NON-INVASIVE RETINAL IMAGING IN MICE WITH FLUORESCENT SCANNING LASER OPHTHALMOSCOPY AND FOURIER DOMAIN OPTICAL COHERENCE TOMOGRAPHY

by

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ABSTRACT

Visualization of the internal structures of the retina is critical for clinical diagnosis and monitoring of pathology as well as for medical research investigating the root causes of retinal degeneration. The aim of this thesis is to develop multi-modal non-invasive imaging technology for studying retinal degeneration and gene therapy in mice. We have constructed a FD-OCT prototype and combined it with a Scanning Laser Ophthalmoscope (SLO) to permit real time alignment of the retinal field of view. Fluorescence detection was integrated with the SLO to enable the \textit{en face} localization of a molecular contrast signal, which is important for retinal angiography and retinal auto-fluorescence imaging. The integrated FD-OCT/fSLO system was investigated for imaging the retina of mice \textit{in vivo}. The results of this research have demonstrated that the state of art FD-OCT/fSLO system OCT is a powerful tool to conduct research retinal degeneration and gene therapy in mice.

\textbf{Keywords:} Optical Coherence Tomography; Scanning Laser Ophthalmoscopy; retinal degeneration; \textit{in vivo} retinal imaging; fluorescent imaging; retinal angiography.
DEDICATION

The encyclopaedia defines Infinity as concept in mathematics and philosophy that refers to a quantity without bound or end. In the philosophy of my life up to this chapter:

- Infinity is how much my parents have done for me, how much they sacrificed.
- Infinity is how much I owe them.
- Infinity is how lucky I am to be a student of Marinko.
- Infinity is how much more left to learn.
ACKNOWLEDGEMENTS

I would like to thank my committee members and all my fellows in the Biomedical Optics Research Group.
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<th>Description</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno Associated Virus</td>
</tr>
<tr>
<td>ADC</td>
<td>Analog to digital converter</td>
</tr>
<tr>
<td>APD</td>
<td>Avalanche Photo Diode</td>
</tr>
<tr>
<td>BM</td>
<td>Bruch's membrane</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CH</td>
<td>Choroid</td>
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<tr>
<td>cSLO</td>
<td>Confocal Scanning Laser Ophthalmoscopy</td>
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<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>EM wave</td>
<td>Electro-Magnetic wave</td>
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<tr>
<td>DAQ</td>
<td>Data acquisition card</td>
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<tr>
<td>DOF</td>
<td>Depth of focus</td>
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<td>FD-OCT</td>
<td>Fourier Domain Optical Coherence Tomography</td>
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<tr>
<td>FFT</td>
<td>Fast Fourier Transform</td>
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<tr>
<td>fps</td>
<td>Frames per second</td>
</tr>
<tr>
<td>fSLO</td>
<td>Fourier Scanning Laser Ophthalmoscopy</td>
</tr>
<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
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<tr>
<td>HM</td>
<td>Hot Mirror</td>
</tr>
<tr>
<td>ICG</td>
<td>Indocyanine Green</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>IS layer</td>
<td>Inner segment of Layer of Rods and Cones</td>
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<tr>
<td>LCA</td>
<td>Leber's Congenital Amaurosis</td>
</tr>
<tr>
<td>LCI</td>
<td>Low coherence interferometry</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>Neodymium-doped yttrium aluminum garnet</td>
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<tr>
<td>NIR</td>
<td>Near infra red</td>
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<tr>
<td>OCT</td>
<td>Optical Coherence Tomography</td>
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<tr>
<td>ONH</td>
<td>Optical Nerve Head</td>
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<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
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<tr>
<td>OPL</td>
<td>Outer plexiform layer</td>
</tr>
<tr>
<td>OS layer</td>
<td>Outer segment of Layer of Rods and Cones</td>
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<tr>
<td>PMT</td>
<td>Photo Multiplier Tube</td>
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<tr>
<td>RDD's</td>
<td>Retinal Degenerative Diseases</td>
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<tr>
<td>RP</td>
<td>Retinis Pigmentosa</td>
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<td>RPE</td>
<td>Retinal pigmented epithelium</td>
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<tr>
<td>SLD</td>
<td>Super luminescent diode</td>
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<tr>
<td>SLO</td>
<td>Scanning Laser Ophthalmoscopy</td>
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1: THESIS INTRODUCTION

1.1 Introduction

Animal models of human diseases are often used for the development of novel pharmaceutical and genetic therapies. Non-invasive imaging techniques have the potential to reduce the number of animals required to evaluate therapies, and monitor the progression of successful techniques. The goal of this research is to develop non-invasive imaging technology for vision researchers studying animal models of retinal degeneration and gene therapy. In this thesis, two imaging modalities, Optical Coherence Tomography and Scanning Laser Ophthalmoscopy, are combined to provide both structural and molecule contrast images for diagnostic mouse retinal imaging.

In this introductory chapter, retinal hereditary diseases and the concept of gene therapy as a potential treatment are introduced. The imaging technologies used for non-invasive retinal imaging are described and compared. The remainder of this chapter is a synopsis of the thesis organization.

1.1.1 Introduction to gene therapy for Retinal Degenerative Diseases (RDD’s)

Hereditary disorders result from defective genes that are unable to perform their expected functions, and synthesize wrongly coded proteins or no protein at all. Gene therapy is an exciting branch of research with the potential to
permanently reverse the effects of the disorders. There are several approaches for correcting or replacing faulty genes.

A common approach to gene therapy is to insert a normal copy of the gene into a cell to replace a non-functional gene. Insertion of the therapeutic gene into the unhealthy cell is a very complex procedure. Healthy genes are delivered to the faulty cells by the vectors. The two most commonly used vectors are synthetic vectors and viruses, in which the viral genome has been replaced by the therapeutic gene [1]. A vast multitude of hereditary disorders exists, affecting one or multiple genes. Retinal Degenerative Diseases (RDD’s) are particularly attractive candidates for research in gene therapy because they are often caused by failures of a single gene.

The retina is located at the back of the eye and is responsible for detecting light, then processing and transmitting the signal to the brain via the neurons of the optic nerve. The position of the retinal relative to the other optical components of the human eye is presented schematically in top panel of Figure 1-1.
Successful gene therapy experiments have been performed on animal models of RDD through vectors based on the Adeno Associated Virus (AAV) [3]. The gene therapy vectors were delivered to the subject's eye by injection into the
sub retinal space *in vivo* (in the living organism). The needle is passed through a hole punctured on the sclera just behind the iris, permitting access to the retina without damaging the lens. The procedure is observed through the dilated pupil using a surgical microscope. This type of gene therapy delivery restricts the therapeutic activity to the retina because the blood-retina barrier does not allow the agents to enter the general circulation [4]. Early clinical trials of gene therapy in humans have concentrated on curing patients with RPE65-associated Leber's Congenital Amaurosis (LCA). The results indicate permanent visual improvement in all of the treated subject [5]. Research is currently focussed on applying gene therapy to other RDD’s, such as Stargardt’s disease.

### 1.1.2 Introduction to Stargardt’s disease

There are various types of inherited retinal diseases such as Retinitis Pigmentosa, macular degeneration, and Cone-rod dystrophy (CORD). Macular degeneration, a medical condition that results in damage to the retina, has brought up a considerable amount of attention to the governments, health organizations, and researchers in recent years.

Stargardt's disease is the most common hereditary form of macular degeneration, targeting minors in contrast to the other forms that affect the elderly. German ophthalmologist, Karl Stargardt in 1901, first identified the disease. Disease surveillance suggests that 1 in 10,000 adolescents are affected by the disease worldwide. With Stargardt's disease, very centre of the retina, called the macula, and its surrounding area are affected. Any damage to the
macula results in loss of central visual acuity (sharpness of vision), and decreased colour vision with small blind spots [6].

1.1.3 Clinical description

The early stages of the Stargardt's disease start before the age of twenty. Patients usually face difficulty with reading and seeing in dim lighting. They also suffer from blurred and distorted vision. In this stage, a lipid-containing residue, which is also known as lipofuscin, starts to accumulate in the Retinal Pigment Epithelium (RPE) layer beneath the macula (Figure 1-1 bottom panel). Lipofuscin deposits appear as yellowish spots on the retina. As the disease progresses, more deposits accumulate increasing the coverage of larger areas. However, they are not likely to appear outside of the area between the optic disc and the macula. Lipofuscin excess results in atrophy of the macula and the underlying RPE [6]. Patients with Stargardt’s disease have a very low visual acuity at this stage and are mostly considered legally blind. In some cases colour vision impairment also appears leading to imperfection of the red –green colour detection.

1.1.4 Genetic Description

The cause of Stargardt's disease can be explained by understanding the cellular physiology of the eye related to vision. The vertebrate eye contains two types of photoreceptor cells: rods, cones. In mammals there is an additional class of photoreceptors called photosensitive ganglion cells, which are non-visual receptors located mostly in the inner retina [7]. Cones and rods are located
beside retinal pigment epithelial cells, forming a layer often called the Inner and Outer Photoreceptor layers. The outer segment of the cell is responsible for producing the electrical signal according to the received light. Multi disc shaped layers stacked on each other are surrounded by a plasma membrane to form the outer segment of both cone and rod photoreceptors.

Stargardt’s disease is hereditary, and has been connected to a mutation in the ABCA4 gene. The protein encoded by the ABCA4 gene is localized to the outer segment disk edges of rods and cones. The ABCA4 gene is member of a super family of genes that encode trans-membrane proteins which transport lipid substrates that contain vitamins, fatty acids, phospholipids, glycolipids, cholesterol, bile salts, steroids and drugs across the cell membrane [8]. Mutations in the ABCA4 gene produce a dysfunctional protein that cannot perform its transport function. As a result, the toxic lipofuscin deposits accumulate causing the photoreceptor cells degenerate, ultimately resulting in vision loss[6]. Stargardt’s disease is an autosomal recessive disorder [8]. This means that two copies of the defected gene are needed, one from each parents, in order to develop the condition. Diagnosis of Stargardt’s disease can be performed by non-invasive medical imaging technology that can visualize lipofuscin in the retina.

1.2 Non-invasive imaging modalities

The development of techniques for medical imaging is a rapidly evolving field of research. Microscopy, ultrasound, MRI and Computed Tomography (CT)
are well known imaging techniques in medicine and biology. Breakthroughs in medical imaging provide not only the ability to visualize the structure but also the function of the body.

The location of the retina at the back of the eye makes non-invasive imaging of challenging. The following is a brief review of non-invasive imaging modalities used in ophthalmology for visualizing the retina in humans. The design of these systems is facilitated by the relatively large size and high visual acuity of human vision. The following section introduces the ophthalmic imaging techniques used in this research

1.2.1 Fundus photography vs. Scanning Laser Ophthalmoscopy (SLO)

The ophthalmoscope, invented by Herman von Helmholtz in 1851, was the first instrument intended for inspecting retina. It works simply by illuminating the retina by a white light source while looking at it through a specially designed magnifying lens.

The fundus camera was introduced in 1935 to photograph the pattern of blood vessels in the retina and use it as method of identifying criminals [9]. The fundus camera is a move advanced version of the ophthalmoscope and contains two main optical components: the ophthalmoscopic lens and camera optics. Its operation is very close to that of the ophthalmoscope except that it has to form images on the film or CCD camera and that means additional constrains that needs to be issued. An additional aperture at the camera’s entrance pupil along with the field lens system helps to avoid blurred images. Due to the curvature of
the retina the corresponding image will not be formed on one plane. This problem is overcome by designing the ophthalmoscopic lens with specific refractive indices, shapes and sizes to obtain a flat image plane.

In 1980, Webb et al, first introduced the concept of Scanning Laser Ophthalmoscopy (SLO) as “flying spot TV ophthalmoscope” [10]. The technique behind SLO is to raster scan a beam of laser, focused to a tiny spot of the fundus, over the surface of the retina. Back reflected light is travelling the same optical path out of the eye is directed towards the photo detector. The intensity of the back reflected light from the corresponding retinal spot is translated to pixel intensity. Later on, the contrast of the system was improved by utilizing confocal apertures. The operation of the Confocal Scanning Laser Ophthalmoscope (cSLO) is fully explained in chapter 2.

1.2.2 Fluorescence retinal imaging

Fluorescence is the emission of light from specific types of molecules (fluorophores) shortly after the absorption of the light with a shorter wavelength. Fluorescence provides molecular contrast in biological specimen and can be using in medicine, molecular biology and pharmacology. In 1961, HR Novotny took photos of Fluorescein (a FDA approved Fluorophores) passing through the retinal vessels in a human with a fundus camera to study retinal blood flow [11]. Scanning laser ophthalmoscopy has also been widely used for Fluorescein and Indocyanine Green (another FDA approved fluorophore) angiography in order to get higher contrast in vivo fluorescent data of the human retina that possible with fundus photography.
Other than angiography, fluorescent detection is a powerful method for the detection of molecular contrast signals in the retina to diagnose and control disorders. Identifying the auto-fluorescence signals of the retina is one of these applications that have also been performed on humans. For example, utilizing the auto-fluorescence from lipofuscin deposits in the retina associated with Stargardt macular degeneration.

Unfortunately, fluorescent images taken by fundus camera or SLO system are limited to two dimensional topographic images. They cannot be used for morphological studies since they do not provide detailed structural images of deposits in retinal layers.

1.2.3 OCT

Three well known depth resolved imaging modalities, CT and MRI and ultrasound are not well suited for ophthalmology. The resolution and system implementation of these techniques do not fulfil the need of high resolution sub-surface imaging specifically required in ophthalmic imaging. The fundus camera does not provide any depth information and optical sectioning and sub-surface layer imaging of cSLO is minimal. Non-invasive *in vivo* imaging is crucially important when it comes to the retina. The human retina is nominally half a millimetre thick and consists of multiple layers. The relative thickness of each retinal layer is important in the diagnosis and understanding of retinal degenerative diseases.
In 1990, A. F. Fercher and C. K. Hitzenberger presented the non-invasive depth resolved images of human retina acquired using a dual beam low time coherence interferometric technique [12]. A year later, a fibre optic version of Low Coherence Interferometry (LCI) was presented by Fujimoto’s group [13]. This technique, called Optical Coherence Tomography (OCT) led to significant clinical application.

The state of art OCT systems are capable of providing less than 10µm axial resolution [14]. However, OCT can only penetrate through a couple of millimetres of tissue even under ideal conditions. The strength of OCT is the ability to generate high resolution volumetric images of the retina. However, even modern implementations of OCT have drawbacks. For example, two dimensional en face image acquisition is much slower with OCT that SLO.

Furthermore, OCT is limited to the detection of coherent light, and thus is insensitive to fluorescence. OCT is able to provide three dimensional high resolution images but the image contrast is based on a structural basis, relying on refractive index differences in the tissue for contrast, and does not provide information on the molecular content of the sample. As a result auto-fluorescence of lipofuscin deposits cannot be detected with a standard OCT or FD OCT system. A combination of both OCT and fSLO imaging modalities is required in order to image both the retinal structure and fluorescence molecular contrast.
1.3 Thesis objectives and goals

Currently, evaluation of attempts to provide gene therapy to the experimental animals is performed through histology. A time course study of retinal degeneration in mice cannot be performed on a single specimen since invasive histological techniques require euthanizing the animal. Non-invasive visualization of the internal structures of the retina is critical for monitoring pathological changes or rescue by gene therapy over time. With non-invasive imaging, fewer animals are required for time course studies. Another benefit of non-invasive imaging is that it eliminates a large extent of the artefacts due to tissue dehydration, shrinkage, and stretching during the processing involved in histology.

The specific aim of this thesis is to develop non-invasive imaging technology for studying retinal degeneration and gene therapy in mice. Observation of structural deposits will be facilitated by integration of both structural and functional imaging modalities. The integrated system will be specifically designed for non-invasive imaging of Stargardt's diseases.

This thesis is based on a collaborative research project with Dr. Robert Molday, professor of Molecular Biology and Biochemistry (MBB) and Director of the centre for Macular Research (CMR) at UBC. His research group’s primary focus is on vertebrate retinal photoreceptor’s intercellular structures, their membrane protein and the encoded proteins they synthesize. A portion of their research is directed towards engineering new viral vectors to successfully perform gene therapy on ABCA4 knocked out mice.
1.3.1 Customization of FD-OCT/fSLO for mice

Systems are commercially available for human eye imaging that can acquire morphological and functional data simultaneously. However, adopting these systems to image mice eye is challenging and results in non-optimized imaging. The small radius of curvature of the mouse eye results in higher refraction of the perfectly collimated incident light and introduction of stronger aberrations. Instead of using the mouse cornea for focusing a beam on the retina (the technique used in human imaging) we wanted to cancel out the refraction at the cornea, and use the sample arm optics to focus through to the retina. The crystalline lens in the mouse eye introduces some level of additional refraction.

1.3.2 Integration of OCT+SLO

We have developed a custom imaging system through the combination of a prototype OCT imaging system and a prototype fSLO imaging system. Combination of SLO with OCT is a natural pairing in which the unique advantages of each imaging modality complement the other. The two systems operate at different wavelengths (OCT in the near infrared and SLO in the visible) permitting the use of heat control mirrors and dichroic filters to separate out the various signals according to wavelengths. A custom optical setup was constructed to permit co-linearization of the SLO and OCT interrogating beams from the two sub-systems. Images are acquired subsequently with each sub-system and would be located at the same position on the retina. This permits rapid alignment of the field of view with the SLO sub-system, followed by high resolution imaging with the OCT system.
The following two chapters of this thesis concentrate on the theoretical concepts behind the proposed system. Chapter two gives an overview of the Scanning Laser Ophthalmoscopy (SLO) followed by an introduction to fluorescence and fluorescence imaging. Chapter 3 provides a brief background on the Optical Coherence Tomography (OCT). The optical design for a hybrid system for small animals will be introduced in Chapter 4. The rest of the chapter presents multiple experiments to validate and quantify the proposed design criteria. A longitudinal study of retinal imaging in mice models using the hybrid system is presented in Chapter 5. This chapter provides biometrical information of the mouse eye followed by presenting SLO, OCT and fluorescence images acquires using the proposed system of wild type and transgenic mice. This thesis concludes with a discussion of future work.
2: CONFOCAL SCANNING LASER OPHTHALMOSCOPY

2.1 Introduction

Scanning Laser Ophthalmoscopy (SLO) is an ophthalmic imaging technique to obtain high resolution \textit{en face} images of the retina, including the retinal vasculature system. The two dimensional image of the retina is called a fundus image. Scanning laser ophthalmoscopy uses a different approach from the conventional indirect ophthalmoscopy (fundus photography) which uses a flood illuminated imaging method [15]. The scanning laser ophthalmoscope focuses a narrow band laser on a single spot on the fundus. Back reflected light retraces the same optical pathway back towards the optical system. The intensity of the back reflected light from the spot is incident on a photo detector, and corresponds to the brightness of a pixel corresponding to the physical location on the display. A two dimensional image of the sample surface is obtained by scanning the spot over the retina and synchronously detecting the back reflected intensities. The fusion of confocal imaging techniques with SLO, called Confocal Scanning Laser Ophthalmoscopy (cSLO), provides a strong \textit{in vivo} imaging tool for ophthalmologists. Confocal SLO provides higher contrast by limiting the field of view and revealing structure that cannot be observed in traditional SLO [15]. A schematic of cSLO system is provided in Figure 2-1.

In this chapter, the generalized concepts behind Gaussian optics and confocal SLO are reviewed. The formulations developed here will be used to
design the actual specifications of the system constructed in this research as discussed in Chapter 4. The chapter concludes by introducing fluorescent imaging in the retinal with cSLO.

Figure 2-1 A schematic of a simplified the cSLO system used to image human eye. A laser beam from a laser source is focused onto the retina. The corresponding back reflected light passes through the pinhole and is detected by a photo-detector. Meanwhile, excited fluorescent on the retina generates signals at a longer wavelength. Fluorescence is separated from the excitation beam by a Dichroic Mirror (DM) and is directed towards a designated photo detector.

2.2 Overview of Gaussian optics

The intensity profile of a laser beam is described by a Gaussian function. The radius of the beam measured perpendicular to the propagation direction is [5]:

\[
\omega(z) = \omega_0 \left[ 1 + \left( \frac{\lambda z}{\pi \omega_0^2} \right)^2 \right]^{0.5}. \tag{2-1}
\]

The minimum beam radius is called the beam waist \(\omega_0\), which corresponds to the output of the laser. When focused through a lens, the minimal beam waist is represented as the focal waist \(\omega_f\). \(Z_R\) is called the Rayleigh range, and the
depth of the focus of the focused beam is commonly taken as two times the $Z_R$.

The depth of focus corresponds to the distance on either side of the waist where the spot size is equal to $\sqrt{2}\omega_0$ is called the Depth Of Focus (DOF) in Figure 2-2. The DOF is the extent of the region around the image plane in which the image will appear to be sharp. In the case of SLO, the sharpness of the image will be related to the strength of the reflection. The waist and Rayleigh range are described schematically in Figure 2-2. The equations are listed below:

$$Z_R = \frac{\pi \omega_0}{\lambda}, \quad \text{Eq 2-2}$$

$$DOF = 2Z_R = \frac{2\pi \omega_0 f^2}{\lambda}, \quad \text{Eq 2-3}$$

$$\omega_f = \frac{f \lambda}{\pi \omega_1}. \quad \text{Eq 2-4}$$

In Eq 2-4 the assumption was made that a collimated beam was incident on the lens, thus $R_1 \rightarrow \infty$. 

Figure 2-2 This schematic shows the Gaussian beam profile at the focus where $\omega_0$ is the beam waist, DOF represents the depth of focus and $\omega(z)$ is the beam radius at an arbitrary distance $z$. 

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2.3 Concepts behind Confocal Scanning Laser Ophthalmoscopy (cSLO)

2.3.1 Wavelength selection and tissue penetration

A wide range of lasers is used in ophthalmic imaging and treatment. To choose the optimum wavelength for imaging, the type of the tissue has to be considered along with the tissues, which the light has to traverse along the way. Different tissues affect image quality and information because they absorb and scatter light differently. For example, to perform retinal imaging the light has to traverse through nominally an inch of vitreous humour prior to reaching the retina. The vitreous, which occupies the posterior section of the eye, is a clear liquid and is 99% water. The absorption spectrum of water shows that wavelengths in the range of ultra-violet and infrared are highly absorbed. As a result, wavelength choices roughly lower than 400nm or higher than 1100nm are not reasonable choices for retinal imaging, as they would highly attenuate. Consequently, common wavelengths used for cSLO imaging are in the visible and near infrared portions of the spectrum. The longer wavelengths are used for deeper tissue imaging because of lower scattering in the tissue.

2.3.2 Axial and lateral resolution

The focusing optics of the human eye refract incident beams of light onto the retina which is nominally 22mm away from the air-cornea interface[16]. The crystalline lens of the eye provides focusing accommodation to enable a clear view of objects at different distances by changing its radius of curvature, resulting in focal length variations between roughly 14mm to 17mm [16].
Due to the natural focusing elements of the eye, a collimated beam incident on the cornea is focused on the retina and there is no need for external focusing optics. The control of the attainable resolution and depth of focus would be limited by the incoming beam waist. The limitations to the focused spot size at the retina come from aberrations in the cornea and lens, and the entrance pupil size determined by the iris. Pupil diameter is usually 2mm but can be dilated up to 9mm. It is very common to focus the light to a spot on the retina on the order of $<10\mu m$ for SLO and 20-30$\mu m$ for OCT.

Due to diffraction, lateral resolution is not equal to the predicted Gaussian spot size of the focused beam. Instead, the resolution is limited by a circular bull’s eye diffraction pattern with a bright central disc. This is called the Airy disc and its size depends on the wavelength and numerical aperture of the focusing elements (in microscopy this would be the objective lens). The radius of the central circle and the first dark ring around it is estimated by [17]:

$$r_{\text{Airy}} = 0.61 \frac{\lambda_o}{NA_{\text{obj}}}$$

Eq 2-5

The image of two neighbouring spots with the same intensity is only resolvable when their distance is more than the Airy disc radius. This is called the Rayleigh criterion.

Depth resolved resolution is also referred to as axial resolution since it is on the same axis as scanning beam. The diffraction image of a small point object that is resolvable along the scanning axis inside the depth of focus region is called axial resolution. However, in practical applications of ophthalmic imaging,
achieving such a precise depth sectioning is almost impossible. The back reflected light from multiple axial positions inside the depth of focus accumulates at each instance and adding a confocal aperture can only minimally improve the axial resolution [17].

2.3.3 Confocal imaging

Confocal imaging is essentially a geometric filter to improve resolution in the axial direction as well as lateral. The source illumination is focused through a pinhole to reduce the field of illumination. Another pinhole is placed before the detector to reduce the field of view by eliminating the light scattered from depths other than the desired focal plane. Images acquired from a thin volumetric section can thus be produced by confocal imaging. By displacing the detector pinhole along the optical axis different depth sections of the sample can be revealed and combined to reconstruct a depth resolved image of the sample. By rejecting scattered light outside of the depth of focus, confocal techniques provides images which are sharper than ones taken using regular SLO.

2.3.4 Pinhole size

The size of the pinhole and its position in space relative to the focusing lens can dramatically change the quality and the nature of the confocal image. The system resolution is dependent on the pinhole size, and with smaller pinhole diameters corresponding to better sectioning. However, as the aperture is made smaller, the amount of light passing through it is reduced. This result in a decrease of signal to noise ratio and can dramatically spoil the quality of the
images especially when it comes to weak signals, such as fluorescence imaging [18]. The signal to noise ratio cannot always be compensated by increasing the source power especially when it comes to \textit{in vivo} imaging [18]. Theoretically, the best choice of aperture diameter is slightly larger than the Airy disc (all sources). However, getting enough light through this diameter is not possible in practice. The best axial resolution that can be acquired using typical cSLO systems used on human eye nominally 350µm at 780nm [19]. However, 100µm depth sectioning resolution has been reported after utilizing adaptive optics [20].

### 2.4 Fluorescence and auto-fluorescence

When exposed to light, fluorescent molecules get excited by absorbing the energy of the incident photons. The absorbed energy results in an electron jump from the ground state to an excited state. A small portion of the energy is lost which results in vibration or heat, while most of this energy loss is through the emission of a photon as the electron drops to the ground state. As a result, the photons of the emitted light have less energy and thus a longer wavelength (Stoke’s shift) [21]. The process of fluorescence is classically presented by Jablonski energy diagram a shown in Figure 2-3.

Fluorescence imaging is used for both structural and functional live-tissue imaging. Fluorophores can be mixed with antibodies and fluoresce while bound to certain parts of an organism, giving signals that can be used to detect the location and functionality of the respected organism.
Fluorescence imaging arguably provides the strongest signal to detect molecular contrast and better understand the molecular content of a living cell. Some molecules of living tissue contain fluorescent components and do not need external compounds attached to them to produce light. The emitted light from these endogenous molecules is called auto-fluorescence. This phenomenon can be used as a diagnostic indicator, however, it can also interfere with the signal from exogenous fluorophores when the emission ranges overlap. Exposure to a high power excitation source, or excitation over a long period of time, can result in a fading of the fluorescence intensity, a phenomenon commonly known as photo bleaching [21].
2.5 Fluorescent SLO

In vivo fluorescent confocal angiography has found common application in ophthalmology and is performed using FDA approved dyes such as fluorescein and indocyanine green. The most important application of retinal angiography is to detect vascular leakage especially in the choroid [20].

Confocal scanning laser ophthalmoscopy can be readily augmented for detection of fluorescence. The only difference in the system design to implement fluorescent cSLO is in the detections of the multiple wavelengths in the light back reflected from the sample. A dichroic mirror is usually used to split the two concurrent back reflected wavelengths, i.e. reflecting the excitation wavelength and passing the longer wavelength of the fluorescent emission.

The signal from the fluorescence light is much weaker than the excitation intensity. Quantum yield refers to the ratio of absorbed to emitted photons. A quantum yield of 0.1 is considered to be strong fluorescence. Furthermore, the emission from an excited fluorescent molecule is isotropic. Consequently, even at the maximum quantum yield a very small portion of the fluorescence traverses the path back to the detector. As a result, the weak fluorescent signal requires highly sensitive detectors such as a Photo Multiplier Tube (PMT) for detection.

2.6 Summary

Confocal scanning laser ophthalmoscopy permits detection of back reflected and fluorescence signals in the retina. The concepts developed in this chapter is used to design a custom cSLO system for mouse imaging in Chapter
4. However, even cSLO is incapable of providing sufficient axial resolution to resolve the retinal cell layers. In order to acquire high resolution three-dimensional images and OCT sub-system can be integrated with the SLO. The subject of OCT and concepts behind it is presented in the following chapter.
3: OPTICAL COHERENCE TOMOGRAPHY

3.1 Introduction

Optical Coherence Tomography (OCT) was first introduced in 1991 [13] and rapidly found its place in biomedical imaging, especially in the field of ophthalmology. OCT facilitates cross sectional imaging with micron-scale resolution approaching that of histology. A considerable advantage of OCT is that it can readily be performed in vivo. OCT is one of the few imaging techniques which derive a benefit from the coherence properties of light to produce high resolution tomographic images in highly scattering biological tissues [14].

In this chapter, the basics of low coherence interferometry are presented. Following the basics, the concepts behind the OCT imaging in tissue are introduced, with an emphasis on ophthalmic applications.

3.1.1 Interferometry

OCT is a form of interferometry, and is based on the coherent interference of light. When two electromagnetic waves interfere, the consequential intensity contains more terms than just the straightforward accumulation of the two components. For example, consider a Michelson interferometer in which a beam of monochromatic light is split into two arms using a beam splitter as shown in Figure 3-1. When the two electromagnetic waves $\psi_1$ and $\psi_2$ are recombined at
the detector, they interfere with the resulting intensity at the detector $I_{\text{interference}}$ determined by:

$$
\psi_1 = E_1 Re\left\{e^{i(k2z_1-\omega t+\phi_1)}\right\}, \quad \text{Eq 3-1}
$$

$$
\psi_2 = E_2 Re\left\{e^{i(k2z_2-\omega t+\phi_2)}\right\}, \quad \text{Eq 3-2}
$$

$$
I_{\text{interference}} = e_0 (\psi_1 \cdot \psi_2) = ((\psi_1 + \psi_2)(\psi_1 + \psi_2)^*), \quad \text{Eq 3-3}
$$

$$
I_{\text{interference}} \approx 0.5(E_1^2 + E_2^2) + E_1 E_2 Re\left\{e^{i(k2z_1-\omega t+\phi_1)-i(k2z_2-\omega t+\phi_2)}\right\}. \quad \text{Eq 3-4}
$$

Note that $k$ represents the wave number, defined as $2\pi/\lambda$, where $\lambda$ is the corresponding wavelength. The last term in Equation 3-4, known as the interference term, exists only if the waves are coherent. Equation 3-4 can be simplified to [16]:

$$
I_{\text{interference}} \approx 0.5E_1^2 + 0.5E_2^2 + E_1 E_2 (cos(2(z_2 - z_1)k - \omega t + \phi(t)_{2-1})). \quad \text{Eq 3-5}
$$

The interference of the reflected beams produces the fringes which are related to the relative optical path length difference between the two mirrors. The intensity pattern at the detector can be described as a cosine term dependent on the path length difference:

$$
I_{\text{detector}} = I_R + I_S + 2\sqrt{I_R I_S} \cos(2\Delta z k), \quad \text{Eq 3-6}
$$

where $I_{\text{detector}}$ is the intensity of light from each arm to the detector and function is in terms of wave number $k$. Since the EM wave was split into two at the beam splitter, the time average of the term $\phi_{2-1}$ will be zero. By moving one of the mirrors, intensity at the detector changes constantly from a maximum of $4I$ to a minimum of zero.
3.1.2 Coherence and low coherence

Two waves are said to be coherent if their phase difference stays constant throughout their propagation. The possibility of keeping a fixed phase difference along the propagation axis is called “temporal coherence” and depends on the bandwidth of the propagating beam. Lasers are a source of coherent light. The distance that a laser beam travels before it goes through a phase change is called coherence length and the duration of this stability is called the coherence time [16].

By increasing the bandwidth of the light, temporal coherence length decreases. Sources with significantly small coherence lengths are called low
coherence beams. The coherence length can be represented by the bandwidth of the source:

\[ l_c = \frac{2 \ln 2}{\pi} \cdot \frac{\lambda_0}{\Delta\lambda} \]  

Eq 3-7

where \( \Delta\lambda \) the bandwidth of the source, \( \lambda_0 \) is the central wavelength of the source bandwidth and \( \Delta\lambda \) is the full width half max bandwidth. Equation 3-7 is based on the assumption of a Gaussian spectral profile, as is normally used for OCT imaging. When a broad spectral source is incorporated into Equation 3-7, it is commonly referred to as Low Coherence Interferometry (LCI). If the path length difference between the sample and the reference in the interferometer is more than the coherence length, the two beams would not interfere. This is because the sample beam will have a different phase term than that of the beam coming from the reference arm. Waves that travelled through the sample longer than the coherence length do not contribute to the fringes, which is why this technique is also referred to as coherence gating.

The equation at the detector can be re-written to include the source spectrum. In Equation 3-6, we consider that the detector to be wavelength resolved:

\[ I_{detector} = 2S(k)(I_R + I_S) + S(k)[2\sqrt{I_R I_S} \cdot \cos(2k\Delta z)] \]  

Eq 3-8

where \( S(k) \) is the intensity profile of the source spectrum in wave number.
3.1.3 Optical Coherence Tomography

Optical Coherence Tomography (OCT) is a non-invasive medical imaging modality based on low coherence interferometry (LCI). Different types of OCT have been developed based on the techniques they use to extract the depth information from the interferometric signal. The earliest introduced method is referred to as Time Domain OCT [13]. In this method, the intensity information of each depth location in the sample is extracted by translating the reference mirror. The depth intensity profile along the scanning axis is constructed by moving the reference mirror position.

An advancement in OCT technology called Fourier Domain detection was introduced in 2003 [22] [23] [24]. In FD OCT, the reference arm remains fixed, and it is possible to acquire data from multiple depths at once by spectrally resolving the signal at the detector and applying the Fourier-transform. The topology of a simplified spectrometer based FD-OCT system is presented in Figure 3-2.

![Figure 3-2 Fourier Domain OCT interferometer topology.](image-url)
The data acquisition and processing steps in FD OCT are schematically described in Figure 3-3 for the case of a single reflector in the sample arm. The interference fringes superimposed on the spectrum generate the FD OCT signal, commonly called an interferogram. Due to the nature of the spectrometer, the interferometric signal acquired is sampled linearly in wavelength. However, it is the frequency of the fringes in wavenumber units which is related to the path length difference between the reference mirror and the scatterer location in the sample. In order to process the signal with the discrete Fast Fourier transform, the interferogram needs to be re-sampled to be linear in wavenumber space. After calculating the Fourier transform of the interference spectrum, the resulting signal consists of “spikes” around the DC component, which represent the locations of the scatters and their complex conjugate:

$$\Im[I_{detector}] = \Im(\sum 2S(k)(I_R + I_S) + S(k)[2\sqrt{I_R I_S} \cdot \cos(2k\Delta z)]) \quad \text{Eq 3-9}$$

$$\Im [S(k)(I_R + I_S)] = 2\hat{S}(z) \ast \delta(z) = 2\hat{S}(z), \quad \text{Eq 3-10}$$

$$\Im \{\cos(2k\Delta z)\} = \delta(z + 2\Delta z) + \delta(z - 2\Delta z). \quad \text{Eq 3-11}$$
In addition to fewer moving parts, FD OCT has a significant sensitivity advantage over Time Domain OCT [22] [23] [24]. The remainder of this thesis will refer exclusively to Fourier domain detection in OCT.

3.2 Imaging with FD OCT

3.2.1 OCT and tissue model

Photon interactions in tissue can be categorized into three general possibilities: most photons are scattered, some are absorbed, and very few do not go through scattering of any type and are called ballistic photons. In OCT only the singly back scattered photons are detected. Multi-scattered photons are filtered at the coherence gate since they are out of phase with the reference beam. As a result, they do not contribute to the interferometric fringes [14].
The back reflected light from the sample arm is not a simple phase delayed version of the reference arm. When light propagates into the tissue, due to absorptions and scattering, its amplitude, polarization, and phase change [14]. If the initial intensities at the sample and reference arm are the same, the backscattered sample arm beam intensity from a depth section \( z \) at the wavelength \( \lambda \) would be [25]:

\[
I_s(\lambda,z) = I_r e^{-2 \int_{0}^{z} (\mu_a(\lambda,\xi_z) + \mu_s(\lambda,\xi_z)) d\xi_z} R(\lambda,z),
\]

where \( \mu_a(\lambda,z) \) is the absorption coefficient of the sample at depth \( z \) and \( \mu_s(\lambda,z) \) is the scattering coefficient and \( R(\lambda,z) \) is the fractional reflectivity.

### 3.2.2 Retinal OCT

OCT images are generated by acquiring a depth (axial) scan of data at a point on the sample. A two dimensional scan, referred as a B-scan, is obtained by scanning the beam across the sample. The primary application for OCT is in ophthalmology, specifically for imaging the light sensitive retina at the back of the eye. The retinal layers can be observed \textit{in vivo} using OCT, appearing as bright and dark alternating bands corresponding to the backscattered intensity of light from the various layers in the sample. Image contrast is also generated from refractive index changes at tissue boundaries in the sample and the boundary locations. Figure 3-4 is an example of an OCT B-scan a human retina near the Optical Nerve Head (ONH) taken \textit{in vivo} at SFU.
3.2.3 Speckle

The OCT image in Figure 3-4 contains a granular or “salt and pepper” pattern superimposed on the retinal layers. This is referred to as speckle, and is caused by sub-voxel sized scatterers in the sample. Scattered wave fronts coming from closely spaced particles can interfere since they are from the same source and the distance between them is much closer than the coherence length of the light source. These sub-wavelets are different in phase since they are not exactly from the same spatial coordinates and their interference signals create the bright and dark speckled appearance. Additional sources of speckle are: signals from multiple scatterings that went through a delay smaller than the coherence time, backscattered waves from different sides of a large particle, and scatterings from closely packed sub-wavelength particles [26]. In OCT, speckle is
often considered a form of noise because it detracts from the visibility of the layers, but it can also be used in speckle correlation tracking as in ultrasound.

3.2.4 Lateral and axial resolution and wavelength selection

A notable feature of OCT as a non-invasive imaging technique is the independence of its axial resolution from the sample arm optics, or more precisely, the lateral resolution. As discussed in Section 3.1.2, axial resolution is determined only by the spectral bandwidth of the source and is equal to the coherence length of the source. Sources with broader spectra produce higher resolution images, corresponding to a shorter coherence length as given by Equation 3-7. In contrast, the lateral resolution in OCT is dependent on the sample arm optics. A particular constraint of OCT imaging is that the depth of focus of the sample arm beam must be long enough to contain the entire sample. This enforces an additional constraint of image resolution versus imaging depth.

Wavelength selection for OCT imaging is based on the relative levels of water absorption and tissue scattering. As explained in Chapter 2, the wavelength selection for SLO system depends the type of the tissue that the light has to traverse along the way to the target tissue. OCT is a depth resolved imaging modality so light penetration in the tissue that is imaged also has to be considered. Depth penetration is dependent on both the sample itself as well as the wavelength of choice. In Chapter 2, it was explained that sources with wavelength ranging between 400nm to 1100nm are reasonable choices for retinal en face imaging due to low absorption in vitreous humour. However, OCT retinal imaging sources usually have central wavelengths of 830nm or 1060
(especially for choroid imaging) because the longer wavelengths penetrate further into the retinal tissue than the visible spectrum.

In conclusion, the resolution, contrast loss and depth penetration in OCT is dependent on the choice of the source wavelength in addition to the tissue components of the sample.

3.3 Summary

Optical Coherence Tomography is a low coherence interferometric imaging modality that provides highly detailed depth resolved tissue information. OCT images are limited to providing structural information and does not provide information on the molecular content of the sample. We have integrated OCT and SLO techniques to acquire fast en face structural and functional images along with slower but depth resolved structural images. The rest of this thesis presents the design criteria of the hybrid FD-OCT/SLO system and demonstrates its validity for retinal imaging. The next chapter concentrates on the optical design and the electronics and integration concerns of the proposed system.
4: INTEGRATED FD-OCT/fSLO SYSTEM

The preceding chapters have discussed two different retinal imaging modalities. One is suitable for *en face* molecular contrast imaging (SLO) and the other one is used to reconstruct depth resolved structural images (OCT). In this chapter we describe the hybrid system that integrates both techniques into a single multimodal imaging system and explain the design criteria in order to customize it for mouse retinal imaging.

4.1 FD-OCT/fSLO design detail

We have developed a custom multimodal retinal imaging system through the combination of a prototype FD-OCT and fSLO sub-systems. The two systems operate at different wavelengths (OCT in the near infrared and SLO in the visible) permitting the use of heat control mirrors and dichroic filters to separate out the various signals according to wavelengths. A custom optical setup was constructed to permit co-linearization of the SLO and OCT interrogating beams from the two sub-systems. A schematic diagram of the combined system is shown in Figure 4-1. A photograph of the system is also presented in Figure 4-2. The Near Infra Red (NIR) and green beams are combined at the hot mirror (HM) and directed through a unit magnification beam expander and a focusing lens. Hot mirrors are special mirrors/filters designed to transmit visible wavelengths while reflecting near-infrared wavelengths. The heat control mirror we are using
is designed to work at the incidence angle of 45 degrees. The heat control mirror reflects the OCT beam toward the sample while transmitting the light for SLO.

The combined OCT and SLO beams were raster scanned across the sample using galvanometer mounted mirrors, so registration of the images is anticipated if the beams are co-linear. A dual axis galvanometer based optical scanner was used (GM Figure 4-1). A mirror galvanometer is an electro-mechanical device that senses electric voltage, except that instead of moving a needle, it moves a mirror. An analog command input of up to ±10V DC results in ±20° mechanical degrees of scanner rotation. The X-Y galvos we used had a 3mm clear aperture (Cambridge Technology 6210H).

Although the SLO and OCT systems are not required to operate concurrently, images acquired subsequently with each subsystem would be located at the same position on the retina. This permits rapid alignment of the field of view with the SLO sub-system, followed by high resolution imaging with the OCT system.
Figure 4-1 Optical layout of the combined SLO/OCT. The infrared beam from the OCT system is combined with the green beam from the SLO at the hot mirror (HM). The fiber delivering the NIR light for OCT also acts as the collection port for backscattered light. A beam splitter is used to direct backscattered green light from the sample to the avalanche photodiode (APD) used for SLO detection. Orange fluorescent light generated at the sample is transmitted through the dichroic mirror (DM) and detected at the photomultiplier tube (PMT). A final optical filter (OF) is used to reject the excitation beam and transmit the fluorescent signal.

4.1.1 OCT Sub-system

The OCT sub-system was used to acquire three dimensional images of the mouse retinal structure, providing the ability to measure retinal layers thicknesses and observe deformations in morphological size and shape. OCT provides a combination of long depth of focus with high axial resolution. The lateral resolution is determined by the sample arm optics, but the axial resolution is determined by the optical bandwidth of the source. In this experiment, a near infrared a superluminescent diode (SLD) was used as the source. The unique property of SLD is the combination of laser-diode-like output power and
Figure 4-2 A photographic representation of the FD-OCT/fSLO system brightness with broad LED-like optical spectrum. The SLD used in this thesis had a central wavelength of 826nm and a FWHM bandwidth of 72nm. This is a common operating wavelength in retinal OCT applications balancing minimal attenuation in the water based vitreous humor with maximum tissue penetration in the retinal layers. A spectrometer was used in the detection arm, making this a Fourier Domain (FD) OCT system. The average optical power at the sample was below 500microwatts, in accordance with the ANSI recommend limit for ocular exposure at this wavelength range.

For OCT detection, a high speed custom designed spectrometer was used. The spectrometer was made of a 1200 lines/mm transmission diffraction...
grating, a 1024 element high speed Gigabit Ethernet (GigE) camera with 14\(\mu\)m square pixels. The camera was operated at a speed of 20kHz, corresponding to the line rate of the system.

4.1.2 SLO sub-system

Scanning Laser Ophthalmoscopy (SLO) provides high resolution *en face* images of the retina, including the retinal vasculature system. The two dimensional image of the retina is called a fundus image. The scanning laser ophthalmoscope focuses a narrow band laser on a spot on the fundus. Back reflected light is carried out through the same optical pathway towards the photo detector. The intensity of the back reflected light from the spot is incident on a photo detector, and corresponds to the brightness of a pixel corresponding to the physical location on the display. A two dimensional image of the sample surface is obtained by scanning the spot over the retina and synchronously detecting the back reflected intensities. Given that FD OCT is also a raster scanning technique, integration of these two imaging modalities is a natural pairing. The light source used for the SLO sub-system was a frequency doubled neodymium-doped yttrium aluminum garnet laser which produces green light at a wavelength of 532 nm. The green light undergoes the minimal attenuation when it passes through the optics of the eye, but is more strongly absorbed than the infrared light in the retinal tissue layers. For retinal imaging, the light intensity incident on the sample was reduced to 200 microwatts, below the ANSI recommended limits for long duration exposure.
The monochromatic green beam was also used as the excitation source for fluorescent retinal imaging. With reference to Figure 4-1, the green beam from the SLO is coupled into the OCT sample arm using a hot mirror at HM. Dichroic filters are designed to reflect a small range of wavelengths, and transmit the remaining portion of the spectrum. Thus, dichroic mirrors are used in optical systems for color separation applications. The dichroic filter used in our setup is designed to reflect the green excitation light and to transmit the longer wavelength fluorescent emission.

To improve the resolution and contrast of both SLO and fluorescent images both beams were passed through confocal apertures prior to the detector. An optical multimode fibre was used instead of a pinhole to carry the SLO signal toward the APD. The back-reflected green SLO signal light was coupled into the fibre using a 25mm lens. We used several different fibres, with core diameters ranging from 50 to 100µm, during the imaging experiment, which is described in detail in chapter 5. A 100µm diameter aperture (Edmund Optics) was placed in the optical path before the PMT. The orange beam was focused with a short focal length lens (11mm EFL) to make sure enough photons passed through the spatial filter to the PMT detector.

4.2 fSLO electro-optics detection

There are three main optical sensor choices for low signal detection: PIN photodiodes, avalanche photo diodes and photo multiplier tubes. Photodiodes have unity internal gain. Even though a photodiode has a very short response time, its sensitivity is too low to detect lower than microwatt intensity back
reflected light from the retina. For this reason, the PIN photodiode may not be the right choice for neither SLO nor fluorescence detection.

4.2.1 SLO detection

We used a silicon Avalanche Photo Diode (APD), S5343 from Hamamatsu to detect the SLO signal light. The APD’s sensitivity for 620nm light is 0.42 A/W at the gain of unity which shows its incredible ability of low level light detection. The APD gain (also called APD multiplication ratio) is dependent on the reverse voltage applied to it. Increasing the voltage will increase the gain up until the point that voltage drop occurs due to current flow in the device. In contrast to the PIN photodiode which has fixed gain and sensitivity, the sensitivity of the APD can be controlled by changing the reverse voltage (as a result changing the gain). For the best results, we increased the APD gain to 50, corresponding to a sensitivity of 25 A/W according to the specification sheets. According to the manual provided by the manufacturer to obtain this gain, a 150 V reverse voltage is required at nominal room temperature of 20º C. Another feature of APD is their relatively high response speed, in our case the cutoff frequency of S5343 is 250MHz.

The avalanche photodiode needs to be placed on an operational circuit. This circuit is designed to feed the reverse voltage to the APD, as well as amplify the signal. Figure 4-3 shows the schematic of the peripheral circuit that we have designed. The optimum APD bias voltage is in the range of 150 to 180 V. Higher than 180V supply voltages result in lower APD gains. We used batteries as the voltage source because they introduce a minimal noise to the system compared
to the ripple in power supplies. The gain is also temperature dependent; however, the lab temperature is fixed to within a reasonable range and there was no need for a temperature offset on the circuit. To avoid current flow from the source to the APD, a $1\, \text{M}\Omega$ current limiting resistor was setup prior to the diode ($R_{\text{lim}} = 1\, \text{M}\Omega$ in Figure 4-3).

APDs are current generating devices, but digitizers usually work with voltages, and this was the case with the digitizer that we used. A trans-impedance amplifier was used to convert current to voltage independent from the load resistor (digitizer’s input resistance). Several considerations had to be taken into the account before choosing the trans-impedance resistor. A high impedance resistor was desired for low noise readouts; however, the generated voltage should not exceed the supply voltage. This can reverse the performance of the circuit and damage the diode. Also, we anticipated that the intensity of the back reflected light from the mouse’s eye is in the order of tens of nano-Watts. Based on the current that is generated at this intensity, a range of resistors from $100\, \text{K}\Omega$ to $1.2\, \text{M}\Omega$ was chosen to provide readout voltages more than $100\, \text{mV}$ for the digitizer. The $R_{\text{CV}} = 800\, \text{K}\Omega$ was experimentally chosen based on the image quality of light backscattering from the mouse retina after qualitatively comparing multiple possibilities. At this impedance, the combination of best visual image quality along with the best response speed was obtained.
4.2.1.1 Fluorescent detection

The back reflected auto-fluorescent signal would not be intense enough to be detected by an APD. The Photo Multiplier Tube (PMT) which is a much more sensitive and sophisticated photosensor is the ideal choice for this purpose. We used a Hamamatsu PMT module H5784 series. Again, like an APD, the gain of the PMT can be adjusted by using the control voltage. For example, when control voltage is at 0.8 volts, the PMT would detect at a gain = $10^5$. At this gain detection sensitivity at the peak sensitivity wavelength would be 30 V/nW. Since the current to voltage conversion is done through an amplifier feedback resistance of 1MΩ with a conversion factor of 1 V/µA, detection sensitivity can be transformed to 30 µA/nW, over thousand times more sensitive than the APD. However, the
PMT has variety of drawbacks and is not a good choice where APD can be used. One has to consider that PMT has lower quantum efficiency relative to APD and it is a slower detector. For example the PMT we used has a cutoff frequency of 20 kHz.

4.3 Data acquisition and display software

The galvanometer used to scan the spot across the sample was limited to a repetition rate of maximum 1.5 kHz in practice. The step response time of the galvanometer for small angle scan was 140µs and 800µs for large angles. For all SLO experiments on mice, images of the maximum field of view of the retina were taken. For the focusing optics that we used (described in Section 4.5) and in order to view the full retina, the X axis mirror of the galvo was driven through an angle of roughly 4°, corresponding to a 2mm scan length. This distance was mathematically calculated and validated when physically tested on a resolution target. The sampling interval when 800 samples are taken from 2mm long of the sample is about 2.5µm. In practice, for angular scans of 4° the repetition rate was limited to nominally 500-1000 Hz.

Analog to digital conversion was performed using a multifunction NI-PCI 6251 board with a maximum sampling frequency of 1 MSPS (kilo-samples per second). Each line consisted of 800 samples, and each frame consisted of 500 lines. The frame rate for SLO was nominally 1 fps, which was displayed in real time using a custom written software application. The frame rate for SLO could easily be set to 2 fps. However, during the fluorescent image acquisition, the frame rate had to be reduced to 1 fps for best results. The remaining parameters
stayed identical to that of only SLO. The ADC acquired data on both channels simultaneously.

FD-OCT data could not be acquired with the same lateral sampling rate as SLO’s. The line rate provided by the FD-OCT was nominally 20 kHz, and a 500 line B-scan was acquired in nominally 25ms. Scanning a 2D surface for volumetric acquisition required roughly 10 seconds, over an order of magnitude slower than the SLO system. For this reason, the SLO sub-system was used to align the position of the system to the sample, followed by OCT acquisition of a data volume.

The NI-PCI 6251 was used for both generating the analog output to galvos and for digitizing the signals from each of system outputs. This made synchronization of the system relatively simple because the clock signal for the input and output applications are on the same board. Another very important element of synchronization is triggering. The trigger is hardware or software event that initiates an action; in our case it starts the acquisition. Even though we are using a common clock, physically moving galvos results in a lag in data acquisition due to multiple factors such as mechanical mirror inertia. We used digital pulse trigger to make sure that data acquisition starts when the mirror is returned to its start position and is ready to scan the next line.

Custom software written in C++ was developed to acquire, process, and display OCT or SLO images in near real time. The software controlled the beam scanning and digitization concurrently. The FD-OCT processing was able to implement re-sampling the data when transferring from wavelength to
wavenumber space and the FFT in real time. By implementing a few changes and augmenting of the additional threads, the original FD-OCT software was enhanced to a multithread real time environment for use in SLO and fluorescent simultaneously. The fluorescence and SLO signals are digitized simultaneously using the same high speed digitizer. Through the software, the number of lines per image was user controllable, and could be adjusted to higher numbers for better sampling, or reduced for faster frame rates. The speed of the mirror could also be easily controlled using the software. Since the OCT and SLO sub-systems operate at significantly different speeds, it was not crucial to integrate the software for the two components immediately. Under normal imaging conditions, the SLO system was first used to align the field of view of the retina. Once the correct orientation was obtained, the software was closed and the OCT acquisition was started.

4.4 Human data

The SLO software and optical detection system was first evaluated by imaging a human subject. SLO is a common clinical imaging modality, so the image quality of the custom-built system could be evaluated. The healthy human eye is a high quality optical system that focuses a beam of collimated light on the retina with minimal aberrations. As a result, SLO images can evaluate the performance of the system electronics in the absence of focusing issues and optical aberrations anticipated for mouse retinal imaging.

We used a collimated beam incident on the cornea and the natural focusing optics of the eye focused the beam on the retina. This optical setup is
commonly used by the BORG laboratory for human retinal OCT imaging. To adapt the SLO sub-system for imaging the human eye we also replaced the green laser source with a CW laser diode (LQA830-140C, Newport) which produces NIR light at a wavelength of 830 nm. Because the eye is less sensitive to the NIR beam wavelength, it is more tolerable for imaging the volunteer’s eye. Imaging was performed under the human imaging protocols in the BORG lab. The beam power at the cornea was set at 500µW which below the maximum permissible long term exposure set by ANSI standards. The subject’s eye movement resulted in motion artefact in the images which were acquired at the rate of 2 frames per second. An example of this motion artefact is indicated with an arrow in the top left panel of Figure 4-4.
Figure 4-4 Representative images of human optic nerve head acquired with the custom built SLO system. All four human ONH images above are taken in one session. Zoom in was performed optically by changing the scan length of the galvanometer mirrors. The ability to resolve the retinal nerve fibres is a sign of high image quality.

The image quality of the human SLO images was comparable to that of commercial systems. This experiment validated the quality performance of the system electronics and data acquisition software.
4.5 Optical design for imaging mouse retina

The design criteria used to design the sample arm optics for rodent retina imaging were determined from the following imaging concerns. For the combined rodent retinal SLO/OCT, the design was dominated by the requirements of the SLO system. Our collaborators demanded high resolution en face images which caused us to choose the optics accordingly. Even though the OCT system was the second priority, a depth of focus of ~300µm was required in order to image the complete thickness of the retina. One of the system constraints was that the collimated beams hitting the galvo mirrors were limited to 3mm diameter. Table 4-1 lists the optical design parameters used for SLO and OCT sub-systems. A combination of three lenses with focal lengths of $f_1=25$, $f_2=30$ and $f_3=40$mm was used as shown in Figure 4-5 in order to design the system according to the values in the Table 4-1. Figure 4-6 is a photograph of the focusing optics in the setup.

<table>
<thead>
<tr>
<th></th>
<th>SLO Sub-system</th>
<th>OCT Sub-system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collimated beam waist</td>
<td>1.30 mm</td>
<td>1.39 mm</td>
</tr>
<tr>
<td>Focal waist</td>
<td>4.34 µm</td>
<td>6.28 µm</td>
</tr>
<tr>
<td>Depth of focus</td>
<td>222 µm</td>
<td>300 µm</td>
</tr>
</tbody>
</table>
Figure 4-5 Focusing optics in common between both sub-systems. The labels $f_1$, $f_2$, $f_3$ are the focal lengths of each of the lenses. Red lines represent the 830nm beam for OCT and the green lines are for 532nm SLO beam, and the orange lines are for fluorescence. Minor adjustment of the axial position of the second lens was used to adjust the focus.

Figure 4-6 A photograph of the focusing optics in common between both sub-systems.
4.6 Demonstration of imaging on test targets

4.6.1 USAF resolution target

The ability of imaging system to identify structures within the depth section is determined by the lateral resolution, which in turn depends on the focused spot size. The calculated beam size from the previous section is an estimate of the spot size, but does not incorporate various unknowns, such as: the exact numerical aperture of the source fibre, perfect collimation of the beam, and aberrations introduced by the lenses. In order to quantitatively measure the SLO system’s lateral resolution, images of the 1951 USAF Resolution Target were acquired.

The SLO images of a USAF resolution target are shown in Figure 4-7. Both images in the figure has the same number of acquired points, but physically different scan area. The system was able to acquire clear images of line-space elements of group 5. The Modulation Transfer Function (MTF) of group 5, element 1 in Figure 4-7 was plotted since the system was able to acquire clear images of the line-space pairs. From MTF plot of this element, we measured that the maximum and minimum intensity was $I_{\text{max}} \approx 2200$ and $I_{\text{min}} \approx 1300$, respectively. The lower and higher bounds of the intensity valued start changing at the third element of group 5. The local contrast minimum and maximum around element 3 are $I_{\text{min}3} \approx 1500$ and $I_{\text{max}3} \approx 2100$. This change in intensity indicated that spot size is commensurate with the line/spaces of this element, which is nominally 12µm. This experimental result agrees with the previously calculated resolution (\(\sim 9 \mu m\)).

In the calculation of the waist in Gaussian optics, we used the $1/e^2$ of the peak
intensity, meaning that the reported waist of 9um is only ~93% of the actual beam size. Also, the image could have been acquired anywhere within the DOF and still appear focused but have a radius up to \( \sqrt{2} \) calculated waist.

Figure 4-7 Images taken from a USAF resolution target using optics described in section 4.5. Features that are smaller than the first elements of group seven cannot be resolved.

4.6.2 Fluorescence Phantom

The system was designed to be compatible with detection of lipofuscin deposits in ABCA4 knock out mouse in future research. Lipofuscin fluoresces orange light when exposed to 532nm excitation. Fluorophores such as Rhodamine 6G (maximum absorption at 531 nm and emission at 580nm) or Alexafluor 555 (maximum absorption at 555 nm and emission at 565nm) are suitable replacements of lipofuscin for experimental purposes. Retinal angiography images using Alexafluor 555 in mice is presented and explained in Chapter 5. The system schematic in Figure 4-1 contains a dichroic filter and an orange filter which is located in the optical path prior to the PMT to isolate the
fluorescent signal and reject the rest of the spectrum, especially the 532nm excitation beam.

In order to test the fluorescent channel and to validate the efficiency of the filters used, a fluorescent sample was imaged. A phantom was constructed from a grid of cotton string. The string in one orientation was pre-soaked in a low concentration of Rhodamine 6G, and imaging was performed at the intersection of strings with dual channel SLO and fSLO. The image acquisition results are presented in Figure 4-8. The SLO channel shows both sets of regular and fluorescent soaked string. However, the fSLO channel only shows the fluorescent signal from one string. Note the high level of saturation of the fluorescence signal. The bottom panel of Figure 4-8 shows an overlay of the fluorescence signal on the SLO image, indicating the source of fluorescence correlates to the correct string.
Figure 4-8 Dual channel image of intersection of two cotton strings, one of which soaked in Rhodamine 6G. The top left is the image from the SLO channel and shows both sets of string. Top right image was acquired from the fluorescent channel simultaneously, and only the signal from the fluorescent string was detected. The bottom panel is the overlay of the fluorescence signal on the SLO image.

4.6.3 Mouse retina histology sections

After successfully testing the fluorescent channel, we needed to verify that we can detect the auto-fluorescence from a histological section before attempting it in vivo. A glass slide with multiple ABCA4 knock out mouse eye histological cross sections from a collaborator’s lab (Dr. Robert Molday, UBC) was used for this measurement. Figure 4-9 shows a schematic of the cross sectional plane of
a mouse eye and the corresponding region from which the images were acquired.

![Diagram of a mouse eye and a sample SLO image of the mouse eye histological cross sections.](image)

**Figure 4-9** A schematic cross sectional model of the mouse eye and a sample SLO image of the mouse eye histological cross sections. The image indicates the part of the eye from which the image was acquired.

Images of the retinal histology were acquired by the collaborator using a fluorescent confocal microscope, emphasizing molecular contrast in the retinal signal from the lipofuscin concentrated in the RPE. In Figure 4-10 a SLO image of one of these cross sections is compared to a micrograph of the histology acquired by the collaborator. The two images are positioned over each other so that the correlated layers coincide, and the layers were labelled based on the histology micrograph. However, in the fluorescent image from our system, there is a strong auto-fluorescence signal from the photoreceptor inner segment in addition to the RPE. The histology micrographs were taken with 488nm excitation, while we used a 532nm source for imaging. The additional fluorescence observed with the SLO may correspond to the interaction of the fixatives used in histological processing with the intra-retinal layers. As will be
presented in the next chapter, retinal auto-fluorescence acquired \textit{in vivo} from excitation at 532nm was not detected. The excitation spectrum of the fluorescence in the IS layer likely has a maximum near 532nm, and falls off to an insignificant value at the excitation wavelength using in the histology micrograph.

![Image](image_url)

\textbf{Figure 4-10} Top: An SLO image of the retinal section is compared to a microscopic image from the same sample. The retinal layers (labelled) of both images coincide. Bottom: a fluorescent image of the histology was taken with the 488nm excitation filter (coloured image) and placed beside a fluorescent image acquired from the PMT channel of the SLO system such that retinal layers coincide.

When imaging the histological sections with both the APD and PMT detectors turned on, artefacts were observed in the fluorescence channel from objects which were not expected to be fluorescent. Top left panel in Figure 4-11 is taken from the fluorescent channel when both detectors were on. The circular
object in the image was not anticipated to be fluorescent. Based on the following analysis, we deduced that the artefact was due to cross talk between the channels of the ADC. First, we turned off the power to the PMT, but left the APD on. The resulting image acquired in the fluorescence channel thus corresponded to the cross talk in the APD channel of the ADC from the SLO channel. The circle artefact is present in the top right panel of Figure 4-11. The image is the same even when the light going to the PMT is physically blocked. With the APD turned off, and only the PMT turned on, the acquired image is shown in the bottom left panel of Figure 4-11. Note that the circle artefact is not observed. That means circle artefact does not fluoresce and is only detected by the APD. Using Matlab, we also subtracted the cross talk artefact image from the image taken from the fluorescent channel when both detectors were on. The resultant image is presented in the bottom right panel of Figure 4-11, and correlates to the image acquired at the PMT with the APD turned off.

Based on the fact that the circle artefact was not observed in the image acquired with the PMT on and the APD off, we concluded that no green light was leaking to the PMT. Consequently, this indicates that if the signal in the PMT is very low, then we have to acquire data with the APD turned off. Alternatively, we have to turn up the gain on the PMT so that we get a large enough signal which overpowers the cross talk.
Figure 4-11 Four images of one of the histology samples all taken from the fluorescent channel. The top left image is taken when both detectors were on. The top right image is when only the APD is on. The bottom left image is acquired when only the PMT is on. We subtracted the top right image from the top left one using Matlab, and the resultant image is presented in the bottom right panel.

To better observe the intensity profile and distribution pattern of the autofluorescence we overlaid an image taken from the PMT channel with its correlated SLO image. Figure 4-12 represents the result of this overlay. For
better representation, the SLO image was false coloured green, and the fluorescent signal was false coloured red in this image.

![Image 4-12](image_url)

Figure 4-12 Image of the histological sample of the mouse retina that is acquired by overlay of fluorescent and SLO data. Orange represents auto-fluorescent lipofuscin deposits on the green retinal layers.

4.7 Summary

The optical design and the electronics and integration concerns of the proposed system were addressed in this chapter. The prototype system was successfully tested on various phantoms. The calculated resolution of the system was validated after comparison with a resolution target. It was demonstrated that the system is capable to extract fluorescent information independent of the structure, but suffers from electronic crosstalk between the channels when one signal is significantly larger than the other.

In the following chapter, the performance of the system is investigated for multimodal imaging of the mouse retina. Fluorescent detection ability is
examined by performing retinal angiography in mouse. Images acquired from each sub-system are presented and compared.
5: MOUSE EYE IMAGING

5.1 Mouse’s eye

In this chapter, the hybrid FD-OCT/fSLO is adapted for mouse retinal imaging. Images of the mouse eye acquired with the SLO, and with the hybrid FD OCT system are presented.

5.1.1 Biometric information

The mouse is a common experimental model of ophthalmic diseases. Like most other mammals, the basic structure and function of the mouse eye is very similar to that of humans. An advantage of using mice for research over other animals is that they can be bred in short periods of time and have relatively low maintenance costs.

The size of the mouse eye increases by the age up to 60 days post-natal [27]. The axial length and ocular dimensions of the eye are also correlated with the bodyweight as well as the gender [28]. The amount of variation in the mouse eye from specimen to specimen makes it virtually impossible to design a schematic that perfectly represents the mouse eye. However, a suitable schematic model can be developed representing a typical mouse older than 60 days, after which age related morphological changes are negligible.

The refractive properties of wild type mouse eyes has been measured by Schmucker and Schaefer [28], and indicates poor optical performance as a result
of hyperopia, or far sightedness. Schmucker et al. indicated that the least hyperopic refractions in C57BL/6 mice optics is at 32\textsuperscript{nd} day (mean refraction $\pm$ SD: +4.1 $\pm$ 0.6 D) [28]. They also indicate that the imperfection might be as a result of “the small eye artifact”, discussed in the following section, rather than hyperopia. Optical imperfection increases with age reaching its maximum after 55 days from the birth [27, 28].

5.1.2 Comparison of mouse and human eyes

The mouse’s eye is much smaller than that of humans. The axial length and retinal arc of the human eye are roughly eight and ten times longer, respectively, than that of mice. Another very visible difference relative to the human eye is the size of the lens, which is proportionately quite large, rounded, and occupies about 75% of the intraocular space. For reference, a schematic of the human eye was presented in the top panel of Figure 1-1, and the mouse eye in Figure 5-1. These huge differences in the relative dimensions of the optics result in multiple challenges for retinal imaging. The small radius of curvature of the mouse eye results in higher refraction of collimated incident light and introduction of spherical aberrations. The result is that the mouse eye has a very low visual acuity, nominally 1/100 that of the human eye [29]. In addition, according to the measurements performed by Remtulla et al., the eyes of mice and other rodents introduce chromatic aberration due to the dioptric difference in the far points for red and blue light [30]. Refractive errors and aberrations are mostly introduced by cornea rather than the crystalline lens. This is because of
the large difference in refractive index between cornea and the external environment (air).

Figure 5-1 A schematic of the mouse eye

5.2 Mouse Retina Imaging

5.2.1 General info:

All mouse imaging experiments were performed under protocols compliant to the Canadian Council on Animal Care, and with the approval of the University Animal Care Committee at SFU. The mice were anesthetized prior to imaging in order to minimize sample motion. However, there was still some motion due to the mouse breathing, and vibration in setup, which was observed during image acquisition. The imaging performance of the hybrid OCT and fSLO system was investigated for alignment to the optic nerve head of the mouse. From an imaging perspective, the ONH provides a fixed reference location on the surface of the retina.
During the anaesthesia the number of blink reflexes reduces dramatically (effectively to zero) and the surface of cornea starts to dry out. Failure to preserve cornea’s moisture results in the development of cataracts and cloudiness of the cornea. Cataracts obstruct the light passage and reduce the quality of the images. During all the experiments natural tear drops were routinely applied to both eyes to keep them moist and healthy.

5.3 Results

Flattening the cornea with a cover slip contact lens reduces the amount of spherical aberrations and it is routinely performed by Dr. Sarunic’s group during OCT retinal imaging experiments in rodents. This method improves the simplicity of retinal imaging systems and the quality of the images acquired dramatically. The system alignment is faster, and increases the animal throughput during imaging (fast imaging with fewer modifications between animals).

In the remainder of this thesis, the mouse retina images were acquired using corneal refraction cancelation using a flat cover slip contact lens. Data acquired from both pigmented and albino mice using the fSLO/OCT system are presented. The imaging performance of the hybrid system for imaging mice was investigated in three stages: SLO only, fluorescence SLO, and SLO/OCT. Optical imaging of the retinal blood vessels in albino mice is significantly easier than in the pigmented wild type counterparts. The Retinal Pigment Epithelium (RPE) is a highly pigmented layer which absorbs light strongly. In the albino mice, the RPE is not pigmented, so less light is absorbed, providing a brighter, higher contrast signal.
5.3.1 SLO imaging of albino mice

Retinal imaging was first performed with the single channel SLO on the non pigmented eye of a CD1 mouse. Image acquisition started with the largest field of view possible with the system (limited by the scan speed of the galvos) and optical zoom was routinely changed by applied by varying the scanning amplitude through the SLOViewer software. The effects of size of the confocal aperture on image quality were experimentally investigated. As discussed in Section 4.1.2, a multimode optical fibre was used both as the pinhole and as the light conduit to the APD. Switching between different fibres of known fibre core diameter facilitated investigation of the effects of different pinholes.

Figure 5-2 shows two images taken with two different fields of view and a 50µm fibre used as the confocal aperture. Figure 5-3 images are taken from the same mouse using a 100µm fibre confocal aperture. Retinal arteries are sharp and clear and concavity of the ONH cup is clearly feasible in both set of images; however, ONH cup and neuroretinal rim images have a better contrast when the smaller aperture is used. Image sharpness and visibility of small features such as small vessels, micro capillaries and nerve fibres are indications of high quality images. These features are pointed out with red arrows on higher magnification images in Figure 5-4 which were acquired with a 50µm fibre confocal aperture, and in Figure 5-5 which were acquired with a 100µm fibre confocal aperture.
Figure 5-2 Two retinal images from an albino mouse. The first image is zoomed out to cover the largest area of retina possible. Both images were taken with a 50µm fibre used as the confocal aperture.

Figure 5-3 Two retinal images from an albino mouse. The images cover two different areas of the retina around the ONH and were taken with a 100µm fibre used as the confocal aperture.
Figure 5-4 Two zoomed in images of two different areas of an albino mouse's retina. A 50µm fiber used as the confocal aperture. The retinal arteries are sharp and clear in both images and many of the inner vessels are visible. The yellow arrows point to some of the micro capillaries.
Figure 5-5 Two zoomed in images of two different areas of an albino mouse's retina. A 100µm fibre used as the confocal aperture. Retinal arteries are sharp and clear in both images. However, neuroretinal rim images have a better contrast and micro capillaries are more visible when the smaller aperture is used. The red arrows point to some of the micro capillaries.

Nerve fibres are not well resolved in either imaging configuration. Micro capillaries are more visible when the smaller aperture is used. This is because in
the albino mouse, back reflection from the choroid is strong and the absorption in the RPE is low; consequently the smaller confocal aperture significantly improves the contrast.

5.3.2 SLO imaging of wild type pigmented mice

Next, SLO images were acquired from a pigmented wild type BDF1 mouse. Figure 5-6 shows two of the images taken from retina of this mouse. To acquire images from the pigmented mouse, a wider confocal aperture of 100\(\mu\)m was used. Although the larger confocal aperture resulted in a lower resolution, it was required to collect sufficient back scattered light intensity to generate an image.

![Image](image-url)

**Figure 5-6 Two images from a pigmented wild type. The first image is zoomed out to cover the largest area of retina possible.**

Without the strong back reflection from the choroid layer, the retinal images have a higher contrast for the superficial layers like the retinal nerve fiber layer. Retinal tissue pattern and nerve fibres which could not be observed in the
albino mouse can be resolved in pigmented mouse images such as Figure 5-7 in which red arrows point to regions where nerve bundles are discernable. The absence of choroid reflection due to strong absorption in the RPE also has a down side. Unlike images of albino type, small capillaries of inner retina cannot be seen in the SLO images of the wild type mice.

Figure 5-7 SLO image zoomed into the ONH cup area. Small vessel are not as visible as in albino mouse images. In pigmented mice, the weaker back reflection from the choroid helps to see nerve fibres better. The red arrows point to nerve fibres.

A dark spot was seen on the retinal area adjacent to the ONH during image acquisition. This was thought to be due to cataracts developed on the cornea rather than representing a feature on the retina. This was investigated by changing the imaging angle of the eye, and seeing if the position of dark spot moves. Figure 5-8 shows two images of the fundus acquired at different angles. The red boxes indicate the position of the black shadow. Since the shadow changes positions, this indicates that it is not on the surface of the retina.
5.3.3 SLO with fluorescence detection

The ability to acquire fluorescence data in combination with SLO is demonstrated through retinal angiography in Figure 5-9. The frame rate was again 1 fps for this acquisition, and the optical power of the excitation beam at the sample was maintained at 200 microwatts. The fluorophore used was Alexafluor 555 conjugated to BSA which was injected to a pigmented mouse via the tail vein. The blood vessel pattern observed in the fluorescent SLO (fSLO) image corresponds to the dark absorbing blood vessels in the regular reflectance SLO image. Higher contrast along with lower background intensity was obtained by implementing confocal detection for the fluorescent signal.

Images with the best contrast were the ones taken right after the injection before the fluorescent signal start fading away. We terminated the image
acquisition when we faced a relatively strong amount of background intensity. This was thought to be due to a fluorescent signal from deeper in the choroid and the strong emission from the label.

Figure 5-9 Representative images of fSLO. In this image, SLO is on the left, fluorescence in the middle, and the overlay is on the right. The focus was deeper that is why the superficial vessels are out of focus.
5.3.4 Combined SLO and OCT

Figure 5-10 presents the SLO and OCT fundus images acquired on a wild type mouse using the hybrid system. First the SLO system was used to orient the field of view on the ONH. The SLO image was acquired in ~0.5s. The system was switched over to the OCT, and a volume was acquired at the same location, requiring ~10s (500 lines per B-scan and 400 B-scans per volume at a line rate of 20kHz). Motion artefact was observed in the OCT fundus image reconstruction – the regular undulations in the blood vessels are due to movement of mouse during volume acquisition. The SLO image of the ONH contains a higher level of lateral detail, and the contrast of the blood vessels is stronger than in the OCT. An overlay of SLO and OCT images are also presented in Figure 5-10. Note the excellent overlap of the blood vessel pattern, indicating good correlation between SLO and OCT image acquisitions.

Figure 5-10 SLO and OCT images acquired at the same location. The blood vessels are more readily observed in the SLO image. Motion artifact can be observed as undulations in the blood vessels. ONH, Optic Nerve Head.
The SLO fundus image only contains the information of the surface reflection from the retina. Although some level of depth sectioning can be obtained from the confocal detection, the calculated axial resolution is on the order of the thickness of the whole retina. On the other hand, an entire volume of resolution data is contained in the OCT fundus image, and a depth profile of the retinal cell layers is contained high underneath each pixel in the reconstructed fundus image. Figure 5-11 demonstrates an example of an OCT volume acquired superior to the ONH after alignment with the SLO system, and a typical B-scan of the retinal cell layers.

Figure 5-11 (Top) Reconstruction of the fundus image from the OCT imaging sub-system. (Bottom) a B-scan of subsurface data can be extracted from the volume of data.
5.4 Summary

High quality multimodal images were acquired from the retina in mice. Retinal features such as micro capillaries and nerve fibres were resolvable in SLO. Retinal angiography was performed using Alexafluor555, permitting visualization of the micro capillaries even when not observed in the SLO images of pigmented mice. Lastly, co-registration of the SLO and OCT was demonstrated.

In the next chapter, the acquired results are reviewed and discussed. Investigation on the performance of the FD-OCT/fSLO system will come to a conclusion.
6: CONCLUSION AND FUTURE WORK

This thesis presented a hybrid imaging system which integrated SLO and OCT imaging modalities. It has also been demonstrated that the system provides images of sufficient quality to assist researchers on monitoring retinal degenerative diseases.

6.1 Discussion

Optimization of the multimodal imaging system was compromised between high quality SLO imaging and high quality OCT imaging. The optics were optimized for SLO, and the OCT imaging sub-system was adapted accordingly by changing the waist of the incoming beam from the source. Because the axial resolution of the OCT system is dependent on the source and not the sample arm optics, the resulting visual image quality was not affected. The frame rate for the SLO was limited to 2 fps, an order of magnitude slower than attainable with rapid polygonal rotating mirrors. This is because we wanted to use scanning galvos compatible with OCT. However, the SLO frame rate was an order of magnitude faster than fundus reconstructions with the OCT sub-system. As a result, aligning the field of view of the system on the optic nerve head of a mouse was substantially faster and easier than with OCT alone. The rapid alignment is crucial in order to minimize the time spent imaging a single specimen, enabling higher throughput and use of a lighter anaesthesia. The latter is important when performing frequent imaging of the sample animal in time
course studies, or when imaging older specimen which are more sensitive to anaesthesia. However, alignment of the OCT system was not the only governing criteria. If it was, then the same (or similar) wavelength could be used for OCT and SLO, significantly reducing system complexity [31]. Integrating a green beam for the SLO, as opposed to using the NIR wavelength of the OCT system, also permitted fluorescent SLO image acquisition. This is important for complementing studies of retinal angiography and retinal auto-fluorescence in addition to OCT.

As discussed in section 5.1.2, the anatomy of the human is similar to that of mice, but the high radius of curvature in smaller eyes makes the imaging challenging. The quality of the images we attained confirms the system overcomes the constraints introduced by animal’s eye anatomy. However, the system can benefit from additional efforts to improve image acquisition and processing.

Throughout this research, it was demonstrated that the prototype system is sufficiently tested and is ready to be transferred to the next stage. At this point we believe that the system has reached to a sufficient level of maturity and is ready to be transferred to the collaborator’s laboratory to conduct the time course study on mice. The first step of the next stage is to perform validation studies comparing multimodal images from the system with histology.
6.1.1 Time course study of Stargardt’s disease in mice

fSLO retinal angiography was demonstrated with the system, and the path to the detection of auto-fluorescence is a future work. This system provides the groundwork for future studies investigating the rodent models of retinal diseases which lead to accumulations of auto-fluorescent deposits, such as Stargardt’s Macular Dystrophy. Investigation of lipofuscin deposits of Stargardt’s disease in particular is going to be conducted in the collaborators laboratory.

Possible continuation of research on the multi-modal rodent retinal imaging system is outlined in the next section.

6.2 Future work

The system can benefit from minor improvements of imaging acquisition for use in future vision based research applications. Most importantly, the issues to resolve include motion artefact, faster SLO imaging, and interfacing between the mouse and the imaging system.

6.2.1 Scanning patterns for image acquisition

At the moment, the fast scan axis which is in the horizontal direction is performed in a saw tooth pattern. For a fast scan, the voltage amplitude to the galvo is increased incrementally to provide steady mirror movement until it reaches the end of the scan. At this point, mirror is rapidly returned back to its starting point with a sudden change in control voltage. This procedure is repeated until the complete frame is acquired. The resulting sawtooth scan pattern is sub-optimal because the time during which the mirror is reset to the
starting position is “dead time,” and the large angle rapid change in position results in mirror ringing, requiring additional time to settle.

We anticipate that image acquisition can be improved by implementing different scanning patterns. For example, a triangle scan pattern will possibly provide smoother transition by avoiding the need to rapidly reset the mirror position. The triangle scan pattern is compared to the sawtooth in Figure 6-1. Preliminary investigation of the sawtooth scan pattern indicated that significant changes to the OCT/SLO Viewing software would be required for registration of forward and backward scans resulting from the non-uniform turn-around time for the mirror from line to line.

![Figure 6-1 A sawtooth raster scanning pattern and a triangle raster scanning pattern.](image)

### 6.2.2 Stereotactic head fix

During the anaesthesia, the mouse does not blink and its movements minimizes. However, neither motion nor the corresponding motion artefacts in the image were completely absent during the *in vivo* imaging. Motion artefacts still occurred as a result of the mouse breathing and heart beats. The SLO imaging speed of 1 frames per second was fast enough to avoid significant artefact on
many images. However, it was not possible to avoid motion artefact while acquiring two frames at a fixed position. This is important for averaging frames in order to improve the quality of images, and increase the sharpness for better visualization of smaller features and higher contrast.

A simple solution is to design better physical immobilization of the mouse, such as a bite bar, to reduce the effect of motion. The stereotactic head fix can be augmented to a multiple degree of freedom stage to permit rotation around the vertical and horizontal axes. The ability to adjust the mouse orientation relative to the imaging beam in free space facilitates alignment and provides a larger field of view.

Another possible benefit of a head fix allows imaging without anesthetizing the specimen. Paques et al. reported acquisition better quality images in the awake mouse compared to anesthetized in the literature [32].

6.2.3 Contact lens

As explained in Chapter 5, we applied refraction cancelation at the cornea by using a cover slip to flatten out the curved surface. Gently pressing out the cover slip on the cornea is not likely to affect the results of retinal examination nor damage the eye. However, during the investigation of disorders such as glaucoma it is important to avoid changing the pressure of the eye. In addition, the technique of flattening the cornea could be only applied on anesthetized specimen.
To overcome these issues, a custom designed plano-convex contact lens could be investigated as a permanent replacement for the cover slip. This can be a simple solution to improve the system’s capability to image all kinds of disorders on both anesthetized and awake mice. A challenge however is to adapt the contact lens to various sizes of the eye and different radiiuses of curvature of the cornea in different mice. We anticipate overcoming the challenge by using ophthalmic lubricants such as hydroxypropyl methylcellulose as an index matching gel. The custom optics will also need to have broad-band optical anti-reflection coatings in order to reduce back-reflection in the system.
REFERENCE LIST


