup. The neural retina was lifted off from the retinal pigment epithelium, which was still attached to the underlying choroid and sclera, and both tissues were whole-mounted in 70% glycerol and coverslipped. Both neural retina samples and RPE/choroid samples were imaged using a white light laser confocal microscope (Leica TCS SP8) in reflection mode with a 20x multi-immersion objective. The samples were illuminated consecutively with light from 470 – 670 nm in 10 nm intervals throughout the whole sample at 0.5 µm intervals in depth, over an area of 0.6 mm x 0.6 mm. The hyperspectral spectra were then analyzed on a layer-by-layer basis in order to be able to characterize the wavelength-dependent spectra in each retinal layer.

**Results**

Hyperspectral data cubes (the raw intensity of which can be found in Fig. 5.1A for OCT and Fig. 5.1B for HCM) were manually segmented into layers and the normalized mean intensity for each layer was found for every wavelength. As the RNFL/GCL layer and the RPE both reflect more light than the rest of the neural retina, the gain of the confocal microscope was adjusted accordingly when imaging these layers with HCM.

Figure 5.2A shows the normalized spectra as seen in vivo by OCT. The general trend reveals that all retinal layers exhibit a dependence on wavelength, with higher reflectivity at longer wavelengths. This data does not agree with what we see in the ex vivo case, as in Fig. 5.2 B. Here the most interesting feature is the peak at 580
Fig. 5.1. A) 3D OCT reconstruction (log scale). B) 3D HCM reconstruction (linear scale with each of the three sections acquired with a different gain). As the retinal nerve fiber layer/ganglion cell layer (RNFL/GCL) and the retinal pigment epithelium (RPE) both reflect more light than the rest of the neural retina, the gain of the confocal microscope was adjusted accordingly when imaging these layers.

nm which is observed in all layers. It is possible that this is related to the off-white color we observed in the tissue following fixation. In Fig. 5.2 C, the ex vivo HCM data from the pigmented mouse was compared to the albino. As expected, there are very large differences in spectral signatures from the RPE (not shown), however it is also possible to differentiate spectroscopically between the plexiform and nuclear layers. In order to be able to visualize the data, OCT (Fig. 5.2 D) and HCM (Fig. 5.2 E) data were combined into an RGB image, with R = 610 – 670 nm, G = 540 – 600 nm and B = 470 – 530 nm.

Conclusion

The spectral profiles of the mouse retinal layers were investigated with both OCT and HCM. As a preliminary study, it would appear that the in vivo data does not yet correlate well to what is observed ex vivo, and therefore caution should be exercised when considering both in vivo and ex vivo spectral profiles. Future work is required to identify the source of these discrepancies, and therefore to find spectral data for the retinal layers which can be agreed upon. Since preclinical in vivo imaging methods
Fig. 5.2. A) Normalized spectral data as acquired in vivo by OCT. B) Normalized spectral data as acquired ex vivo by HCM. C) Spectral differences between the pigmented and albino mice (ex vivo). The plexiform layers can be separated from the nuclear layers. D) RGB image representation of an en face projection of 3D OCT data (pigmented). Blood vessels are highlighted in red. E) RGB image representation of an en face projection of 3D HCM data (pigmented, not including RPE). It can be observed visually that the neural retina exhibits a weak wavelength dependence. **RNFL** retinal nerve fiber layer. **GCL** ganglion cell layer. **IPL** inner plexiform layer. **INL** inner nuclear layer. **OPL** outer plexiform layer. **ONL** outer nuclear layer. **RPE** retinal pigment epithelium.

are often later correlated to histology, this study could be a step towards finding corrections which allow reliable in vivo depth-resolved hyperspectral retinal analysis.
6. MULTI-CONTRAST OPTICAL COHERENCE TOMOGRAPHY

In parallel to the work with white light optical coherence tomography, a longitudinal study was being conducted into the retina of an APP/PS1 mouse model of Alzheimer’s disease using the system first described in [128]. With a central wavelength of 840 nm and a FWHM bandwidth of 100 nm, this system offered an axial resolution of 3.8 µm in retinal tissue. The multi-contrast nature of this system provided standard reflectivity tomograms, polarization-sensitive tomograms and OCT angiograms of each mouse retina. With a single image acquisition (lasting ~15 seconds), simultaneous information on retinal layer thickness and structure, presence of hyper-reflective foci (HRF), phase retardation abnormalities and retinal vasculature was collected. OCT images of both eyes of 24 transgenic mice and 15 age-matched wild-type littermates were analyzed both quantitatively and qualitatively, providing a detailed documentation of the in vivo observations in this mouse model. The tri-fold OCT images were then compared 1:1 to histological analysis involving an advanced preparation and staining protocol, and also to the Aβ plaque load in the brain.

All results were then combined into a manuscript entitled “Retinal analysis of a mouse model of Alzheimer’s disease with multi-contrast optical coherence tomography”. At the time of submission, this manuscript has been submitted to a peer-reviewed journal, but is still under review. In the meantime, a pre-print version can be found on arXiv [129], the PDF file of which can be found on the following pages.
Retinal analysis of a mouse model of Alzheimer’s disease with multi-contrast optical coherence tomography

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Abstract. Recent Alzheimer’s disease (AD) patient studies have focused on retinal analysis, as the retina is the only part of the central nervous system which can be imaged non-invasively by optical methods. However as this is a relatively new approach, the occurrence and role of pathological features such as retinal layer thinning, extracellular amyloid beta (Aβ) accumulation and vascular changes is still debated. Animal models of AD are therefore often used in attempts to understand the disease. In this work, both eyes of 24 APP/PS1 transgenic mice (age: 45-104 weeks) and 15 age-matched wildtype littermates were imaged by a custom-built multi-contrast optical coherence tomography (OCT) system. The system provided a combination of standard reflectivity data, polarization-sensitive data and OCT angiograms. This tri-fold contrast provided qualitative and quantitative information on retinal layer thickness and structure, presence of hyper-reflective foci, phase retardation abnormalities and retinal vasculature. While abnormal structural properties and phase retardation signals were observed in the retinas, the observations were very similar in transgenic and control mice. At the end of the experiment, retinas and brains were harvested from a subset of the mice (14 transgenic, 7 age-matched control) in order to compare the in vivo results to histological analysis, and to quantify the cortical Aβ plaque load.

1 Introduction

It is hypothesized that cerebral changes precede Alzheimer’s disease (AD) symptom presentation by over 20 years.1 Since the beginning of the millennium, the number of deaths from AD in the United States has increased by 145%. For comparison, the number of deaths caused by heart disease (the number one cause of death in the United States) has decreased by 9% in the same time period.2 AD is a chronic and irreversible neurodegenerative disorder with no current cure. The time delay between the start of the disease and the presentation of symptoms means that the disease is already at an advanced stage before it can be detected, and even when a patient presents with AD symptoms, a definitive AD diagnosis still remains challenging.

Post mortem diagnosis of AD is realized by the positive histological identification of both extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tau tangles, which are both found in the brains of AD patients.3,4 A key step along the road to an early AD diagnosis would be an in vivo identification of Aβ plaques. However as the plaques are small (ranging from 10-200 µm3) and located within the brain, this presents some logistical difficulties.

One recent idea to circumvent these difficulties is the use of the eye “as a window to the brain”. The retina and the brain are derived from the same embryological origin; they both extend from
the neural tube. The retina is therefore the only part of the central nervous system which can be imaged non-invasively by optical methods. However the question still remains whether the retina can hold the key to early AD diagnosis as there are few studies which directly correlate findings in the retina to those in the brain. Those studies which have been done before have found a correlation between the amplitude of retinal vascular pulsatility and neocortical Aβ scores (measured using florbetaben positron emission tomography), and also between fluorescent components (measured using fluorescence lifetime imaging ophthalmoscopy) and both p-tau181-protein concentration in the cerebral spine fluid and the mini-mental state examination score.

Recent studies have also focused on the identification of extracellular Aβ accumulations in the retina of AD patients, however there are conflicting reports on this topic. Some reports have identified extracellular Aβ in the retina, however other groups have demonstrated that there was no Aβ to be found. Another topic of current debate is whether AD could also be considered a vascular disorder, and therefore the retinal vasculature of AD patients has also recently been studied. Patients with AD may exhibit more tortuous retinal vessels. Narrowing of retinal blood vessels and reduced venous blood flow rates have also both been found in AD patients, and an overall more sparse retinal microvascular network has been observed. A recent OCT angiography (OCTA) study has shown a reduced vessel density specifically in the superficial capillary plexus. It has also been suggested that blood flow changes may precede neurodegeneration.

While the aforementioned studies have all demonstrated a reduction in blood circulation in the retina in late stage AD, recent studies have indicated that the retinal vessel density and vessel diameter both seem to be increased in individuals suffering from preclinical AD. Such results are consistent with the theory that an inflammatory response occurs in the retina in the early stages of AD - a theory which has also been proposed for neurovasculature.

Retinal layer thinning, particularly in the retinal nerve fiber layer (RNFL), is also present in the retina of AD patients. However looking forward to a marker for diagnosis, RNFL thinning is not specific to AD and it is not only associated with other diseases such as glaucoma and Parkinson’s disease, but also more generally with increasing age.

With many contradictory observations, it is clear that there is still a great deal of research to be performed in order to fully understand the effects of AD on the retina. Some attempts to do this have focused on the use of mouse models of the disease. When performing studies on animal models, it is important to know where the similarities and differences to the human disease lie. While there are many mouse models of AD, this work focuses on a doubly transgenic model which expresses a chimeric mouse/human amyloid precursor protein (APP) and a mutant human presenilin 1 (PS1). The following paragraphs describe the retinal changes observed in this transgenic APP/PS1 mouse model so far.

Much like in the case of the human, while well documented in the brain, the appearance of extracellular deposits of Aβ in the retina is disputed. Several studies have reported no extracellular deposits of Aβ in the retina, despite plaques being present in the brain and an increased expression of APP in the retina, similar to what has been found in humans. This has been reported in mice of several ages: 9 months old, 7-12 months old and 13 months old. It has been suggested that the nonamyloidogenic pathway may endogenously limit Aβ formation in the retina. A further extensive histological analysis of the retina concluded that no identifiable retinal pathology exists in these mice. Conversely, extracellular deposits of Aβ were found at the age of 27 months old in the choriocapillaris and the nerve fiber layer, but not in the other layers. In another study, plaques were found in the inner plexiform layer and in the outer plexiform layer. These plaques ranged
in size from 5-20 µm in mice of 12-13 months old, and larger with increasing age.\textsuperscript{44} This study also reported no observed changes in retinal layer thickness. Deposits were also found distributed throughout the retina of transgenic mice at the age of 9 months and 17 months, but were identifiable as young as 2.5 months, even before the plaques appeared in the brain.\textsuperscript{9}

There is therefore a need for more studies linking the retina and the brain in both AD patients and in animal models of AD,\textsuperscript{45} and more work needs to be done to assess and quantify the presence of $A\beta$ in the retina.\textsuperscript{46} Optical coherence tomography (OCT)\textsuperscript{47} may be a useful tool to employ for this purpose. As a non-contact, non-invasive imaging modality, OCT has become part of clinical routine for in vivo retinal diagnostics. Functional extensions of OCT have made it possible to not only visualize contrasts based on backscattered intensity (reflectivity), but also motion (OCTA)\textsuperscript{48–50} and polarization properties (PS-OCT) such as birefringence.\textsuperscript{51–54} The birefringence of $A\beta$ plaques has been studied in detail using polarimetry,\textsuperscript{55–58} and also with PS-OCT.\textsuperscript{59,60} The plaques appear as hyper-scattering structures in standard reflectivity OCT images,\textsuperscript{51–63} but the addition of polarization-sensitive detection provides an additional tissue-specific contrast. Furthermore, the combination of PS-OCT with OCTA allows a simultaneous analysis of this tissue-specific contrast and changes in the retinal vasculature.

In this work, the appearance of the retina of an APP/PS1 mouse model of AD was evaluated by multi-contrast spectral domain OCT. By observing retinal changes over a range of ages (45-104 weeks) in intensity-, polarization- and motion-based contrast modes, the observations in the retina were documented, mapped directly to histology, and compared to the $A\beta$ plaque load in the brain.

\section{Materials and Methods}

\subsection{Optical coherence tomography}

A modified version of a PS-OCT system described elsewhere was used in this study.\textsuperscript{64} In brief, the system operated at a central wavelength of 840 nm with a full width at half maximum bandwidth of approximately 100 nm, resulting in an axial resolution of around 3.8 µm in retinal tissue. Light incident upon the mouse eye was of a known polarization state, and the polarization-sensitive detection allowed for the differentiation between polarization-preserving tissue and polarization-altering tissue.

An additional refocusing telescope was added to the system to correct for aberrations induced by poor focus of the mouse eye itself.\textsuperscript{65} A diagram of the modified version of the system can be found in Fig. 1. Two additional achromatic doublet pairs ($2 \times$ AC254-080-B, Thorlabs and $2 \times$ AC254-050-B, Thorlabs) were mounted on a translational stage, allowing the focus to be manually optimized for each individual mouse eye while reducing the beam diameter incident on the pupil from 0.8 mm to 0.5 mm. Each mouse eye was aligned with respect to the 2.85 mW measurement beam to ensure the optic nerve head (ONH) was at the center of the 1 mm $\times$ 1 mm field of view. With an A-scan rate of 83 kHz, five repeated B-scans (consisting of 512 A-scans each) were acquired at 400 unique locations. Such a scan pattern allowed for an increased signal-to-noise ratio (SNR) in the reflectivity and PS-OCT images, and also the ability to produce OCTA images.

\subsection{Mice}

A breeding pair of APP/PS1 mice (APPswe, PSEN1dE9 MMRRC stock number 34829-JAX) was purchased from The Jackson Laboratory (Bar Harbor, ME, USA),\textsuperscript{34–36} and a breeding colony was
established at the Division of Biomedical Research at the Medical University of Vienna. Hemi-
zygous mice were bred with wildtype siblings and subsequently kept under controlled lighting
conditions (12 hours light, 12 hours dark) with food and water ad libitum. Both eyes of 24 mutant
mice (17 female, 7 male) and 15 wild-type littermates (9 female, 6 male) were imaged using the
multi-contrast OCT system. At the time of imaging, the mice ranged in age from 45 weeks to
104 weeks. During the experiment, the animals were anesthetized using an inhalational isoflu-
rane/oxygen mixture (4% isoflurane for 4 minutes in an induction chamber to induce anesthesia,
2% delivered via a nose cone thereafter). To facilitate the OCT imaging, pupils were dilated using
topically applied tropicamide and phenylephrine. The cornea was kept moisturized using artificial
tear eye drops, and heating pads were placed underneath the mice to prevent a reduction in body
temperature. All experiments were performed in accordance with the ARVO Statement for the Use
of Animals in Ophthalmic and Vision Research and Directive 2010/63/EU. Ethics protocols were
approved by the ethics committee of the Medical University of Vienna and the Austrian Federal

2.3 OCT image analysis

The analysis performed in this work was based on a previously-published multi-contrast image
processing pipeline including standard intensity-based reflectivity contrast, polarization-based con-
trast and motion-based angiographic contrast. Prior to analysis, the images were corrected for ax-
ial motion and the retina was flattened with respect to the retinal pigment epithelium (RPE)/choroidal
complex as detected by the cross-polarized channel, a technique made possible by the polarization
sensitive detection. Any dataset for which the retinal flattening failed (due to poor signal-
to-noise ratio) was excluded, resulting in a total of 72 datasets for evaluation (44 eyes from 24
transgenic mice and 28 eyes from 15 wildtype control mice). A graphical visualization of the
Fig 2  Histogram representations of the number of eyes used for analysis. A total of 44 eyes from 24 APP/PS1 transgenic mice (a) and 28 eyes from 15 wildtype littermates (b) were imaged within an age range of 45 weeks to 104 weeks.

age of the mice at each measurement can be found in Fig. 2. A flow chart of the overall post-processing pipeline can be seen in Fig. 3, and a description of the analysis can be found in the following sections.

2.3.1 Retinal thickness analysis

To allow for a comprehensive retinal thickness analysis, layer segmentation of the retina was first performed using a previously-described algorithm. The distance between the inner limiting membrane (ILM) and the posterior surface of the RPE was defined as the total retinal thickness. The posterior surface of the outer plexiform layer (OPL) served as the boundary between the inner and outer retina, as defined in this manuscript. An annulus around the optic nerve head was created with an inner diameter of 500 µm and an outer diameter of 900 µm, and the mean total, inner and outer retinal thicknesses were evaluated within this annular 3D volume. The annulus was then cut in half transversely and the mean thicknesses were again calculated in the two resulting regions, corresponding to the superior and inferior retina. Due to mouse eye alignment, it is estimated that this boundary line is accurate to within ± 30°. Coordinates of the segmentation lines and the ONH annuli were stored for later use in OCTA analysis.

To test for statistical significance, the retinal thickness measurements were plotted as a function of age for both the transgenic and the wildtype mice. A linear regression pre-test was performed to determine if the thickness measurements were dependent on age. If the results were deemed significant ($p < 0.05$), an analysis of covariance (ANCOVA) was performed to test for a difference in the trend between the two groups. In the case where the pre-test was deemed statistically insignificant ($p \geq 0.05$), regular analysis of variance (ANOVA) was performed to test for a difference in the group means. The gradient of the slope of the regression lines were also documented for both transgenic and wildtype mice, corresponding to a measurement of reduction of retinal thickness in units of µm per week.
Fig 3 A flow chart of the multi-contrast optical coherence tomography (OCT) post-processing pipeline which consists primarily of reflectivity, phase retardation and angiography data. HRF Hyperreflective foci.
2.3.2 Hyperreflective foci analysis

All 72 reflectivity datasets (44 eyes from 24 transgenic mice and 28 eyes from 15 wildtype control mice) were manually screened for hyperreflective foci in the posterior layers of the retina, spanning the region from the posterior OPL border to the posterior RPE (i.e. the whole outer retina). The hyperreflective foci were then manually segmented using ITK Snap. Using the data from this segmentation, the number and location of hyperreflective foci was evaluated for each eye.

2.3.3 Polarization properties

The phase retardation images were calculated for every B-scan by

\[ \delta = \arctan \left( \frac{A_V}{A_H} \right) \]  

(1)

where \( A_V \) and \( A_H \) correspond to the signal amplitudes of the co- and cross-polarized channels, respectively.\(^{51, 69}\) In the healthy mouse retina, high retardation values are only expected in the melanin-containing regions, namely the RPE, the choroid, and the remnants of the hyaloid artery near the ONH. Depolarization occurs due to the fact that the melanin granules scramble the polarization state of the incoming light beam, resulting in random phase retardation values. Polarization-preserving tissues, i.e., the rest of the retina, do not retard the phase of the incident beam, and therefore the phase retardation values are low. All retardation B-scans were manually inspected for abnormally high retardation signals from outwith these retinal layers. The number of these abnormally high retardation signals was evaluated, and the sources were investigated with retinal histology.

2.3.4 OCT angiography

The B-scan repetition allowed for the computation of OCTA images, revealing locations of motion contrast. After bulk motion compensation and removal of frames with uncorrectable motion, OCTA images were computed by calculating the averaged magnitude of the complex differences between consecutive repeated B-scans. The time delay between the acquisition of repeated B-scans (\( \approx 7.7 \) ms from one B-scan to the next) which provides the angiographic contrast in the first place also makes the method very susceptible to motion. All datasets were therefore manually visually screened, and data which included severe motion artefacts or regions of poor angiography signal were excluded. The angiography analysis was therefore performed on 39 transgenic eyes and 16 wildtype eyes.

An automated OCTA processing pipeline was created which consisted of several steps. The superficial vascular plexus (SVP) and the deep capillary plexus (DCP) were segmented from the retina using the layer segmentation coordinates obtained from the reflectivity data (SVP corresponds to the RNFL; DCP corresponds to the OPL). A maximum intensity en-face projection was then calculated over the SVP and the DCP independently, and the histograms of each image were equalized using contrast limited adaptive histogram equalization \(^{70}\) before image binarization. The binary images were then morphologically opened and closed (disk-shaped structuring element with a radius of 1), and skeletonized to remove speckle noise and enhance the vessel connections. A square averaging filter with a 5-pixel side length was then applied to the images to create the final binary vessel representations of “vessel” vs. “non-vessel”. The annuli around the ONH which had
been calculated using the reflectivity data were then applied to these vessel maps. The vessel density was then calculated as the percentage of pixels which were marked as “vessel” in the whole annulus, as well as in the superior and inferior retinal regions.

After this image processing was performed, a modified version of the Weber contrast was calculated to test the relationship of the OCTA vessel intensity to the background, using the binary image as a mask. The mean intensity value of all pixels determined both “vessels” \( T_v \), and “non-vessels” \( T_b \), was calculated, and the modified Weber contrast, \( C_W \) was calculated as

\[
C_W = \frac{T_v - T_b}{T_b}
\]

and the results were plotted as box-and-whisker diagrams for both transgenic and wildtype mice.

### 2.4 Histology and immunostaining

After OCT imaging, a sub-group of the mice (14 transgenic, age: 54-104 weeks, 7 wildtype control, age: 54-103 weeks) were euthanized by cervical dislocation. Immediately after sacrifice, the brains were extracted and the eyes were enucleated for histological analysis.

#### 2.4.1 Brain

The mouse brains were sagittally cut into two hemispheres, and one hemisphere was prepared for histopathological workup. The samples were fixed in 4% formalin and processed through graded alcohols and xylene into paraffin. Sagittal brain sections with a thickness of 2.5 \( \mu m \) were cut on a microtome, deparaffinized, rehydrated, and stained immunohistochemically using an anti-A\( \beta \) antibody (clone 6F/3D, diluted 1:100, Dako). The sections were evaluated using a slide scanner (Hamamatsu NanoZoomer 2.0 HT) and saved for digital pathology. The images were analyzed using Fiji. First, the cortex was manually selected and the “ColSeg” tool was utilized to segment the plaques by their brown color. The “analyze particle” tool was then used to count the plaque number and calculate the plaque load in plaques per \( \text{mm}^2 \). The plaque load was then plotted as a function of mouse age, and a linear regression analysis was performed on the data.

#### 2.4.2 Retina

To obtain vertical histological slices of the retina, six left eyes from wildtype (n=3) and transgenic (n=3) mice diagnosed with and without abnormal OPL banding (see Section 3.5) were immersed unopened in Davidson’s fixative for 24 hours at 4\(^\circ\)C and processed through graded alcohols and xylene into paraffin. Three-micron-thick sections were then cut, mounted onto slides, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E).

For wholemount preparation and A\( \beta \) immunostaining, eyes were immersed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.4. Some eyes were fixed unopened. In the others, cornea and lens were removed and the eyecups fixed in PFA for at least 24 h at room temperature. After rinses in PBS, the retina was dissected free from RPE, choroid and sclera, cryoprotected in ascending sucrose concentrations (10%, 20%, 30%), and snap-frozen and thawed three times to increase antibody penetration. From each mouse, the left retina was treated with 70% formic acid for 10 minutes and then rinsed repeatedly in PBS, while the right retina was left untreated. Retinal wholemounts were processed free-floating in 24-well plates and all incubations and rinses were done with gentle rotation on a rocker table at 4\(^\circ\)C. Blocking of non-specific binding was
performed in 3% normal donkey serum in 0.1 M PBS, 0.25% Triton X-100 and 0.05% sodium azide (medium), followed by incubation with mouse anti-human Aβ (Abcam, ab11132, clone DE2B4, 1:400 in medium) for 72 h. After washes in PBS, retinas were incubated in donkey anti-mouse Fab fragments conjugated with Alexa Fluor 488 (Jackson ImmunoResearch Laboratories, 1:500 in medium) for 24 h, rinsed, and coverslipped (retinas ganglion cell side up) in Aqua/Polymount (Polysciences). To serve as positive and negative controls, respectively, brains from transgenic APP/PS1 mice and their wildtype littermates were harvested after enucleation and fixed in 4% PFA for 24 h at 4°C. After washes in PBS, brains were cryoprotected in ascending sucrose concentrations (10%, 20%, 30%), snap-frozen in liquid nitrogen-prechilled isopentane and cut into 100-µm-thick sections using a cryotome. The sections were collected in PBS/0.05% sodium azide and processed under the same conditions applied to retinal wholemounts. H&E-stained sections were examined with brightfield illumination on a Zeiss Axio Imager Z2. Immunofluorescence analysis was performed with a Zeiss LSM880 laser scanning microscope (LSM). A total of 17 retinas from 11 transgenic mice (age: 54-103 weeks) and 11 retinas from 6 wildtype control mice (age: 54-103 weeks) were suitable for detailed histological examination. A 1:1 correlation of the retinal wholemounts to the OCT image data was then performed by mapping the vessel pattern of the SVP as visualized by the LSM to the corresponding OCTA datasets.

3 Results

3.1 Hyperreflective foci

For each retina, the whole 1 × 1 mm² area surrounding the optic nerve head was evaluated. Of the 24 mutant mice, 16 showed HRF in at least one eye. In the wildtype littermate control group, HRF were identified in 12 out of the 15 mice. Figure 4a displays pie charts which document this in terms of eyes: there were an equal number of eyes with and without HRF in the transgenic mice, and a difference of only one in the wildtype mice. Since it is difficult to identify small HRF in the plexiform layers due to the appearance of hyperreflective blood vessels, a normalized probability distribution of all identified HRF in the outer retina alone was plotted (Fig. 4b). A similar HRF distribution in transgenic and wildtype retinas was observed. The number of HRF per eye was also counted for the transgenic (Fig. 4c) and the wildtype (Fig. 4d) mice. With the exception of one outlier in each group, all outer retinas contained less than 10 HRF within the investigated field of view. Qualitatively, the types of HRF also looked very similar between transgenic and wildtype mice, examples of which can be found in Fig. 4e-h. Figure 4e and Fig. 4f show examples of larger HRF located anterior to the ELM in the transgenic and wildtype animals, respectively, while Fig. 4g and Fig. 4h show smaller HRF in the middle of the ONL. Neither the number of HRF nor the size correlated with the age of the mice, for either group.

3.2 Retinal thickness

The total, outer and inner retinal thickness was calculated in an annulus around the ONH, and also for the superior and inferior (180 ± 30) degree sectors, resulting in nine thickness comparisons between transgenic and wildtype mice. The results of this analysis can be found in Fig. 5. When plotted as a function of age, all nine datasets displayed a general trend of decreasing retinal thickness with age for both the transgenic mice and the wildtype controls. Statistical pre-tests revealed that the dependence upon age was significant for all data except three wildtype datasets: the total outer retinal thickness (p = 0.075), the superior outer retinal thickness (p = 0.124) and
Fig 4  Results of hyperreflective foci (HRF) analysis. a) Pie charts indicating the number of eyes with and without HRF for both transgenic and wildtype mice. b) HRF probability distribution displayed with respect to outer retinal layer position for transgenic and wildtype mice. The distributions are very similar, with most HRF appearing near the external limiting membrane (ELM). **ONL** outer nuclear layer. **IS** inner segments. **OS** outer segments. **RPE** retinal pigment epithelium. c) Histogram of HRF occurrence in transgenic mice. d) Histogram of HRF occurrence in wildtype mice. e-h) Some examples of the appearance of HRF in OCT reflectivity images. Each image is a maximum intensity projection over four consecutive B-scans, where each B-scan is already averaged 5 times and plotted on a log scale. e-f) HRF located above the ELM in both the transgenic mouse retina (e) and in the wildtype retina (f). g-h) HRF located in the middle of the ONL in both the transgenic mouse retina (g) and in the wildtype retina (h). Ages of mice in weeks (w) are indicated on sub-figures (e-h).
Fig 5  Analysis of retinal thickness as a function of age for both transgenic and wildtype mice. a-c) Total retinal thickness measured around the whole annulus (a), then subdivided into a superior half (b) and an inferior half (c). d-f) Outer retinal thickness measured around the whole annulus (d), in the superior half (e) and in the inferior half (f). g-i) Inner retinal thickness measured around the whole annulus (g), in the superior half (h) and in the inferior half (i). The corresponding statistical evaluation can be found in Table 1, and the gradients of the slopes can be found in Table 2.

the inferior inner retinal thickness ($p = 0.101$). For these three datasets, the comparison between transgenic and wildtype data were analyzed with ANOVA, while all other analysis was performed with ANCOVA. No statistical significance was found between the retinal thickness changes in the transgenic and control groups, for any of the retinal regions. The results of the statistical analysis can be found in Table 1. Using the gradient of the slope of the linear regression analysis, a measurement of the decrease of retinal thickness was documented in units of µm per week, as shown in Table 2. All trends were negative, therefore the gradient of each slope is the negative of the value in the table.
Table 1 Statistical evaluation for the retinal thickness analysis. All values displayed are p-values, and significance is defined as $p < 0.05$. ■ indicates pre-test p-values for retinal thickness as a function of age for the transgenic group. □ indicates pre-test p-values for retinal thickness as a function of age for the wildtype group. Pre-test p-values were calculated using linear regression analysis. \( \therefore \) indicates p-values from ANCOVA comparing the trends of the transgenic retinal thickness vs. age to the wildtype retinal thickness vs. age. \( \therefore \) indicates values where the initial pre-tests failed, and therefore one-way ANOVA was performed to test for significance between the means of the two groups. There were no statistically significant changes in retinal thickness between the transgenic and the wildtype groups.

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Table 2 Decrease in retinal thickness in units of $\mu$m per week. ■ indicates the values for the transgenic mouse and □ indicates the values for the wildtype mouse.

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3.3 OCT angiography

Following layer segmentation of the SVP and the DCP, the vessel density was quantified in these layers in the total, superior and inferior retina for both transgenic and wildtype mice. Figure 6a shows a typical OCT angiogram from the SVP of a transgenic mouse. Combining its binarized
form (Fig. 6b) with the annulus which was defined from the reflectivity data (Fig. 6c), binary representations of the retinal vasculature around the ONH were obtained (Fig. 6d). Figure 6e-h shows the same processing steps for a wildtype mouse. The binarized annulus (Fig. 6d,h) was then used as a mask on the annular angiogram (Fig. 6c,g) in order to calculate the Weber contrast (Fig. 6i). The contrast is similar between transgenic and wildtype mice. Figure 6j-m and Fig. 6n-q show examples of the same analysis pattern for the DCP in the transgenic and wildtype mice, respectively. The Weber contrast in this case (Fig. 6r) is lower for both groups, although the contrast values remain similar between transgenic and wildtype mice.

The vessel density calculations for all retinas in the SVP and the DVP are shown in Fig. 6s and Fig. 6t, respectively. No significant differences were observed between transgenic and wildtype mice for any of the retinal regions.

3.4 Phase retardation abnormalities

All individual B-scans of all mouse retinas were screened for phase retardation abnormalities, i.e., depolarizing deposits located outwith the known melanin-containing RPE and ONH regions. Such deposits were found in at least one eye of 22 out of 24 transgenic mice and 11 out of 15 controls, and there were no apparent differences between those deposits observed in transgenic and wildtype groups. Figure 7a-b shows the two most common forms of phase retardation abnormalities, which appear as small, round depolarizing deposits beside a vessel wall (Fig. 7a) or beneath the RNFL adjacent to the optic nerve head (Fig. 7b). No Aβ plaques were identified in any of the retinas where the PS-OCT data was correlated to histology (more details in Section 3.6). However in some cases, melanin migration was found to be the source of the contrast, as demonstrated in Fig. 7c-f.

3.5 Double-banded OPL

Abnormalities in the structure of the ONL/OPL were found in the reflectivity OCT images in both eyes in a total of 3/24 transgenic mice (age: 54 weeks, 67 weeks and 81 weeks) and 3/15 wildtype control (age: 67 weeks, 81 weeks and 97 weeks). Examples of the appearance of the double-banded OPL can be found in Fig. 8. Figure 8a shows an example of a “normal” appearance of a retina observed in a transgenic mouse, where the OPL appears as a single hyperreflective band. In contrast, the hyperreflective OPL appears to split into two in Fig. 8b. Similar double bands of hyperreflective OPL signal were observed in the 3 wildtype mice as shown in Fig. 8c. To evaluate potential structural bases underlying the atypical retinal layer contrast, exemplary mouse retinas depicting OPL double-banding in the OCT exam were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Microscopical examination revealed that the double-banding of the OPL precisely correlated with rearrangement of proximal ONL somata towards the outer border of the INL (Fig. 8d).

3.6 Retinal histology

3.6.1 Typical observations

To confirm human Aβ in the retinas of APP/PS1 transgenic mice, indirect immunofluorescent staining of retinal wholemounts was performed with a mouse monoclonal antibody directed against amino acids 1-17 of human Aβ (clone DE2B4). The marker identifies intracellular Aβ and safely
Fig 6  a-h) Example of optical coherence tomography angiography (OCTA) analysis of the superficial vascular plexus (SVP) of a transgenic mouse (a-d) and a wildtype control (e-h). a,e) En-face OCTA depth projection through the SVP. b,f) Binary representation of the SVP with white pixels corresponding to blood vessels. c,g) Annulus around the optic nerve head (ONH) as provided by the intensity-based contrast data. d,h) Binarized annulus, where the yellow dashed line corresponds to the boundary between the superior retina (above) and the inferior retina (below). i) Weber contrast comparing the intensity of the angiogram signal of the blood vessels to the intensity of the background in the SVP. j-q) Example of OCTA analysis of the deep capillary plexus (DCP) of a transgenic mouse (j-m) and a wildtype control (n-q). j,n) En-face OCTA depth projection through the DCP. k,o) Binary representation of the DCP with white pixels corresponding to blood vessels. l,p) Annulus around the ONH as provided by the intensity-based contrast data. m,q) Binarized annulus, where the yellow dashed line corresponds to the boundary between the superior retina (above) and the inferior retina (below). r) Weber contrast comparing the intensity of the angiogram signal of the blood vessels to the intensity of the background in the DCP. s-t) Vessel density analysis. Total, superior and inferior vessel density calculated for transgenic and wildtype mice in the SVP (s) and the DCP (t). Age (a-d, j-m): 93 weeks, Age (e-h, n-q): 76 weeks. Single points in (i) and (r-t) correspond to data outliers. All scale bars = 100 µm.
Fig 7 Depolarizing deposits. a) Example of depolarization along a vessel wall (indicated by orange circle). b) Example of depolarization near the optic nerve head (indicated by orange circle). c) Identification of migrated melanin. After wholemounting the retina, the OCT angiography data (in red) was used to correlate the vessels measured in vivo to the overview of the retina provided by the ex vivo preparation (greyscale). d) PS-OCT image showed location of abnormally high phase retardation in the inner nuclear layer (indicated by yellow arrows). e) A high resolution confocal microscopy scan was acquired at the area of interest marked in (c), at the depth position marked by the yellow arrows in (d). A cluster of melanin is revealed at this location, as seen in (f). Scale bar in bottom right applies to (a), (b) and (d).

Fig 8 Demonstration of retinal layer abnormalities. The outer plexiform layer (OPL) is indicated with arrows. a) A transgenic mouse retina with a typical appearance - the outer plexiform layer appears as one single hyperreflective band. b) A transgenic mouse retina with the OPL disrupted, appearing as a double-banded hyperreflective layer. This effect was observed in 3/24 transgenic mice. c) A similar double-banding effect was also observed in 3/15 wildtype littermates. d) H&E-stained histological slice of the same mouse retina as in (c). The structural correlate of the double-banded OCT signal in the OPL region appears to be rearranged proximal outer nuclear layer somata. RNFL Retinal nerve fiber layer. GCL Ganglion cell layer. IPL Inner plexiform layer. INL Inner nuclear layer. OPL Outer plexiform layer. ONL Outer nuclear layer. IS/OS Inner/outer segment junction. RPE Retinal pigment epithelium. BM Bruch’s membrane. CH Choroid. SC Sclera. Age (a-d): 81 weeks.
detects extracellular A\(\beta\) without cross-reacting with APP\(^{83}\). This is evidenced by the clear labeling of A\(\beta\) plaques only in brain sections from APP/PS1 transgenic animals used as a positive control.

Following the donkey anti-mouse secondary antibody staining protocol outlined in Section 2.4.2, it was expected that in addition to A\(\beta\), sources of endogenous IgG present in the retina would bind to the secondary anti-mouse antibody and be highlighted. Figure 9a shows an example of a retinal wholemount of a transgenic mouse with the peripapillary blood vessels distinctly labeled due to abundant endogenous mouse IgG present in the serum. Figure 9b demonstrates the precise fit of the ex vivo vessel pattern with the in vivo OCTA image. The wavy appearance of some of the vessels is a result of motion artefacts caused by breathing during the measurement. From the positive control of the cortex of the transgenic mouse (Fig. 9c), it can already be observed that signal of a similar intensity to the roundish A\(\beta\) plaques also comes from the capillary network. Figure 9d-f shows the equivalent images for an example of a wildtype control mouse. In the cortex of the wildtype mice (used as a negative control, Fig. 9f), only the capillary network showed fluorescent labeling.

Outwith the blood vessels and capillary network, other sources of fluorescent signal were found in both transgenic and control mice. Examples of such features are shown in (Fig. 9g-l). In Fig. 9a, a blood vessel (indicated by the solid box) appears to be intensely fluorescing. However, by analysing a series of confocal optical sections (z-stacks) throughout this region (Fig. 9g-h), it became clear that this signal derived from aggregates of secondary antibody-fluorochrome conjugate artefactually adhering to the surface of the retinal ganglion cell layer. The signal did not derive from either neuronal or non-neuronal structures within the retina as the overview at 10x magnification made it appear. Similar observations were made in the wildtype retinas too (Fig. 9j-k). Structures signalling intensely from within the retina included areas where branches of retinal capillaries appeared to get close together, reminiscent of micro-aneurysms (Fig. 9i), and microglia (Fig. 9l), identifiable by the dendritic morphology of their processes. Any source of fluorescent signal which did not fall under one of these categories was then considered a candidate for A\(\beta\).

### 3.6.2 Potential deposits of retinal amyloid beta

Of the 17 mice which underwent retinal histology, only one mouse (transgenic, age: 104 weeks) displayed fluorescent signals in the retina which could be attributed to extracellular A\(\beta\). In this mouse, there was one such area of interest in the left eye, and seven in the right eye. Images of all A\(\beta\) plaque candidates can be found in Fig. 10. Figure 10a shows an overview of the left retina. While many bright spots were observed, only the one indicated by the dashed box did not exhibit the characteristics of what was shown in Fig. 9. A z-stack through the retina confirmed that this plaque candidate sat approximately 15 \(\mu\)m below the surface of the retina, extending into the anterior IPL. Examples of z-planes can be found in Figure 10b-g. A similar analysis of the right retina provided images of the further seven plaque candidates, which can be seen in Fig. 10h-n. The locations of three of the eight plaque candidates were also covered in the field of view of the in vivo OCT measurements, however no abnormalities were found in these locations in the OCT data, using any mode of contrast.

### 3.7 Cortical amyloid beta plaque load

In a subset of the mice (14 transgenic mice and 7 wildtype littermates), histological slices of the brain were prepared and immunohistochemically stained against A\(\beta\). Figure 11a shows an example
Fig 9  Representative depictions of the ex vivo retina following fluorescent staining against amyloid beta (Aβ). a) Retinal wholemount of a transgenic mouse and b) its correlation to in vivo OCT data. c) The positive control (the cortex of the transgenic mouse) shows fluorescent labeling of Aβ plaques and capillaries. d) Retinal wholemount of a wildtype mouse and e) its correlation to in vivo OCT data. f) In the cortex of the wildtype mouse (negative control), only capillaries are labeled. g-l) Some typical observations seen throughout transgenic and wildtype mouse retinas. g) When zooming in to the surface of the retina in the location indicated in the dashed box (h), some scattered bright spots appear. The orthogonal views (positions indicated by the white cross-hairs), however, show that these lie only on the surface of the retina. i) Fluorescent signal positioned at a capillary junction. j-k) Similar to (g-h), single, larger accumulations of fluorescent tracer find themselves at the interface of vitreous and retina, but not within the retina. l) Microglia, indicated by ovals, are identifiable by their dendritic processes and are also found throughout the retina. This image was acquired within the ganglion cell layer.
Fig 10  Candidates for fibrillary amyloid beta (Aβ) detected in the retina of one mouse, as identified by confocal microscopy.  a) Overview of the ex vivo retina (left eye) acquired with a 10× magnification objective lens.  b-g) En-face planes at 5 µm intervals at the position identified by the dashed box in (a), where the zero-position is at the interface of vitreous and ganglion cell layer. The fluorescent abnormality, i.e., the Aβ candidate, is indicated by the arrow in (d) and (e). Scale bar in (g) is valid for (b-g). Images were acquired with a 40× magnification objective lens. h-n) Seven further Aβ candidates were identified in the retina of the right eye of the same mouse (all acquired with a 40× magnification objective lens). All structures were detected less than 40 µm from the surface of the retina, i.e., between the retinal nerve fiber layer and the inner plexiform layer. Scale bar in (h) is valid for (h-n).
Fig 11  Quantification of the plaques per mm² in the cortex. a) Histological slice of a transgenic mouse brain, immunohistochemically stained against amyloid-beta (Aβ). Aβ plaques appear as brown deposits. Age: 103 weeks. b) Following the same staining protocol, the wildtype littermates do not show any Aβ plaques in the cortex. Age: 103 weeks. c) Count of plaques per mm² for a subset of 14 transgenic mice (the subset of 7 controls showed no plaques). Linear regression analysis showed a statistically significant trend of an increasing plaque load with age ($R^2 = 0.439$, $p = 0.0098$).

of the staining results for a transgenic mouse, while Fig. 11b shows an example of a similar region in the control brain. In the transgenic mice, brown plaques were identifiable throughout the entire cortex. The mouse depicted in Fig. 11a was 103 weeks old at the time of sacrifice, and Aβ plaques were also visible in abundance in the hippocampal formation and the cerebellum, as well as in other areas of the brain. To the contrary, no plaques were observed in any of the brain regions in the 7 examined wildtype mice (Fig. 11b).

For the 14 transgenic mice, the plaque load was plotted as a function of age. This plot can be found in Fig. 11c. Linear regression analysis revealed an $R^2$ value of 0.439 and a p-value of 0.0098. The gradient of the slope indicated that the plaque load increases by 0.354 plaques per mm² per week over the investigated age range. Such a result demonstrates that Aβ plaque load increases with age in the transgenic mouse brain, and that the trend is statistically significant.

4 Discussion

Since the role of the retina in Alzheimer’s disease is still widely disputed, the aim of this work was to provide a comprehensive overview of what can be observed in the retina of an APP/PS1 mouse model using multi-contrast OCT, and to compare this to histological results. A combination of reflectivity images, PS-OCT images and OCTA were used to investigate the structure and function of the retina. From the in vivo data alone, several retinal abnormalities were successfully identified in this model, however there were no statistically significant differences between the transgenic and wildtype groups. This suggests that the hyperreflective foci, retinal thickness changes, phase
retardation abnormalities and structural differences which were measured with OCT are either strain-related or age-related, rather than being due to the genetic mutation itself.

The total retinal thickness was found to significantly decrease with age in the superior and inferior halves as well as in the whole annulus around the optic nerve head. No difference was seen, however, between the transgenic and wildtype groups. These results provide an in vivo validation for that which was previously observed ex vivo by Perez et al. In future studies, the inner and outer retina could be subdivided further to quantify individual layer thickness. Since RNFL thinning occurs in AD patients, it may also be interesting to quantify the RNFL thickness alone in this mouse model. However, quantifying the RNFL thickness in mice using OCT is difficult, as the peripapillary thickness of the healthy RNFL is only approximately 20 µm, and is interrupted by blood vessels and ganglion cells. Automatic segmentation of the mouse RNFL is therefore challenging, as it can not always be distinguished from the IPL in the OCT images. Owing to its fibrous structure, the RNFL is also birefringent and since it is much thicker in the human retina, the effect of the birefringence is stronger. PS-OCT has already been proposed as a tool for imaging the RNFL in glaucoma, so RNFL measurements in AD patients may be a promising future application for PS-OCT.

The phase retardation analysis performed in this study mainly identified depolarization deposits associated with vessel walls and melanin pigments. No depolarizing deposits were identified in locations which corresponded to candidates for retinal Aβ, although locations of melanin migration which were observed in this study mirror results which were documented in the human in age-related macular degeneration. Future experiments on this topic could also consider HRF in the intensity data, but this becomes challenging in layers which contain vasculature, as the edges of vessels can also appear as hyperscattering features. Previous PS-OCT studies identifying migration of melanin pigments have typically used the metric of degree-of-polarization-uniformity (DOPU) to quantify the polarization scrambling, or depolarization, of the structure in question. In order to calculate DOPU, a sliding window must be applied within the images, sacrificing the overall resolution. In this study, as the depolarizing structures were small, it was decided to use the phase retardation images alone, as they provided higher image resolution.

The OCTA analysis revealed no significant differences in the vessel density between transgenic and wildtype groups in either the SVP or the DCP. This is contrary to what has been observed in human vessel microangiography studies. However, in the previous human studies, the area of interest usually targets the fovea. Since mice do not have a fovea, there is not an equivalent measurement in the mouse retina. The modified Weber contrast was similar between transgenic and control groups; the wider standard deviation for the wildtype can be attributed to the smaller sample size. The Weber contrast in the DCP was lower than in the SVP for both groups, which is to be expected as the blood flow in the DCP is lower than in the SVP and therefore the angiogram signal is weaker due to the more random orientation of the red blood cells. Angiograms with poor signal had already been excluded from the analysis as the imaging of older retinas using optical methods can prove challenging. Older mice generally develop cataracts, which results in a lower transmission of light through the lens and therefore a poorer image quality. The shorter the wavelength which is used for OCT, the more attenuating a cataract becomes. Therein lies a trade-off between the axial resolution of the OCT images (the shorter the wavelength, the higher the resolution) and the maximum SNR which can be achieved in the retinal images. Care must also be taken that eye drops are rigorously applied throughout the experiment to ensure a cataract does not form while the animal is under anesthesia.
The disorganisation of the OPL/ONL structure observed in three transgenic mice and three wildtype mice was not expected from current literature regarding this mouse model. Previous studies in both the mouse\(^{31}\) and the human\(^{35}\) have attributed similar OPL/ONL splitting to mutations in the CACNA1F gene encoding for the L-type calcium channel Ca\(_{v}\)1.4 which is also expressed in the outer nuclear layer of the mouse retina. Without the Ca\(_{v}\)1.4 calcium channel, photoreceptor synapses are lost, and the dendritic sprouting which occurs in the photoreceptor layer (in the second-order neurons) is abnormal.\(^{83}\) Whether this gene is defect in this particular APP/PS1 mouse lineage is a topic which must be explored further.

Regarding the analysis of the immunolabeled wholemounted retinas, strong fluorescent signal appears to derive from a range of sources. Examples of fluorescent signal caused by aggregates of the secondary antibody which adhere non-specifically to sticky remnants of the vitreous on the surface of the the ganglion cell layer of the retinal wholemounts can be seen in Fig. 9g-h and Fig. 9j-k. With the employed immunostaining protocol, non-A\(\beta\) specific signal also derives from binding of the anti-mouse secondary antibody to endogenous IgGs present within, e.g., serum and microglia. This makes it difficult to unequivocally assign biochemical A\(\beta\) specificity to highlighted structures. Therefore, in order to better delineate potential deposits of intra- or extracellular A\(\beta\), the morphology of the fluorescent signal was also considered. In this study, both retinas of only one transgenic mouse were highlighted as containing candidates for retinal A\(\beta\) deposits, displaying intense fluorescence signal and also presenting with a fibril-accumulation-like structure. Of note, A\(\beta\) immunopositive assemblies with similar and distinct fibrillary appearance and a size in the few µm range have been described in human AD retinas.\(^{12}\)

Previous studies have indicated that A\(\beta\) accumulations in the brain are visible with PS-OCT and regular intensity-based OCT,\(^{59,60,63}\) however this study was not able to recreate these findings in the retina. This could be due to the difference in size of the A\(\beta\) plaques - the plaque candidates which are proposed in Fig. 10 are much smaller than those in the brain (example in Fig. 11a). It has also been previously concluded that not all plaques are visible by either contrast modality.\(^{60}\) Hence negative OCT findings do not rule out the presence of retinal A\(\beta\) plaques. Even if plaques could be seen in the retina with OCT, it would be difficult to distinguish them from the HRF and the depolarizing deposits which are already present in these retinas as observed in Fig. 4 and Fig. 7. In our immunofluorescence protocol, our “positive control” is the brain, and not the retina, as no retinal “positive control” sample exists. Despite our best efforts to mimic retinal wholemount conditions in the control samples, the tissues are simply not the same and therefore it cannot be ruled out that the immunostaining protocol is less optimal for the retina than it is for the brain. However, the candidates for retinal A\(\beta\) identified in Fig. 10 would provide an argument that the protocol is indeed suitable.

Given the lack of differences between transgenic and control groups in the in vivo OCT data, and the fact that A\(\beta\) could only be identified ex vivo in 1 out of 11 transgenic animals, the suitability of this APP/PS1 mouse as a model of the human must be called into question where the retina is concerned. As with any other mouse model of AD, it only models some aspects of the disease and not others, and each mouse model will experience different age-related and strain-related changes in addition to anything caused by gene mutation. This study finds itself among the conflicting reports regarding the presence of A\(\beta\) in the retina.\(^{8}\) Our results indicate that extracellular A\(\beta\) may be found in the retina of this mouse model, although not in all, or even most, samples. A much larger study would need to be conducted in order to statistically determine the likelihood of identifying
Aβ plaques in the retina of this mouse model. A topic of future exploration could be a comparison of retinal observations in different APP/PS1 mouse models, also adding a quantification of microglia to the retinal analysis.84

The cortical Aβ plaque load, evaluated alongside the retinal data, showed a statistically significant increase with age in the transgenic mice. Despite not being able to correlate this to any retinal changes, this study has documented the typical observations which could be expected to be found in the retina of this mouse model, both in vivo and ex vivo. The 1:1 mapping of the OCT data to the retinal histology was crucial for this experiment, allowing a detailed quantitative and qualitative analysis of structural and functional features of this APP/PS1 mouse model. Tri-fold OCT imaging contrast coupled with retinal wholemounts is therefore a promising method for the analysis of animal models of many retinal diseases.

5 Conclusion

While the cortical immunohistochemical staining revealed clear, marked differences in Aβ plaque load between APP/PS1 transgenic mice and their wildtype littermates, a similar difference was not observed in the retina. Candidates for retinal Aβ were only identified in 1 out of 17 transgenic mice. Multi-contrast OCT did, however, reveal retinal abnormalities in these mice, including deposits of migrated melanin and a double-banding of the ONL. Owing to the occurrences in both transgenic and control mice, it is likely that these are strain-dependent and not due to the genetic mutation itself. Nevertheless, the combination of multi-contrast OCT with 1:1 mapping of retinal histology allowed for a thorough documentation of what one would expect to see in this APP/PS1 mouse model of AD.

Disclosures

The authors have no relevant financial interests in the manuscript and no other potential conflicts of interest to disclose. Part of this work has been presented at the Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting in Vancouver, Canada, and the published abstract by Baumann et al. can be found in Ref.85.

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References


7. DISCUSSION

The results of this thesis featured multi-contrast imaging of the rodent retina, primarily with two high resolution OCT systems. The first system, its design a part of this work, was a white light system based on a supercontinuum laser as a light source. With its largely unrivalled axial resolution and visible light spectroscopic contrast capabilities, this system offers an insight into the retina which is, to date, unique. The second system, used for a longitudinal study of the APP/PS1, operated in the near-infrared (NIR) wavelength region centered at 840 nm. While also custom-built, this system was previously published [128], and advanced robust post-processing software had already been developed [116,130]. While still offering a high axial resolution (3.8 µm in retinal tissue), this system was faster, had an increased penetration depth and offered better image reproducibility. Both systems clearly have their advantages and disadvantages, many of which will now be discussed here. A short discussion will then follow regarding the imaging results from the systems, and their implications for histological analysis. This section will then end with a brief outlook to the future of high resolution OCT.

7.1 White light OCT vs. 840 nm OCT

7.1.1 Resolution

The most pronounced difference between white light OCT and its 840 nm counterpart and, arguably, the main motivation for construction of visible light based systems, is the resolution. Since the central wavelength in a visible light system is lower, even for the same spectral bandwidth the axial resolution improves dramatically (due to the squared-dependence upon central wavelength). Extending a visible light system (containing any part of the visible light spectrum) to a white light system (containing the whole visible light spectrum, as was the case in this work) also improves the resolution even further due to the inverse linear relationship between the FWHM bandwidth and the minimum resolvable depth difference. The lateral resolution also improves, but the effect is not as pronounced, as the lateral resolution only improves
linearly with decreasing central wavelength. The lateral resolution also varies from animal to animal, depending on the optical quality of the eye. The high numerical aperture of the mouse eye (0.49) [75] makes it theoretically possible to achieve sub-micron resolution retinal imaging laterally as well as axially when using visible light OCT. However, in order to be able to ensure high resolution for every image acquisition, adaptive optics (AO) would be required [131]. AO-OCT uses a feedback loop to correct for aberrations induced by the eye itself. This method is already well established in research at NIR wavelengths [132], but has only recently been demonstrated in the visible light range [133]. Incorporating AO into an OCT system, however, adds complexity and increases the cost of an OCT system, and was not deemed necessary during the course of this work.

7.1.2 Dispersion

One of the major limiting factors in achieving the theoretical axial resolution on OCT is dispersion. Dispersion arises due to the frequency dependence of the propagation of a light wave through a material. In OCT, both hardware and software methods can be used to correct for the resulting pulse broadening and distortions. By physically matching the materials present in the sample and reference arms (i.e., by using the same optical components in each) the dispersive effects can be limited. However, this is challenging when OCT is used for retinal imaging, as the dispersion caused by transmission through the cornea, aqueous, lens and vitreous can only be approximated. This is usually compensated either by placing an optically similar material (such as a water cell [97]) into the reference arm, or by applying a numerical phase correction in post-processing [134]. An approximation of this numerical phase correction frequently takes the form

\[
\Phi(\omega) = -a_2 (\omega - \omega_0)^2 - a_3 (\omega - \omega_0)^3,
\]

where \(\omega_0\) corresponds to the center angular frequency, \(\omega\) to the angular frequency, and \(a_2\) and \(a_3\) correspond to the second- and third-order dispersion imbalance terms, respectively.

This was the method which was applied using the 840 nm system, and this was sufficient to achieve close to the theoretical axial resolution limit. In the case of the white light OCT system, the situation was more challenging. The \(a_2\) and \(a_3\) terms were
themselves wavelength dependent, and therefore this had to be taken into account and corrected for, too. Additionally, the measurements are far more sensitive to even the slightest changes in dispersion mismatch when using visible light. The wavelength-dependent phase correction coefficients had to be changed not only from one animal to the next, but also from one dataset to the next within the same eye. Even the smallest change in path length through the anterior eye was enough to cause visible reduction in the axial resolution. Finding the exact dispersion correction coefficients therefore significantly increases the processing time of the images. In this work, the dispersion correction coefficients were set to improve the B-scans in their entirety. Recently, depth-dependent dispersion compensation has been proposed as a method to ensure as high an axial resolution as possible throughout the whole retinal image [135]. This method compensates for the differences in dispersion mismatch between the inner and outer retinal layers, recognising the dispersion caused by the retina itself. While this would increase the post-processing time even further, this further step will be necessary in future. This is important when quantifying structures which have layer thicknesses close to the resolution limit, for example, Bruch’s membrane [136].

7.1.3 Sensitivity and roll-off

The sensitivity of an OCT system is defined as the exact attenuation of the signal which results in a signal-to-noise ratio of 1. Theoretically, the best performance of an OCT system occurs within the shot noise limit, where the dominant noise contribution originates from the photon noise from the reference arm [48]. Unfortunately it was not possible to reach shot-noise-limited detection of the white light OCT system due to physical limitations. The beam emerging from the spectrometer input diverged too rapidly to be detected by a power meter. This, in combination with the loss of diffraction power of the grating at the edges of the spectrum meant that the shot noise limit could be obtained. The high relative intensity noise of the supercontinuum laser was partially to blame for this [32]. The sensitivity was lower for the white light system than the 840 nm system, even though the acquisition time of the cameras was slower by around a factor of 3. Due to laser safety requirements, the incident power onto the eye was lower for the visible light system. Furthermore, many of the optical components used within the white light system attenuated at least the edges of the spectrum, also leading to a reduction of the SNR.
Common to both systems used in this study, custom-made grating-based spectrometers were used. The mapping of wavelength-to-pixel allows easy wavelength calibration to be performed, simply by utilizing several notch filters with different central wavelengths and then fitting a curve between the measured data points. However, the interference measured in OCT does not scale linearly with wavelength, rather with the wavenumber, $k$. For many standard OCT systems (and also, to a certain extent, the system used in Chapter 6) the biggest disadvantage here is the precise measurement of the spectral resampling function (and then ensuring that the spectrometer is not moved at all). Owing to the geometrical mapping of the spectrum by the spectrometer, the spectrum is much more densely sampled at longer wavelengths. In the white light system, we opted for a line-scan camera detector which had a large number of pixels (16384 pixels with a 5 µm width) to ensure a sampling which was as dense as possible, even in the shorter wavelengths. Even so, there was an inevitable wavelength-dependent sensitivity roll-off, differing from 16 dB/mm for the longer wavelengths (measured at 670 nm) down to 24 dB/mm for the shorter wavelengths (measured at 460 nm). While this effect also occurred with the 840 nm system, it was nowhere near so prominent. The shorter spectral bandwidth also allowed a smaller line scan camera to be used for the 840 nm system, removing the requirement of a custom-designed spectrometer lens.

### 7.1.4 Light penetration

While the depth range in OCT is dependent upon the number of pixels in the line scan detector, this is not usually the limitation of the penetration depth observed during retinal imaging. After passing through the neural retinal layers, the light is the incident upon the melanin-containing retinal pigment epithelium and the choroid. Melanin is a strong absorber for all wavelengths used in retinal OCT, but this effect becomes more pronounced in the shorter wavelengths [137]. In the OCT images acquired by the white light system, very little signal is seen from below the RPE of the mouse retina as most of the light is absorbed in this layer. With the 840 nm system, the signal tends to tail off in the region of the choroid, as the absorption coefficient in this wavelength range is lower. White light OCT is therefore not suitable for choroidal imaging, at least not in pigmented samples. Furthermore, white light OCT is very sensitive to cataract formation in the lens. As the cataracts are white
in appearance, their attenuation coefficient for white light is high [138]. In practise, even small cataracts in the rodent eyes made the image quality too poor to acquire with this white light system. To image the APP/PS1 mice, the study design was such that the mice were allowed to age before OCT imaging. This meant that they were likely to have cataracts already [139], and therefore were unsuitable for OCT imaging in the white light range. The 840 nm OCT system was much less sensitive to cataract formation; good quality images could still be acquired in the presence of mild cataract.

7.1.5 Laser safety

There has been much discussion on the safety of white light OCT for retinal imaging. Not only are the maximum permissible exposure (MPE) levels lower than they are for near infrared light [140], there is also the issue of potential patient discomfort when moving visible light systems forward towards clinics [141]. The laser safety limits were taken into consideration when designing the white light system. In the high power limit, a stationary beam was assumed, and the power spectrum incident on the eye was plotted. As the visible light range spans three MPE wavelength categories, it is not enough to perform calculations for the central wavelength only. The MPE must be found for all wavelengths, and the total power of the spectrum, $P$, must be such that

$$\sum_{\lambda} \frac{P_{\lambda}}{P_{\text{limit},\lambda}} \leq 1$$

(7.2)

and that no individual wavelength exceeds its MPE either. While also documented this way in the laser safety regulations [140], it is important to stress here that this calculation must be performed for every visible light system independently, as the resulting power is highly dependent upon the shape of the spectrum. Nevertheless, when accurately considering the shape of the spectrum, it is definitely possible to construct visible light OCT systems which are safe for human retinal imaging as well as animal imaging.
7.1.6 PS-OCT

For both systems in this work, the phase retardation was chosen as the method of visualization and analysis for PS-OCT measurements. For the white light system, this was because the phase data was too unstable - the system operated at an A-scan rate of only 25 kHz and therefore even the smallest of motion artefacts during in vivo imaging made the phase data noisy and unreliable. Additionally, the same motion causes a greater phase shift at shorter wavelengths, making the white light PS-OCT inherently more sensitive to phase fluctuations. Phase retardation, however, can be calculated from the intensity data alone, providing a more reliable metric. In order to minimise the mismatch of the resampling function or dispersion correction between the signals acquired by the two detectors, a wavelength calibration was performed between the two detectors. By accurately measuring the location of each wavelength in terms of pixel number on both cameras, one spectrum was mapped to the other and then the same remapping and dispersion compensation was performed on both spectra. This would not correct for polarization-based dispersion, but no evidence of this was found within the mouse eye images and therefore it did not seem to pose a problem.

The choice of phase retardation in Chapter 6 was not due to the lack of reliable phase data, but rather to ensure an effective manner of visualization. While searching for depolarizing deposits on a background of polarization-preserving retina, the DOPU is the conventional contrast metric [55]. However in order to calculate DOPU, an averaging kernel is applied to calculate the homogeneity of the depolarization values within. By nature, this reduces the visibility of the effects of small sources of depolarizing signal. As we were searching for small deposits which were not too much larger than the resolution limit of the system, we opted to stick with phase retardation itself. The phase retardation contrast could theoretically have been more readily identifiable by using the white light system, as the same thickness of material corresponds to a larger number of wavelengths at short wavelength ranges. It therefore makes sense to assume that shorter wavelengths would be more sensitive to retarding samples.

This fact would also hold true when considering birefringent samples. At shorter wavelengths, the samples would exhibit stronger birefringence characteristics and therefore the incident light would need to penetrate less of the tissue before the birefringence became detectable [142]. When using NIR light, axis orientation measure-
ments in the mouse eye do not provide much information. The only fibrous, orientated structure in the mouse retina is the RNFL, which is thin. Since birefringence effects are cumulative, the mouse RNFL does not have the depth to substantially alter the polarization state of the incident light within it. A move down towards the blue end of the spectrum may offer viable axis orientation measurements in the future, modelling more closely the layer thickness/wavelength ratio which exists in human RNFL measurements [121].

7.1.7 OCT angiography

OCTA is notoriously prone to imaging artefacts [143, 144], many of which are caused by motion. When phase-based or complex OCTA is performed, the images are subject to the same errors as discussed for PS-OCT. However even in amplitude-based OCTA, as this is based on speckle decorrelation, there is a time delay between the acquisition of two (or more) images from which the angiogram is calculated. This means that it is also very sensitive to motion, far more than in phase retardation measurements where the two frames are acquired simultaneously. In living samples, breathing is one example which causes erroneous OCTA measurement. Imaging should therefore be performed at high speed to limit these effects, and post-processing algorithms should be applied to correct for the remaining artefacts, where possible. OCTA was only performed using the NIR system in this study, and not with the white light system. OCTA measurements performed with the NIR system were reliable in amplitude-, phase- and complex-based forms. Clear segmentation of the superficial vascular plexus and the deep capillary plexus was possible within the retina. The intermediate capillary plexus was more challenging to segment, as it often contained signal from multiply scattered light from superficial vascular plexus above. The reliability of the binarization of the intermediate capillary plexus OCTA images could not be guaranteed, and therefore this vascular layer was left out of the quantitative analysis.

Visible light OCTA has previously been demonstrated [145], but the angiograms are noisier than those which have become standard for NIR OCTA. As the speed of visible light laser sources increases, it may be of interest to image other tissues in order to perform white light OCTA in combination with spectroscopic OCT to differentiate blood vessels from the lymphatic system [146].
7.1.8 Spectroscopic OCT

While visible light optical coherence tomography is becoming much more rapidly adopted, most studies to-date have been limited to the green light range (approx. 500-600 nm) as the systems have been designed for retinal oximetry. By expanding the light range to include the whole visible spectrum, many new opportunities for spectroscopic contrast have been opened up. In section 4, this spectroscopic analysis was used to detect Mie theory scattering. However visible light sOCT has also been used to quantify bilirubin [65], and it could also be possible to perform oximetry at the 415-nm spectral window. As the eye is generally designed for visible light, it is likely that there are many additional applications of visible-light based spectroscopy in the retina. While the white light system designed in Chapter 2 could theoretically be used for retinal oximetry, we decided to focus on some previously undiscovered spectroscopic imaging techniques. In chapter 2, a “hyperspectral OCT” imaging modality was proposed, proving capable of identifying particles within the retina based mainly on their size and shape, thanks to the oscillating profiles as dictated by Mie theory. Moving outwith in vivo applications, this approach could be utilized further in order to assign particular hyperspectral OCT profiles to particles of particular diameters. As also discussed in the discussion session of Chapter 4, in the in vivo case this is largely impossible because the shape, size and relative location of the melanin granules in vivo is, to all intents and purposes, random. This may not be the case for industrial applications, where (for example) it is known that the particles are not simply ellipsoids, but rather spherical [147]. Hyperspectral OCT may also be of interest in art investigations, where spectral profiles of layers beyond the superficial are of interest [148, 149]. It should also be noted here that the concept of sOCT need not be limited to the visible light range. In fact, previous studies have shown spectroscopic contrast in the NIR for applications such as identification of cell nuclei [150]. The advantage of using visible light for this purpose simply lies in the fact that smaller spectral bandwidths are required to maintain the same axial resolution, allowing denser sampling as a function of wavelength, and also that the spectroscopic contrast is directly comparable to that which can be visualized by the eye.
7.1.9 Optics

The design and construction of the white light OCT system pushed the boundaries of the common off-the-shelf optical components. In particular, the lenses, polarization optics and optical filtering all posed particular problems. The imaging telescope configuration was simulated using Zemax (OpticStudio 15.0, Zemax LLC) to find a combination of white-light-suitable lenses with minimal chromatic aberrations. The lenses used for the spectrometers were custom made in order to achieve a spectral line measuring 8.2 cm in width but only 10 µm in height. Selection of the “optimum” quarter wave plates was not possible; the retardance only remained within 2% tolerance between 460 - 644 nm, and values at the edges of the spectrum were not available from any manufacturer. It was therefore not completely known what the polarization properties at the edges of the spectrum were, and this issue needs to be resolved in order to make any quantitative PS-OCT measurements in the white light range.

The supercontinuum laser used in this work produced partially coherent light from 380 - 2200 nm. To build a white light system from this (400 - 700 nm), the excess, unwanted parts of the spectrum were filtered by a homemade filter box consisting of a dichroic mirror, a bandpass filter, a water cuvette and a neutral density filter. Currently available dichroic mirrors and bandpass filters can easily transmit a window in the visible light range, but they break down at longer wavelengths, transmitting light beyond 1350 nm as well. To absorb this excess light, a water cuvette was used, transforming the NIR light into heat. While this combination was suitable for the construction of the white light system, it does not take advantage of the full capabilities of the supercontinuum. If the commercially available optics were capable of supporting broader bandwidths, there would be no reason why the axial resolution of an OCT system couldn’t be improved further. Extending the bandwidth to include both visible and NIR parts of the spectrum may also be a future direction for supercontinuum-based OCT. Many of the limitations discussed here would need to be addressed before this would be possible, but as commercially available optics improve over the years, axial resolutions in the tens-of-nanometers range could be available in the future.
7.2 Histology as a “ground truth”

In Chapter 6, a great effort was made to correlate the in vivo OCT images directly to histological data. An advanced retinal wholemount preparation and staining protocol was carried out, and the ex vivo retina samples were imaged with confocal microscopy in order to preserve the 3D retinal structure. This study highlights the importance of direct histological comparison. Depolarizing deposits were found in the retina using PS-OCT, and upon ex vivo examination it was found that migrated melanin was the source of this, rather than any evidence of retinal Aβ. Had retinal Aβ plaques also been observed with PS-OCT, they would not have been distinguishable from the melanin deposits without the confirmation of histology, at least if they were to appear as they do in the brain [100].

However, it is essential that care is taken with the interpretation of histological data. Within the course of this work, this point has been demonstrated twice: once in the spectral profiles of the individual layers of the mouse retina, and the other in the interpretation of whether Aβ plaques could be observed in the retina or not. For the spectral profiles, it was clear that the fixation and preparation process had altered the wavelength-dependent reflectivity. Further study on this topic is required to formulate true conclusions regarding in vivo retinal spectroscopic image calibration using ex vivo samples, but the preliminary data suggests that the differences between the in vivo and ex vivo data come from more than just the spectral errors caused by the anterior eye/the wavelength-dependent sensitivity roll-off.

In the second case, that of the identification of Aβ plaques, the theme becomes a little more complicated. A discussion should be made over the fact that when trying to establish a new histological staining protocol, the environment of the positive control must be carefully selected. For scientific accuracy, the positive control should contain the target in its known location [151]. In the case of Chapter 6, this would correspond to a retina of the same APP/PS1 mouse model which was known to contain plaques of Aβ. Unfortunately, such a sample was not available as this was exactly one hypothesis that was being tested. The sample used as the positive control in this case was taken from the brain instead. Although every effort was made to ensure the samples were as similar as possible (in thickness, taken from the same mice etc.), the fact that the very environment from which the sample was taken was not the same could cause a serious error in interpretation. However, the fact that candidates for Aβ were found
in both retinas of one mouse suggests that the staining did work, and that Aβ plaques are found in the retina, although very infrequently.

Sources of “noise”, i.e. unspecific staining, can also affect the interpretation of the histology images. In Chapter 6, it was possible to explain the sources of some of the signals as non-specific adherence of the secondary antibody to the sticky remnants of the vitreous, and as endogenous immunoglobulin G present within serum and microglia. Although when faced with a sample which contains many of these noise signals, it is very challenging to identify one single point of interest, in a “needle in a haystack” scenario. A large time investment and a carefully trained eye is necessary to be able to interpret this data, and moving forward to clinical validation, an additional test for Aβ would be desired. Methods based on polarized light imaging have been successful in the identification of Aβ in the brain, either following Congo red-staining [152,153] or in native tissue using PS-OCT [100,154]. Such tests could also be applied in the retina, as a two-fold testing approach. This would, however, be conditional upon successful identification of retinal Aβ with these modalities, which has not yet been proven.

In general, it should be noted that histopathological analysis is also subject to many artefacts [155] and while the comparison between in vivo and ex vivo data is valuable, it should not be taken for granted that the histology case is always correct. A direct example of this was observed by Hanlon et al. [156] who, for example, noted a 35% systematic error in corneal thickness measurement when measured ex vivo. In this case, in vivo OCT measurements were more accurate. This must, therefore, be considered on a case-by-case basis.

7.3 Animal models of Alzheimer’s disease

To create AD mouse models, autosomal dominant genes are used. This is the hereditary, early-onset form of AD, and considering that only 7% of AD cases are early-onset, and only 7% of those are familial [157,158], this is a very rare form of AD in the human population [159]. Despite this, creating mouse models in this way is reproducible. Sporadic AD, on the other hand, is thought to be linked to complex interactions of genetic polymorphisms and environmental risk factors [160], making reproducible modelling more challenging.
It is important to remember that animal models of AD, or any other disease, are indeed only models. While the similarities between the human and (particularly) other mammals make such animal modelling worthwhile, care must be taken to relate the findings back to the human in a manner which does not assume that anything is “the same”. No animal model of AD can exhibit all the features of what is observed in the human. True human AD includes a variety of pathological features, of which Aβ plaques are only one. If the goal of a study is to directly investigate a single mechanism, then the isolation of only this feature can be an advantage.

The retinal analysis of the APP/PS1 model performed in this study is an important demonstration of this fact. While 24 transgenic mice and 15 wildtype littermates were analyzed, only one mouse showed potential Aβ plaques in the retina, and they were not visible in vivo using the NIR PS-OCT system. Since the presence of Aβ plaques in the retina is debated both in this mouse model and in humans, it may be possible that this low number is the reason for the debate, i.e., the plaques do exist but very infrequently. The plaque presence could also be location-dependent, which could present additional challenges for retinal screening. One conclusion which could be drawn from this is that this APP/PS1 mouse model is therefore not a good candidate for investigating retinal Aβ, as huge sample numbers would be required in order to achieve any statistically meaningful results.

7.4 Outlook on the future of high resolution OCT

During this discussion section, there has been a lot of focus on the current limitations of white light OCT, including speed, sensitivity, power limits and optical components. The NIR system used in this work was already well established, and therefore a direct comparison between the two is perhaps a little premature. The five-fold improvement in axial resolution offered by the white light system coupled with its spectroscopic and hyperspectral capabilities offer something new and unique to the field, and given the rate of improvement in supercontinuum laser technology in the last few years, it is very likely that the image quality of white light OCT and its hyperspectral derivatives will improve in the near future. If a swept white light source were to become available, this would help to reduce the roll-off issues caused by the spectrometers which are present within the supercontinuum-based systems. Alternatively, a white light system could
be constructed where the spectrometers sample the spectra linearly in wavenumber [115, 161–163] in order to unify the sensitivity roll-off.

In terms of white light PS-OCT, this work has served only as a proof-of-concept; the full capabilities have yet to be fully explored. In addition to retinal imaging (both rodent and human), white light PS-OCT could be applied to skin imaging, investigating the depolarizing properties of the melanosomes. The hyperspectral aspect could also be applied to PS-OCT, investigating phase retardation, axis orientation, DOPU and even diattenuation as a function of wavelength. This could become particularly interesting at shorter wavelengths, from the blue portion of the spectrum down into the UV. With current supercontinuum technology, further extension past around 350 nm is impossible as the photonic crystal fiber would absorb UV light beyond this wavelength. This would cause the fiber to turn black and break very quickly. However, if there was a commercial demand for OCT light sources at UV wavelengths and no necessity for the infrared region, photonic crystal fibers could perhaps be fabricated from other materials and a shorter laser pump wavelength could be used to create a new type of supercontinuum [164].

It remains to be seen whether supercontinuum-based OCT will proceed in this direction, or whether a visible light swept source laser will be developed and overtake the demand for such a thing. For years, spectral domain OCT technology helped to drive the development of supercontinuum lasers, but now a point has been reached where the supercontinuum can generate wider spectra than could possibly be considered useful for OCT. It is, therefore, an interesting time to be working on such a topic, and I look forward to seeing the development of new optical components in the near future.
8. CONCLUSION

The primary focus of this work was high resolution rodent retinal imaging using OCT, with additional sources of contrast to that offered by traditional reflectivity-based imaging. For this purpose, a white light OCT system was developed with an axial resolution below 1 µm. Using this system, PS-OCT and sOCT was demonstrated in both the healthy and the VLDLR mouse eye, in addition to the high resolution reflectivity images. An experiment was then conducted into the feasibility of detecting melanin in the RPE using the system. Exploiting the spectral dependence of backscattering as dictated by Mie theory with the help of further hyperspectral post-processing, melanin was successfully detected in the RPE of the Brown Norway rat. Hyperspectral OCT, developed during this work, may find future applications in retinal imaging of melanin in diseases such as AMD, but may also find uses outside the eye, including skin imaging, industrial particle sizing, or art investigations.

In parallel to the development of the white light system, a longitudinal study was being conducted on the retina of an APP/PS1 model of Alzheimer’s disease using a multi-contrast system centered at 840 nm. Simultaneous information was acquired on retinal layer thickness and structure, presence of HRF, phase retardation abnormalities and retinal vasculature. The retinas and brains of these mice were also investigated post mortem with an advanced histology protocol to compare the in vivo results to a ground truth. While the cortical immunohistochemical staining revealed clear, marked differences in Aβ plaque load between APP/PS1 transgenic mice and their wildtype littermates, similar retinal changes were not observed. In fact, candidates for retinal Aβ were only identified in 1 out of 17 transgenic mice in histology, indicating that these APP/PS1 mice do not routinely develop Aβ plaques in the retina in the same manner as they do in the brain.

Additional sources of contrast, including PS-OCT, sOCT and OCTA, offer valuable information at both visible light wavelengths and in the NIR range. The increased resolution provided by white light OCT also applies to these additional contrast mechanisms. For this reason, with the improvement of laser technology and optical components, it is likely we will see a rise in the use of white light multi-contrast OCT technologies in the future.
REFERENCES
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Education

- **Medical University of Vienna**
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- **University of St Andrews**
  - MPhys(Hons) Physics, 2:1  
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- **Mintlaw Academy**
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Research Experience

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  Vienna, Austria  
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    - Design and construction of a visible light, polarisation sensitive OCT system for rodent retinal imaging
    - Performing longitudinal studies on animal models of the retina using extensions of OCT
    - Developing post-processing algorithms to improve image quality
    - Hyperspectral confocal microscopy of the *ex vivo* mouse retina

- **University of St Andrews**
  - Master’s Project (Supervisors: Dr. C. T. A. Brown and Prof. K. Dholakia)  
  Fife, UK  
  September 2014-May 2015
    - Optimisation of a Fourier domain OCT system to increase speed and improve image contrast
    - Imaging Antarctic krill with the system after such changes were implemented

- **University of St Andrews**
  - Research Intern (Supervisor: Dr. C. T. A. Brown)  
  Fife, UK  
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    - Assisting with the setup of an OCT system for the imaging of Antarctic krill

- **Purdue University**
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  Indiana, USA  
  January–June 2014
    - Analysis of water-splitting ruthenium catalysts using density functional theory to determine which intermediate states are formed in the short time it takes to split water
    - X-ray absorption spectroscopy of ruthenium and manganese complexes at Argonne National Laboratory, IL

- **Ninewells Hospital**
  - Volunteer Researcher (Supervisor: Dr. G. Gardner)  
  Dundee, UK  
  May–June 2012
    - Measuring staff radiation dose during diagnostic positron emission tomography scans
Awards

ARVO International Travel Grant
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- April 2019

Best Oral Presentation
- Gordon Research Seminar: Lasers in Medicine and Biology, Lewiston, ME
- July 2018

Best Oral Presentation
- 14th Young Scientists Association PhD Symposium, Vienna, Austria
- June 2018

IOP Research Student Conference Fund Bursary
- Institute of Physics
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First Place: Student Oral Presentation
- 2nd Canterbury Conference on Optical Coherence Tomography, Canterbury, UK
- September 2017

Best Poster Presentation
- 13th Young Scientists Association PhD Symposium, Vienna, Austria
- June 2017

Postgraduate Congress Travel Grant
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Early Career Travel Award
- PLOS
- December 2016

International Communication Travel Grant
- Austrian Science Association
- November 2016, February 2018 and July 2019

Science|Art Award
- Young Scientists Association, Medical University of Vienna
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Semester Honors Award
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Selected Committee Roles

Gordon Research Seminar: Optics and Photonics in Medicine and Biology 2020
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- New Cottage Chinese Take Away, Aberdeenshire, UK
  - Counter/Kitchen Assistant
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Peer-reviewed journal publications (as first author)

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2018


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2018


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