Two-Photon Adaptive Optics Fluorescence Lifetime Imaging Ophthalmoscopy

by

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This thesis is dedicated to my family, whose support and guidance has helped me make it this far.
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Biographical Sketch

The author was born in Marshall, MN and raised in Lakeville, MN, a suburb of Minneapolis-St. Paul. He completed his undergraduate studies in Physics at the University of St. Thomas in St. Paul. During the summer between his junior and senior years, he worked in the laboratory of Dr. Timothy Gay at the University of Nebraska-Lincoln. He enrolled in the University of Rochester PhD program at The Institute of Optics in 2011 and worked in the research group of Dr. Jennifer Hunter beginning in 2012. He was awarded the Center for Visual Science Training Fellowship for the 2013-14 and 2014-15 academic years.

The author contributed to the following articles during the course this thesis:


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Abstract

There are many critical processes involved in keeping the retina functioning properly. Two of these, the visual cycle and the metabolism of the cell, are tied together by their conversion of important molecules from one form to another. In the visual cycle, 11-\textit{cis}-retinal is regenerated so that it can combine with a rhodopsin molecule and initiate phototransduction. In cellular metabolism, the cell undergoes many steps to generate adenosine triphosphate, the energy unit of the cell. These mechanisms are critical in maintaining a functioning retina, however they have been difficult to directly interrogate in the living eye. A technique which can quantitatively measure these processes could allow researchers and clinicians to examine them in healthy subjects and how they change under conditions of disease.

The goal of this work is to develop a technique which will allow us to investigate these measures of retinal function quantitatively and in a repeatable way. Advantageously, molecules which are converted during the visual cycle or cellular metabolism are accessible using adaptive optics aided two-photon fluorescence ophthalmoscopy. Furthermore, I develop a new technique, adaptive optics fluorescence lifetime ophthalmoscopy, which provides a robust and quantitative measure of a key property of retinal fluorescence. Initially, this method was deployed in a new two-photon adaptive optics ophthalmoscope designed for imaging mice. Exogenous fluorophores with known fluorescence lifetimes were used to validate the initial measurements, before using the new technique to establish baseline measurements for a sensor of cellular
metabolism in the mouse eye. Following successful implementation in the mouse, the fluorescence lifetime method was translated to a system dedicated to imaging the macaque retina. By measuring the fluorescence lifetime of endogenous fluorescence originating in the photoreceptors, I found that rods and cones exhibit different fluorescence lifetimes. Further development of this technology may advance research in widespread areas including fluorophore identification in the retina, mechanisms of retinal metabolism, and as a clinical diagnostic.
Contributors and Funding Sources

Research reported in this thesis was conducted under the supervision of thesis advisor Professor Jennifer Hunter (Flaum Eye Institute), with support from thesis committee members Professor David Williams (The Institute of Optics), Professor Nick Vamivakas (The Institute of Optics), Professor Richard Libby (Flaum Eye Institute), and defense chair Professor Farran Briggs (Neuroscience).

The system for mouse imaging and the system for primate imaging were the end result of contributions from many people. Robin Sharma built the system for primate imaging and assisted in the optical design of the mouse system. Martin Gira designed and built electronics for the systems, as well as several custom mechanical mounts. Martin Gira and Dan Guarino built a custom mount to hold mice. For all imaging, the adaptive optics control software used was developed by Alfredo Dubra and Kamran Ahmad and supported by Keith Parkins. The image acquisition and registration software and firmware used were developed by Qiang Yang. Work reported in Chapter 2 was supported by funding from the following NIH grants: EY007125, EY022371, and EY001319.

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Chapter 1

Introduction
1.1 Overview

All animals must have means to interact with their environment through sensing, to find sustenance and avoid predation. Of these senses, vision is the most crucial for many species. The cascade of vision begins with the visual pigment, which has a history dating back to the Cambrian period (Land and Fernald 1992). Although differing wildly in design and functional capability, most animals have eyes which allow them to see and interact with their environment. The retina, consisting of light sensitive cells and neurons which process the visual signal, is an important structure for accomplishing this task.

Over the past several centuries, humans have begun to improve their understanding of the retina through techniques such as histology and microscopy. We have learned a great deal about the retina by cutting it into pieces, staining different cell classes, inserting fluorescent molecules, recording with electrodes, chromatography, and more. These techniques have yielded great insight into both the morphology and the organization of the retina. However, the retina must be studied in its intact condition to fully understand how the neurons function individually and interact with each other in the healthy retina, and how that function deteriorates in the case of retinal disease. Furthermore, the ability to track regenerative therapies such as optogenetics and stem cell therapy could be accelerated with the tools to monitor the retina at a cellular level in the living animal.

Ophthalmic imaging gives us the unique opportunity to study the retina in its intact state. With the application of adaptive optics (Liang, Williams et al. 1997),
individual retinal cells can now be resolved in the living eye, allowing scientists and ophthalmologists alike to use the technology to further our understanding of the retina in health and disease. Adaptive optics imaging has provided unprecedented resolution of retinal cell structure, but more recently the focus has shifted to developing new methods to measure cell function. By combining adaptive optics ophthalmoscopy first with two-photon excitation and now, in this thesis, with fluorescence lifetime imaging, I envision a new technique for interrogating retinal cellular processes including the visual cycle and cellular metabolism.

To better understand the work described later in this thesis, the following chapter will briefly describe the retina and some methods with which humans attempt to understand it.

1.2 The retina

The retina is a multi-layered organ located at the back of the eye which initiates the process of vision. A schematic of the retina in Figure 1.1 illustrates the different cell classes and their interconnected nature. Vision begins when light is focused onto the retina by the anterior optics of the eye, where it is absorbed by the photoreceptors. Phototransduction, or the conversion of the light to an electrical signal, occurs and the photoreceptors transfer the signal to retinal neurons which begin visual processing. The bipolar cells, which contact the photoreceptors in the outer plexiform layer, are the first of such cells. The bipolar cells then make contact with ganglion cells in the inner plexiform layer. As the visual signal moves along this chain, it is pooled and modified by
horizontal cells and amacrine cells. The visual scene is then transmitted through the ganglion cell axons to the brain.

**Figure 1.1** – Diagram of the retina illustrating the interconnected nature of the different cell classes. Light travels from bottom to top. A) Electron microscope image of a cone outer segment and B) Electron microscope image of a rod outer segment. Reprinted from Progress in Retinal and Eye Research, Volume 28, Issue 4, Debarshi Mustafi, Andreas H. Engel, Krzysztof Palczewski, Structure of cone photoreceptors, pages 289-302, 2009, with permission from Elsevier (Mustafi, Engel et al. 2009).
The work in this thesis focuses mostly on two cell layers in the retina: the photoreceptor layer and the ganglion cell layer. As such, it is useful to look at these cells in more detail.

### 1.2.1 Photoreceptors

Photoreceptors consist of four main regions: the inner segment, the outer segment, the cell body (which contains the nucleus), and the synaptic terminal, where the visual signal is passed to secondary neurons. For the purposes of this thesis, two of these regions require extra consideration.

The inner segments contain the cell’s machinery, including the mitochondria, and are the location where new opsin molecules are assembled. As the power generating region of the photoreceptor, they are particularly important when considering cellular metabolism.

The outer segments consist of membranes arranged in a stack-like configuration and contain visual pigment molecules. When a photon of light interacts with one of these visual pigment molecules, the phototransduction cascade begins. Consequently, the visual pigment must be regenerated through the visual cycle.

There are two types of photoreceptors: rods and cones. Rods are incredibly sensitive and can detect even a single photon (Rieke and Baylor 2000). Cones, while less sensitive than rods, are much faster and have multiple types with different spectral sensitivity, allowing for color vision. As the names imply, rods are longer and more rod-
shaped, while cones are shorter and shaped like a cone, with the inner segments being thicker than the outer segments.

In the human and primate retina, cones have the highest density at the center of the fovea, which is used for high spatial frequency vision. This density falls rapidly and then levels off in the peripheral retina. There are no rods at the fovea; they increase in density and reach a maximum at ~18 degrees from the fovea and decrease further out (Osterberg 1935).

1.2.2 Ganglion cells

As the output neuron of the retina, the ganglion cells play a critical role in supporting vision. Ganglion cells directly interact with bipolar cells and amacrine cells via their dendrites and transform these signals into electrical spikes that are then sent to the brain via long axons. There are around 30 types of ganglion cell in the retina (Sanes and Masland 2015), each with their own purpose for detecting features in the visual scene including direction and speed.

1.3 The visual cycle

The photoreceptors initiate vision by absorbing photons and starting the process wherein that signal is transferred to the brain for processing. When the opsin absorbs a photon, 11-cis-retinal is converted to all-trans-retinal. The retinal molecule must be regenerated to its initial 11-cis-retinal form in order to bind with an opsin and be ready to absorb another photon. It then dissociates from the opsin and is converted to all-trans-
retinol. The retinol molecule is transported to the retinal pigment epithelium (RPE), which is a layer of cells located behind the photoreceptors that are responsible for maintaining the photoreceptors. After several additional conversions, the molecule has been regenerated to 11-cis-retinal and can diffuse back into the photoreceptor to associate with an opsin. This is how the visual cycle operates for rods, which is visualized in Figure 1.2 and described in more detail in the following reference (Saari 2000). In addition to the standard rod visual cycle, there is evidence that cones undergo an alternate visual cycle involving the Müller cells, which allows for rapid dark adaptation and extended dynamic range (Wang and Kefalov 2009, Wang and Kefalov 2011).

**Figure 1.2** – The visual cycle. 11-cis-retinal is regenerated via a series of conversions in the photoreceptor outer segment and the RPE. Figure created by Dr. Christina Schwarz.
A method to measure the production and clearance of an intermediate molecule involved in the visual cycle would enable researchers to investigate a host of diseases which impact the visual cycle, including Stargardt disease, Leber congenital amaurosis and retinitis pigmentosa. Fortunately, all-trans-retinol is fluorescent, providing a potential avenue to observe a part of the visual cycle with imaging.

1.4 Cellular metabolism

In order to function, cells must be able to utilize an energy source. In the human body, this is achieved through a multi-stage process which converts glucose to adenosine triphosphate (ATP), diagrammed in Figure 1.3 (Campbell, Reece et al. 2008). The first stage of this process is glycolysis. In glycolysis, glucose is split into 2 molecules of pyruvate and there is a net gain of 2 ATP. Some of the energy is stored in a coenzyme called nicotinamide adenine dinucleotide (NADH). This molecule takes part in reduction-oxidation (redox) reactions and can be converted between its oxidized (NAD\(^+\)) state and its reduced state (NADH). In glycolysis, energy is stored by reducing 2 molecules of NAD\(^+\) to NADH. Importantly, glycolysis does not require oxygen to be present. However, it is an inefficient process for generating ATP. Most of the energy present in the initial glucose molecule is still contained within the 2 pyruvate molecules and must be extracted via the next stages of metabolism, which require oxygen. Pyruvate is transferred to the mitochondria of the cell where it undergoes the next stage of metabolism: the citric acid cycle.
Figure 1.3 – The three stages of glucose metabolism. Glycolysis occurs in the cytosol, producing a small amount of ATP and NAD\(^+\) is reduced to NADH. The next two stages occur in the mitochondria. The citric acid cycle produces more ATP and NADH, along with FADH\(_2\), another electron carrier. Finally, the NADH and FADH\(_2\) are oxidized in the oxidative phosphorylation stage, producing a large amount of ATP. Figure adapted from Biology, eighth edition, by Campbell and Reece (Campbell, Reece et al. 2008).

In the citric acid cycle (also known as the Krebs cycle or tricarboxylic acid cycle), a large number of reactions occur, which are detailed elsewhere (Campbell, Reece et al. 2008). The energy outputs include 1 molecule of ATP per pyruvate molecule, 4 NADH molecules, and 1 molecule of FADH\(_2\). FADH\(_2\) is the reduced form of FAD, or Flavin adenine dinucleotide. Like NADH, FAD is a molecule involved in redox reactions and is also an electron carrier. Once this cycle is complete, the net total of ATP per glucose molecule is 4, but a lot of the energy is now contained in NADH and FADH\(_2\) molecules and must be extracted. This occurs during the next stage of metabolism.
The final stage of metabolism in the cell is oxidative phosphorylation, which is also known as the electron transport chain. In oxidative phosphorylation, NADH and FADH\textsubscript{2} are oxidized and ATP is produced. Each molecule of NADH can generate \(~3\) ATP, and each molecule of FADH\textsubscript{2} produces \(~2\) ATP. When we take into account all the NADH and FADH\textsubscript{2} molecules from the previous stages, the total ATP yield from oxidative phosphorylation is \(~34\) ATP. Therefore, the vast majority of the ATP produced occurs during this stage.

In the last several paragraphs, we see that NADH and FAD are crucial to the generation of cellular energy. Additionally, we notice that these molecules are in different redox states throughout the process. During glycolysis and the citric acid cycle, NAD\textsuperscript{+} and FAD are reduced to NADH and FADH\textsubscript{2}, respectively. During oxidative phosphorylation, NADH and FADH\textsubscript{2} are oxidized to NAD\textsuperscript{+} and FAD. If it was possible to measure the state of these molecules, there could be a window into the machinery of the cell’s metabolism. Fortunately, NADH and FAD are both fluorescent, while their counterparts are not. Therefore, by measuring the fluorescence from these two molecules within a cell, we can envision a diagnostic tool for assessing the cell’s metabolic state.

1.5 Fluorescence: a functional measure of the retina

Fluorescence imaging is commonly used in microscopy due to its ability to generate contrast. Fluorescence occurs when a molecular electron is excited by absorbing a photon, and shortly afterward decays to the ground state by emitting a fluorescence photon. Because of the Stokes shift, the emitted photon is typically of lower energy than
the absorbed photon, allowing one to separate the excitation light from the fluorescence. Only some molecules are fluorescent, and of those, there is a range of excitation and emission spectra, enabling the user to target specific fluorophores of interest.

In two of the most crucial processes for understanding the health of the retina, fluorescent molecules are involved. In the visual cycle, there are retinoids, and in cellular metabolism, there are FAD and NADH. By measuring the fluorescence from these molecules, we may be able to gain insight into these processes and/or compare the fluorescence signatures in healthy retina to that of unhealthy retina.

Previous studies in the brain have shown that NADH fluorescence can be measured using two-photon microscopy, with the highest signal coming from the mitochondrial NADH (Kasischke, Vishwasrao et al. 2004, Chia, Williamson et al. 2008). In 2004, Kasischke *et al.* further showed that NADH fluorescence intensity is modulated by neuronal activity, suggesting that it can be used as a functional measure.

In addition to endogenous fluorescent molecules, there are engineered molecules which can be used as fluorescent probes. One such molecule is named Peredox. Peredox measures the NAD⁺: NADH ratio in the cytosol, thereby providing a proxy for the redox state of the cell. Peredox has been used to show differences in the resting metabolic state of neurons and astrocytes in mouse brain slices (Mongeon, Venkatachalam et al. 2016), and has potential to be used to investigate the retina as well.
1.6 Choosing a model of the retina to study

There are a number of different species to choose from to study the retina in health and disease. The most obvious choice is to study the human retina. However, safety of the retina is of utmost concern for studying humans, and therefore safety must be first established in an animal model. In addition, exogenous fluorophores can be inserted into the animal retina which label particular cells or bind to molecules of interest. In light of these considerations, the macaque monkey retina can be studied due to its similarity to the human. Macaques have a fovea, the central area consisting only of cones, to which the animal fixates for high visual acuity. They are diurnal, and therefore have similar cone classes and cone: rod ratio. As such, they can be studied as a substitute to learn about the human retina. However, there are some challenges associated with studying macaques. Most studies have a small number of animals. As of now, there are few models of retinal degeneration in the macaque, making it challenging to study analogs of some human diseases.

A viable alternative to the macaque in some cases can be to study the mouse retina. There are many transgenic mouse models which can be used to either alter the physiology or to insert markers of particular cell classes and even functional indicators. On the other hand, mice have a rod-dominated retina with no fovea, so they are not well-suited for all studies.
1.7 In vivo imaging of the retina

There are a number of different techniques used for investigating the retina in vivo. Fundus photography is used to image the retina en face, allowing the clinician to see the gross anatomy of the retina. This technique can visualize the major parts of the retina, including the optic disc, large vessel structure, and the fovea using reflected light. Confocal scanning laser ophthalmoscopy (cSLO) fills a similar niche, although it can be combined with fluorescence excitation to further investigate the structure of different constituent fluorophores of the retina. Another commonly used technique is called optical coherence tomography (OCT). OCT of the retina is used primarily to measure the thickness of different retinal layers, allowing the clinician or scientist to determine whether these layers are intact.

These techniques have proven useful for evaluating the retina in the clinic, however they lack the resolution to visualize individual cells. As a result, many diseases cannot be identified until they have caused significant retinal degeneration. In addition, it becomes difficult to determine whether therapy has halted the spread of the disease without cellular resolution. To address this need, adaptive optics can be utilized.

1.7.1 Adaptive optics in retinal imaging

The human eye is not a perfect imaging system; it has aberrations which prevent the ability to focus light perfectly at the retina. The major aberrations present are defocus and astigmatism, both of which are commonly corrected with spectacles for vision, but
there are also higher-order aberrations present (Porter, Guirao et al. 2001). Adaptive optics was first used in retinal imaging in 1997, providing a revolutionary method of visualizing individual photoreceptor cells in the intact retina (Liang, Williams et al. 1997). This technique was expanded to the use of scanning laser ophthalmoscopy in 2002, further improving light collection efficiency and resolution (Roorda, Romero-Borja et al. 2002). Since then, the adaptive optics scanning light ophthalmoscope (AOSLO) has been combined with a multitude of techniques from microscopy to visualize cells and cellular processes in the retina. These techniques include fluorescence imaging (single photon or two-photon excitation) and a number of non-confocal detection schemes.

1.7.2 New technologies advance retinal imaging capabilities

Since its origin in 1997, adaptive optics retinal imaging has become a key tool in furthering our understanding of the retina in health and disease. Early on, the technique allowed unprecedented visualization of the photoreceptor mosaic, but was fairly limited in the cell classes it could investigate. Nonetheless, Roorda and Williams were able to determine the arrangement of the three cone classes in vivo using adaptive optics (Roorda and Williams 1999). When adaptive optics was combined with a scanning laser ophthalmoscope, the axial sectioning and improved lateral resolution allowed for the visualization of nerve fibers and blood flow in retinal capillaries (Roorda, Romero-Borja et al. 2002).

In 2006, AOSLO was combined with fluorescence imaging, further improving the specificity with which cells can be imaged (Gray, Merigan et al. 2006). Prior to this
innovation, AOSLO was limited to capturing reflected light, limiting its usefulness to cells which reflected a high amount of light, such as the photoreceptors and nerve fibers. Cells with low reflectance (such as ganglion cells) or those hidden under highly reflective layers (such as RPE cells, which are located below the photoreceptors) could be targeted with a fluorescence AOSLO. Because particular molecules fluoresce at different excitation and emission wavelengths, it was now possible to produce contrast by carefully choosing excitation and emission wavelengths to particular molecules. This could be done using molecules inherent to the cells, as in the case of targeting lipofuscin, which enabled imaging of RPE cells (Gray, Merigan et al. 2006, Morgan, Dubra et al. 2009). Alternatively, cell classes could be transfected with exogenous fluorophores such as yellow fluorescent protein or rhodamine dextran, allowing one to study these cells in vivo (Gray, Merigan et al. 2006, Geng, Dubra et al. 2012). It also opened an important field for study of retinal function, because RGCs could be targeted with genetically encoded calcium indicators, enabling the study of RGC responses to visual stimulation in an in vivo setting (Yin, Geng et al. 2013, Yin, Masella et al. 2014).

A new optical design for the AOSLO, consisting of telescopes arranged in an off-axis, non-planar arrangement was demonstrated in 2011 (Dubra and Sulai 2011). This new design sought to simultaneously reduce astigmatism in both the pupil conjugate and image planes, resulting in significantly better optical performance. Using this new optical design, it was now possible to resolve rods and the smallest cones in the center of the fovea (Dubra and Sulai 2011, Dubra, Sulai et al. 2011). This design form is now common in new AOSLO systems, including the system described in this thesis.
Confocal reflectance imaging enabled a number of cell classes previously unseen to be imaged, but in some ways, it actually limited the information that could be gathered. This is because the confocal pinhole rejects light which is not directly backscattered from cells in the retina. Light entering the retina may be scattered multiple times, or even refracted and then reflected, causing it to reach the detector outside of a confocal pinhole. In 2012, Chui et al. took advantage of this in order to enhance contrast of blood vessels (Chui, VanNasdale et al. 2012). Since, there have been a number of techniques which have built upon this strategy, including split-detector imaging and multi-offset detector imaging, which have provided the necessary contrast to see new structures in the retina. A number of new cell classes or parts of the cell have been seen in this way, including cone inner segments (Scoles, Sulai et al. 2014), horizontal cells (Guevara-Torres, Williams et al. 2015), and ganglion cells (Schallek, Joseph et al. 2016, Rossi, Granger et al. 2017). This family of techniques has great potential to look for structural changes in retinal disease.

Many of the above techniques represented big steps forward for structural imaging of the retina. However, the problem remains that structural imaging is best at identifying changes to the retina after they have already occurred, when in many cases these changes are already irreversible. The next step is to identify unhealthy cells prior to structural damage, which may then allow for interventions to prevent permanent damage. With this goal in mind, we turn to two-photon fluorescence imaging, which allows us to image endogenous fluorophores involved in retinal function.
1.7.3 Accessing endogenous measures of function via two-photon fluorescence imaging

Until recently, few non-invasive measures of function were possible in the *in vivo* retina. Retinal densitometry has been used to measure the recovery of the visual system to light exposure by measuring the reflectance of the retina at a wavelength strongly absorbed by photopigment (Bedggood and Metha 2012, Masella, Hunter et al. 2014). The interpretation of densitometry data can be complicated by the contribution from intrinsic optical signals. Intrinsic signal imaging has been used to study function in both the brain and the retina by assessing reflectance changes which arise from changes in neural metabolism (Grieve and Roorda 2008, Tsunoda 2009). However, it remains challenging to identify biomarkers which connect directly to specific processes the cells are undergoing.

Fluorescent molecules such as all-trans-retinol and NADH are known to have functional roles in the retina (as discussed in sections 1.3 and 1.4). Until recently, they have been impossible to image *in vivo* in primates, though not in mice, because they are excited by light in the ultraviolet, which is prevented from reaching the retina by the optics of the eye (Figure 1.4a; (Dillon, Zheng et al. 2000)). To access these molecules, we can use two-photon fluorescence excitation. Two-photon excitation works by a quantum-mechanical process in which two photons at a longer wavelength, arriving at the molecule simultaneously, can excite the molecule in the same way that a single photon of twice the energy would (Figure 1.4b). For *in vivo* retinal imaging, light in the infrared can
pass through the optics of the eye, excite fluorophores in the retina via two-photon excitation, and the visible light fluorescence can be recorded.

**Figure 1.4** – a) Excitation spectra and ocular transmission window. NADH (orange dashed line) and all-trans-retinol (blue solid line) excitation lie outside the transmission window of the eye, making it impossible to efficiently excite these molecules with single photon excitation. Using two-photon excitation (red vertical line), which provides an equivalent excitation (purple vertical line) at half the wavelength of the light source, these molecules can be excited. b) The mechanism of two-photon excitation. If two photons of light (red arrows), which together have greater energy than the gap between the ground state and excited state, are absorbed simultaneously, the electron will jump to the excited state in the same way as with single photon excitation (purple arrow). The molecule then emits fluorescence in either case (green downward arrow).
Two-photon excitation has several additional benefits which make it an attractive technique for use in retinal imaging. Because two-photon excitation only occurs at an appreciable rate when the photon flux is very high, it only occurs at the focal volume of the light source. The result of this phenomenon is that two-photon excitation provides axial sectioning, reducing the need for a confocal pinhole in the system and allowing the fluorescence to be captured directly as it leaves the eye. Because light is only absorbed in the focal volume, photobleaching is significantly reduced in two-photon imaging when compared to single-photon fluorescence.

Using two-photon excited fluorescence ophthalmoscopy (TPEFO) of the macaque, Sharma et al. have demonstrated the ability to visualize a multitude of cell types (Sharma, Williams et al. 2016). In addition, they measured an increase in fluorescence from photoreceptors with visual stimulation, which is believed to be due to an increase in all-trans-retinol production (Sharma, Schwarz et al. 2016, Sharma, Schwarz et al. 2017). These new capabilities may provide a functional measure of the visual cycle which could be deployed as a diagnostic tool to measure the function of individual photoreceptors. Higher optical power is used in TPEFO when compared to single-photon fluorescence imaging due to the low efficiency of two-photon excitation, resulting in challenges related to light safety (Schwarz, Sharma et al. 2017). However, TPEFO has been used to measure photoreceptor function with no detectable damage caused (Schwarz, Sharma et al. 2016).
1.8 Fluorescence lifetime imaging ophthalmoscopy

The fluorescence intensity, used as the measure in the above work, is only one of several identifying characteristics, including the quantum efficiency (i.e. the fraction of fluorescence photons emitted for every photon absorbed), excitation and emission spectra, and the fluorescence lifetime. In particular, the fluorescence lifetime can be a very useful tool both for differentiating fluorophores and for functional studies. This is because the fluorescence lifetime is a measure of the rate at which fluorophores transition from the excited state to the ground state (i.e. spontaneous emission of photons), which is independent of overall fluorescence intensity (Chang, Sud et al. 2007). This allows for a more robust measurement, where changes in fluorescence lifetime can be attributed to the fluorophore itself and its environment, rather than system instabilities.

Fluorescence lifetime imaging ophthalmoscopy (FLIO) has been implemented to measure the fluorescence lifetime of the retina in vivo using a modified cSLO (Schweitzer, Hammer et al. 2004). FLIO has been used to investigate changes in fluorescence lifetime in diseases such as age-related macular degeneration (Schweitzer 2009), diabetic retinopathy (Schweitzer, Deutsch et al. 2015), Stargardt disease (Dysli, Wolf et al. 2016), and central serous chorioretinopathy (Dysli, Berger et al. 2017). While promising, there are a number of challenges associated with the FLIO technique. Because these measurements are taken using a modified cSLO, they do not provide cellular resolution of the fluorescence lifetime. In addition, these systems are not able to separate fluorescence originating in different layers of the retina, and suffer from additional
artifacts from crystalline lens fluorescence (Schweitzer 2005). Due to the aforementioned transmission window of the eye, the fluorescence excitation source is typically at 446 or 473 nm. Not only is this wavelength not able to excite fluorescence from molecules such as NADH or all-
trans-retinol, but it also elicits safety concerns and is highly stimulating to the retina.

In this thesis, I work to overcome these technological limitations to expand the applications of fluorescence lifetime measurements in ophthalmoscopy by:

1) Combining FLIO with adaptive optics, which will allow for measurement of the fluorescence lifetime of individual cells and retinal layers. This new technique, which I name adaptive optics fluorescence lifetime imaging ophthalmoscopy, or AOFLIO, will be more sensitive to localized changes in abnormal retina and will more capably disambiguate the fluorescence lifetime in different retinal layers due to different fluorophore contributions.

2) Using two-photon excitation, which will allow endogenous fluorophores involved in cellular metabolism (NADH) and the visual cycle (all-
trans-retinol) to be targeted using 730 nm excitation. I retain the flexibility to image the same fluorophores (lipofuscin, FAD, and exogenous fluorophores such as green fluorescent protein and sodium fluorescein) as in single-photon FLIO by using two-photon excitation at 900 nm.
1.9 Thesis synopsis

The introductory material in this chapter was written to provide a backbone for the advances described in the rest of the thesis. It is my hope that this chapter has provided not only the background that this thesis rests on, but also the motivation for why I have worked toward these achievements.

Chapter 2 will provide an overview of the first aim of this thesis, which was to build a new two-photon AOSLO to image the mouse eye. This system was then used as a development platform for the AOFLIO technique.

Chapter 3 includes the necessary information to implement fluorescence lifetime imaging in an AOSLO. Additionally, this chapter details the necessary steps in order to properly analyze fluorescence lifetime data.

Chapter 4 discusses the first implementation of AOFLIO in the two-photon AOSLO for mice. In this work, two fluorophores with known fluorescence lifetimes were inserted into the mouse retina and imaged to validate the technique. Through this study I also found that fluorescence lifetime measurements may be useful for differentiating spectrally similar fluorophores.

In Chapter 5, the AOFLIO technique was translated to image the macaque retina. During this study, I imaged endogenous fluorescence from photoreceptors in the macaque retina using AOFLIO. I found that rods and cones have different fluorescence lifetimes.
Finally, chapter 6 describes future work that stems from this thesis. Engineering improvements to the two-photon mouse AOSLO, which will improve its utility, are discussed. I have only scratched the surface of the applications for AOFLIO, and some future scientific studies are also suggested in this chapter.
Chapter 2

Two-photon adaptive optics ophthalmoscopy - overview and implementation in the living mouse eye
2.1 Rationale for building a two-photon AOSLO for mouse

Two-photon excited fluorescence ophthalmoscopy is a promising technique for imaging both structure (Sharma, Williams et al. 2016) and function (Sharma, Schwarz et al. 2016) of the intact retina in a living animal. This technique was demonstrated first in an AOSLO designed to image macaques and modified for two-photon imaging (Hunter, Masella et al. 2011). Building upon this successful demonstration, two new AOSLOs were proposed to be dedicated solely to two-photon imaging: one for primate and another for mouse. As detailed in section 1.6, there are reasons why one would want to have the capability to image both of these animals. Briefly, although they have physiology much more similar to humans, macaques are expensive and there are few options to study disease models in macaque. Mice are much cheaper, and there are a myriad of manipulations that can be done to the genome which allow the mouse to be used as a model for complex systems (Hanahan 1989). In addition, fluorescent proteins are routinely inserted either genetically or via a viral vector into the mouse retina. From an optical point of view, mice provide an additional benefit due to their higher numerical aperture eye. Mice have a numerical aperture of ~0.5, which is approximately twice that of the macaque eye. As a result, an AOSLO for imaging the mouse eye can theoretically see structures that are twice as small as that of an AOSLO for the macaque eye. The theoretical lateral resolution that can be achieved with the system described here is 0.9 µm.
Initial two-photon fluorescence measurements from the mouse eye using a modified single-photon fluorescence AOSLO suggested that building an AOSLO dedicated to two-photon fluorescence imaging in the mouse was feasible (Sharma, Yin et al. 2013). In this work, Sharma et al. found that they were able to capture two-photon fluorescence from extrinsically labeled ganglion cells to resolve cell somas and dendrites and to track fluorescence from a genetically encoded calcium indicator under stable conditions. Compared with previous measurements of intrinsic fluorophores in macaque retina, they were able to rapidly acquire images by combining the signal advantage gained from the mouse eye and extrinsic fluorophores.

There have been some efforts to use microscopy through the pupil of the eye in living mice as an alternative approach to ophthalmoscopy (Palczewska, Dong et al. 2014, Wahl, Jian et al. 2016). In these systems, the refractive power of the mouse cornea is canceled via a plano-concave lens, and a microscope objective is used to focus light on the retina. I chose to forego this approach in favor of an ophthalmoscope for several reasons. First, a microscope objective is unnecessary because the animal’s eye already focuses light on the retina. Adaptive optics can correct for aberrations introduced by the optics of the eye as opposed to a microscope objective, which has better optical quality. In fact, both of the reports of live animal microscopy listed above required the use of adaptive optics to provide high resolution. The use of a microscope adds additional complexity to system alignment – now the eye has to be aligned to the microscope such that the cone of light fills the pupil, rather than the relatively simple task of aligning the mouse eye to the exit pupil of the system. Additionally, the microscope objective must
now be aligned to the optical system. Finally, for two-photon fluorescence imaging, the microscope objective introduces additional dispersion to the femtosecond light pulses, which must then be compensated. For these reasons, I chose to build an AOSLO rather than an adaptive optics microscope, for mouse imaging.

2.2 System design

2.2.1 System specifications

With the above listed advantages in mind, I sought to design and build a two-photon AOSLO to image mice \textit{in vivo}. To achieve the best optical design, the specifications of the system were first established. Typically, in an imaging system, the primary specifications are the aperture (i.e. how much light the system can collect from an object), the field of view (how large of an object can be seen), and the wavelength(s) of light used.

In the case of an AOSLO, the system aperture is limited by that of the animal eye itself, since the lens of the animal is the final imaging component. The system must couple light through the pupil of the eye, and therefore making a larger exit pupil to the system will have no benefit for imaging. Therefore, the mouse pupil effectively acts as the aperture stop for the system. The diameter of the dilated mouse pupil is 2 mm, and the focal length is approximately 2.6 mm (Schmucker and Schaeffel 2004). In order to effectively couple light from the AOSLO into the mouse eye, the exit pupil was specified to be 2mm (see Table 2.1).
Table 2.1 - System specifications and design performance goals.

<table>
<thead>
<tr>
<th>System specifications for mouse two-photon AOSLO design</th>
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<tbody>
<tr>
<td>Exit pupil diameter</td>
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<td>Field of view</td>
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<tr>
<td>Wavelength</td>
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<td>Vergence range (at exit pupil)</td>
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<th>Design goals for mouse two-photon AOSLO design</th>
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<tr>
<td>Retinal plane</td>
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The field of view (FOV) is determined by the size (either in a linear dimension or the corresponding angular dimension) of the object to be imaged. These dimensions can be related by the equation \( h = f \tan \theta \), where \( h \) is the object radius (in this case the retina), \( f \) is the focal length (in this case of the mouse eye), and \( \theta \) is the angular half FOV. In order to image a sufficiently large region of the retina at one time, I decided to set the FOV such that I could image a 175 \( \mu \)m x 175 \( \mu \)m field. This field size setting allows me to image a sufficiently large field of view to navigate the mouse retina, and to image a large number of cells at once (cells in the mouse retina range from \( \sim \)2 \( \mu \)m for rod photoreceptors to \( \sim \)20 \( \mu \)m for ganglion cell somas). Translating to angular coordinates, this corresponds to a 5° x 5° FOV. Therefore, the FOV specification was to achieve diffraction-limited performance for a 5° x 5° FOV.

The determination of the wavelength specification can be complicated in an AOSLO. These systems typically use multiple light sources for different functions (e.g.
wavefront sensing, reflectance imaging, fluorescence imaging). In addition, it is beneficial to be able to vary the excitation wavelength for two-photon fluorescence excitation in order to target different fluorophores. The wavelength specification for this system was chosen in order to excite key endogenous retinal fluorophores. 730 nm was chosen as the first excitation wavelength, which allows for targeting of molecules such as NADH, FAD, and all-trans retinol. 900 nm was chosen as the other excitation wavelength in order to target lipofuscin. Additionally, 900 nm can efficiently excite sodium fluorescein and enhanced green fluorescent protein (EGFP). These extrinsic fluorophores can be useful for targeting vessels (fluorescein) or labeling retinal cells (EGFP). The wavefront sensing source and the reflectance source were chosen to reside between those wavelengths to limit the overall bandwidth that the system needed to be well-corrected over. In general, the wavelength specification was less of a concern for system design than the system aperture and FOV because the system consists mostly of reflective elements.

An additional consideration for an AOSLO system is to have the ability to focus through the optically thick retina. In the case of the mouse retina, this is particularly important because the retina has a similar thickness to that of the human, but a focal length that is almost ten times shorter (2.6 mm for the mouse, versus 22.3 mm in the Gullstrand-Le Grand model of the human eye). To change focus in the retina, the vergence of the beam can be changed prior to entering the optical system (Figure 2.1). A fiber light source on a translation stage can be moved to make the ingoing wavefront collimated, converging, or diverging prior to entering the system. The change in retinal
focus is then related to the change in position of the fiber by the longitudinal magnification of the system \( m_L = \frac{n'}{n} m^2 \), where \( m \) is the magnification between the entrance and exit pupils of the system. This need to section the whole retina requires that the optical system be well-corrected over a large range of vergence. For the mouse, the system must be able to change optical power by \(~50\) diopters (1D = 1m\(^{-1}\)) in order to section through the retina (Geng, Dubra et al. 2012). Therefore, the system was designed to be able to change optical power by \( \pm25\)D (with 0D corresponding to collimated light entering the mouse eye in the design).

**Figure 2.1** - Changing the focus in the retina by changing the vergence of the ingoing beam. This is achieved by moving the optical fiber (point source) to change curvature of the wavefront. In **blue** (solid), collimated light is sent into the system; collimated light leaves the optical system and focuses in the middle of the retina. In **red** (wide dashes), light is converging as it enters the mouse eye, causing it to focus toward the inner retina. In **green** (narrow dashes), light is diverging as it enters the eye, causing it to focus in the outer retina. This change in focus necessitates that the optical system be well-corrected over a range of focus depths.

### 2.2.2 System layout

The system design was loosely based on a previous design used for the first mouse AOSLO built in Rochester (Geng, Dubra et al. 2012). In the present design, a block diagram of which is shown in Figure 2.2, 5 afocal telescopes relay light through the
system to planes that are conjugate to the mouse pupil (“pupil planes”). Placed at two of these pupil planes are scanners which raster scan the light to form a rectangular FOV. At a 3rd pupil plane is the deformable mirror, which corrects for the aberrations of the mouse eye as well as the optical system. The 4th pupil plane is empty, although it can be used as a trial lens plane, allowing for a lens to be placed at this location to change the vergence of the beam as it enters the mouse eye. The 5th pupil plane is where the mouse eye is placed. The system is mostly reflective, with 9 of the telescope elements being spherical mirrors. The final element is an achromatic lens, which is necessary to achieve diffraction-limited performance over a large FOV (Geng, Dubra et al. 2012). The details of the optical system are shown in Table 2.2.

**Table 2.2 – Details of the tilts and angles of key components in the mouse two-photon AOSLO.** Angles listed are the mechanical angle with respect to the optical axis. Angles of the reflected beam will be double those listed.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Focal length (mm)</th>
<th>$\theta_x$</th>
<th>$\theta_y$</th>
<th>Beam size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entrance pupil</td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Mirror 1</td>
<td>1000</td>
<td>2.07</td>
<td>-1.10</td>
<td></td>
</tr>
<tr>
<td>Mirror 2</td>
<td>500</td>
<td>0</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>Horizontal scanner</td>
<td></td>
<td>0 ± 1</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>Mirror 3</td>
<td>600</td>
<td>-0.46</td>
<td>-1.3</td>
<td></td>
</tr>
<tr>
<td>Mirror 4</td>
<td>1200</td>
<td>1.4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Vertical scanner</td>
<td></td>
<td>-3</td>
<td>-2 ± 0.5</td>
<td>5</td>
</tr>
<tr>
<td>Mirror 5</td>
<td>750</td>
<td>1.3</td>
<td>-1.61</td>
<td></td>
</tr>
<tr>
<td>Mirror 6</td>
<td>1050</td>
<td>-3.19</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Deformable mirror</td>
<td></td>
<td>3.5</td>
<td>-1.5</td>
<td>7</td>
</tr>
<tr>
<td>Mirror 7</td>
<td>1050</td>
<td>-3.8</td>
<td>-1.74</td>
<td></td>
</tr>
<tr>
<td>Mirror 8</td>
<td>600</td>
<td>1.05</td>
<td>-2.81</td>
<td></td>
</tr>
<tr>
<td>Mirror 9</td>
<td>800</td>
<td>-2.1</td>
<td>-3.23</td>
<td></td>
</tr>
<tr>
<td>Flat mirror</td>
<td></td>
<td>49</td>
<td>-1.3</td>
<td></td>
</tr>
<tr>
<td>Lens 10</td>
<td>400</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mouse eye</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 2.2 - System diagram of two-photon AOSLO designed for the mouse eye. PMT: Photomultiplier tube. SHWS: Shack-Hartmann wavefront sensor. 80/20: 80/20 beam splitter.
2.2.3 Polygon scanner – rationale and specifications

In this system, I used a polygon scanner for the horizontal scan. All other AOSLO systems in Rochester use a resonant scanner. The resonant scanner, while small and able to change its scan angle, introduces a sinusoidal distortion to the FOV because it does not rotate at a constant velocity. Typically this sinusoidal distortion is corrected digitally, either in post-processing or in real-time. The commercial fluorescence lifetime software I used to capture images does not have the capability to correct for a sinusoidal scan. Therefore, I decided to forego this challenge by instead using a polygon scanner, which travels at a constant velocity and does not require any sinusoidal correction.

The polygon scanner used in this system was significantly larger than the resonant scanner used in the previously designed mouse AOSLO (Geng, Dubra et al. 2012), necessitating a re-design because of the requirement that the beam pass over the top of the scanner on both passes (Figure 2.3). Because of the optical aberrations induced by this requirement, the design was modified to compensate by adjusting the mirror angles in both the vertical and horizontal planes downstream in the system.
Figure 2.3 – Shows the result when attempting to reflect the beam past the horizontal scanner with a) the resonant scanner used in most AOSLO systems, and b) the polygon scanner used in the two-photon AOSLO design for mouse. Typical AOSLO systems can use a small angle reflecting in the horizontal plane of the resonant scanner to reduce system aberrations such as the configuration shown in a). Because of the large size of the polygon scanner, this configuration will not work, as shown in b). The beam must be directed above the polygon scanner on both passes, introducing a large amount of aberration which must be compensated downstream in the design.

Choosing the correct polygon scanner was critical to the overall success of the system because it determines factors such as the FOV and frame rate of the video capture. The chosen parameters for the polygon scanner were driven by the needs of the optical
design: 2.5 mm diameter beam at the polygon scanner (B), 2 mm diameter beam at the mouse eye (D), and 5 degree FOV in the mouse retina. The magnification (M) is then \( M = \frac{D}{B} \), resulting in \( M = 0.8 \). The scan angle from the polygon is \( \beta = M \times FOV = 4^\circ \).

One of the key parameters chosen for the scanner was the number of facets. This number affects the duty cycle (C - the percentage of time the beam illuminates the FOV), the required velocity of the polygon scanner to achieve a set framerate, and the size of the scanner. With more facets, the scanner will have a higher duty cycle for the same scan angle, but it will also have a larger size. The relationship is \( \frac{\beta}{\alpha} = \frac{\beta \times n}{720^\circ} \), where \( \alpha \) is the maximum scan angle and is determined by the number of facets (n). I decided to choose 36 facets as a compromise between size and duty cycle. With 36 facets, the resultant duty cycle is 20%.

Having chosen the number of facets in the polygon scanner, the overall diameter of the scanner can now be calculated. The necessary length of each facet is related to the size of the beam and the duty cycle by the equation \( L = \frac{B' + 1}{1 - C} \), where \( B' = \frac{1.5B}{\cos(\frac{\beta}{2})} \). In this equation, \( \theta \) refers to the angle with respect to the center of the scan that the beam coincides with the scanner. For this system, that angle is 0°. The factor of 1.5 is to ensure that the intensity is consistent throughout the field of view. Using these two equations, the length of each facet of the polygon scanner is 5.94 mm. The diameter of the scanner can then be calculated by \( Diam = \frac{L}{\tan(\frac{180}{n})} = 67.9 \text{ mm} \).
2.2.4 System performance

The system was designed using CodeV optical design software (Synopsys, Mountain View, California, USA). All surfaces including mounts were included in the model to ensure beam clearance at all points in the optical path and for all angles. The system was optimized by allowing the program to iteratively vary mirror angles in the horizontal and vertical directions to achieve the best optical performance. Constraints were introduced on mirror angles to prevent the field of view being blocked by optical components or mounts, and to keep the system as compact as possible. The system performance was evaluated by measuring the wavefront error at 27 retinal positions, shown in Table 2.3.

**Table 2.3** - Shows the wavefront error (measured in fractions of a wave) at the 27 retinal positions where optical performance was evaluated. These positions were chosen both for optical reasons (the edge of the FOV typically has the worst optical performance) as well as mechanical (to ensure the beam is not getting blocked). Top cell at each position in FOV: -25D; Middle: 0D; Bottom: 25D. *Above diffraction limit.

<table>
<thead>
<tr>
<th>Vertical Scan Angle</th>
<th>2.5°</th>
<th>0°</th>
<th>-2.5°</th>
</tr>
</thead>
<tbody>
<tr>
<td>.0777*</td>
<td>.0711</td>
<td>.0854*</td>
<td></td>
</tr>
<tr>
<td>.0591</td>
<td>.0610</td>
<td>.0616</td>
<td></td>
</tr>
<tr>
<td>.0451</td>
<td>.0399</td>
<td>.0306</td>
<td></td>
</tr>
<tr>
<td>0°</td>
<td>.0473</td>
<td>.0650</td>
<td>.0410</td>
</tr>
<tr>
<td>.0598</td>
<td>.0618</td>
<td>.0627</td>
<td></td>
</tr>
<tr>
<td>.0382</td>
<td>.0495</td>
<td>.0300</td>
<td></td>
</tr>
<tr>
<td>-2.5°</td>
<td>.0788*</td>
<td>.0507</td>
<td>.0427</td>
</tr>
<tr>
<td>.0585</td>
<td>.0608</td>
<td>.0621</td>
<td></td>
</tr>
<tr>
<td>.0688</td>
<td>.0511</td>
<td>.0254</td>
<td></td>
</tr>
</tbody>
</table>
In addition, the wavefront error was minimized at each pupil conjugate plane. The performance of the final optical design, evaluated at 730 nm, is shown in table 2.4. All pupil planes were diffraction-limited by the Marechal criterion (<0.0714 RMS wavefront error). All retinal locations were diffraction-limited except for the 3 specified in table 2.3. Distortion at all retinal positions was below 1.5%.

**Table 2.4** - Performance of optical design at the specified pupil planes and the 27 retinal locations evaluated. The design was diffraction-limited at all pupil planes. The wavefront error at 3 retinal locations was above the diffraction limit, with the maximum error being 0.0854 waves. The remaining locations were diffraction-limited.

<table>
<thead>
<tr>
<th>Plane</th>
<th>Maximum Wavefront RMS (waves)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast Scan</td>
<td>.0270</td>
</tr>
<tr>
<td>Slow Scan</td>
<td>.0293</td>
</tr>
<tr>
<td>Deformable Mirror</td>
<td>.0421</td>
</tr>
<tr>
<td>Mouse pupil</td>
<td>.0625</td>
</tr>
<tr>
<td>Retinal</td>
<td>.0854</td>
</tr>
</tbody>
</table>

**2.3 Implementation of two-photon AOSLO**

**2.3.1 Light sources**

The two-photon AOSLO for mouse imaging was built and aligned based on the design discussed above. Four light sources were implemented in the system. Coupled into the system via optical fibers were a 633 nm helium neon laser (Research Electro-Optics, Boulder, Colorado, USA), used for alignment, an 850 nm laser diode (Qphotonics, Ann Arbor, Michigan, USA), used for wavefront sensing, and a 790 nm superluminescent diode (Superlum, Cork, Ireland), used for reflectance imaging. The fibers were mounted
on motorized stages to allow for independent focusing. A pulsed laser (Mai Tai XF-1 DS; Newport Spectra-Physics, Santa Clara, California, USA) with central wavelength tunability from 710 nm – 920 nm was used for two-photon fluorescence excitation. The laser operated at an 80 MHz repetition rate with output pulses of <70 fs. Dispersion was compensated through the use of a prism pair placed on motorized stages within the DeepSee attachment of the Mai Tai XF-1 laser. An additional glass light pipe (Part #48584; Edmund Optics) was placed at the output of the laser to bring the dispersion compensation of the DeepSee into the range required for the mouse eye. Light was focused through a spatial filter to produce a clean Gaussian beam and collimated by an achromatic doublet lens before being coupled to the entrance pupil of the AOSLO. The collimating lens was placed on a motorized translation stage to change the focus of the pulsed laser without changing the focus of the other light sources. The four light sources were combined into the system with a custom dichroic mirror which transmitted 630 – 640 nm and 760 – 870 nm light, but reflected light from 710 – 750 nm and 880 – 920 nm (zt633_795-850tpc; Chroma Technology Corp., Bellows Falls, Vermont, USA).

2.3.2 Adaptive optics correction

In order to correct the aberrations present in the eye, one must first measure them. This is often done with wavefront sensing, typically using a Shack-Hartmann wavefront sensor (SHWS) (Liang, Grimm et al. 1994). The general principle is to place a lenslet array of very small lenses conjugate to the pupil of the eye. The lenslet array is placed one focal length (F) away from an array sensor such as a CCD camera. For a plane wave,
these spots would be sharply focused in a regular array. During calibration, a plane wave is sent into the SHWS and the resulting pattern stored as a reference. Due to aberrations, the spots from the wavefront, written as $W(x, y)$, returning from the eye are offset from the locations they would be located if the wavefront were planar. These local deviations are measured as a lateral displacement ($\Delta x$ and $\Delta y$), which can then be used to reconstruct the wavefront (Porter, Queener et al. 2006) using the following equations:

$$\frac{\partial W(x, y)}{\partial x} = \frac{\Delta x}{F} \quad \text{and} \quad \frac{\partial W(x, y)}{\partial y} = \frac{\Delta y}{F}$$

Once the wavefront aberrations of the eye are measured, they can be corrected. The two most common devices used to correct the wavefront are spatial light modulators and deformable mirrors. Of these, spatial light modulators are less flexible because they have wavelength-dependent effects. In addition, they operate only on light that is polarized along the axis of the liquid crystals, forcing the user to sacrifice efficiency by placing a linear polarizer in the beam path or use multiple spatial light modulators (Porter, Queener et al. 2006). Deformable mirrors have high stroke and high light throughput, and are typically the choice in research-grade instruments. There are a number of different choices available when choosing a deformable mirror, including microelectromechanical systems as well as membrane-based mirrors. In this thesis, a membranous design was used for all work due to the high light throughput and high dynamic range afforded by this design type.
There are many algorithms which have been used to impart the optimal surface upon the wavefront corrector, first in astronomy and later in vision science. The software used for adaptive optics correction in this thesis was developed by Dr. Alfredo Dubra and Kamran Ahmad and is based on the direct slope control algorithm (Porter, Queener et al. 2006). During calibration, the software records the change in the wavefront when each of the actuators is moved to determine the influence function of each actuator. This influence function, which can be written in vector form as the Δx and Δy displacement for each lenslet is then placed into a matrix which includes the influence function of each actuator. This is called the influence matrix.

When the adaptive optics is turned on, the pseudoinverse of this matrix is multiplied by the matrix of the measured wavefront to determine the voltage to input on each actuator of the deformable mirror. This process is iterated in order to achieve the best correction and to account for dynamic changes in the wavefront. The deformable mirror can also be used to change the axial position of the retinal image by purposely imparting a curvature on the mirror. In this way, the user can image different layers of the retina.

The adaptive optics correction was achieved using a Shack-Hartmann wavefront sensor. A lenslet array (203 μm pitch, 7.8 mm focal length; Adaptive Optics Associates, Cambridge, Massachusetts, USA) was placed at a plane conjugate with the mouse eye, and a CCD camera (Rolera XR; QImaging, Surrey, British Columbia, Canada) was placed one focal length behind the lenslet array. The beam size at the wavefront sensor
was 5 mm, which is 2.5 times larger than at the mouse eye and the same size as the entrance pupil.

With these parameters, the dynamic range of the wavefront sensor was 0.013 rad (Porter, Queener et al. 2006). The sensitivity is limited either by the pixel size of the CCD camera, the diffraction limited spot size of the lenslets, or the centroiding algorithm. The CCD camera used here had a pixel size of 12.9 µm, whereas the spot size at 850 nm of the lenslets is 78 µm. The sensitivity of the wavefront sensor is therefore 0.01 rad, but this can be improved by about 5x with an efficient centroiding algorithm. The software used in this thesis measures the centroid by calculating the image moment on a sub-pixel level.

The monochromatic aberrations were determined by a custom program developed in house (TsunamiWave). The same program then shaped the deformable mirror (DM97-08; ALPAO SAS, Grenoble, France) to correct the monochromatic aberrations and change focus. The deformable mirror used in this system was a new model, which has a smaller overall size (diameter = 7.2 mm), but the same number of actuators, resulting in a smaller magnification between the deformable mirror and the lenslet array than the previous mouse AOSLO (magnification = 1.4 vs. 2.7 in the previous system). The deformable mirror had 97 actuators with a maximum stroke of 80 µm and a pitch of 0.8 mm. The ratio of lenslets to actuators used in this wavefront sensor was >6.

Previous work in the mouse has found that using an annular illumination for wavefront sensing can reduce unwanted reflections from retinal layers that make it easier
to calculate the centroid of the wavefront spots (Geng, Schery et al. 2011). I therefore placed an annulus which blocks the center portion of the wavefront sensor source at a pupil conjugate in the illumination pathway. With these specifications, the adaptive optics system should be able to correct for the aberrations of the mouse eye (Geng, Schery et al. 2011).

### 2.3.3 Light collection

The system was built to allow for two types of light collection. Light emitted from the superluminescent diode is reflected from the mouse retina, descanned through the system and captured by a photomultiplier tube (PMT H7422-50; Hamamatsu Corporation, Shizuoka-Ken, Japan).

Excitation light emitted from the ultrafast laser is absorbed by molecules in the retina and emitted as fluorescence. A dichroic mirror (T660lpxrxt; Chroma Technology Corp.) re-directs this light to a PMT detector (HPM-100-40; Becker and Hickl, Berlin, Germany or H7422-50; Hamamatsu Corporation). Filters transmitting light from 400-550 nm (E550sp-2p; Chroma) or 400-680 nm (ET680SP-2P8; Chroma) were used to capture fluorescence. The system was completely covered to reduce stray light, which can increase the noise floor and overwhelm the dim two-photon signal.

Detected light photocurrents from the reflectance and fluorescence channel PMTs were converted to voltage and amplified using a new voltage amplifier (C6438-01; Hamamatsu Corporation), made by the same company which produced the PMT detectors used, and custom inverting amplifiers (1X gain for the reflectance channel and
5X gain for the fluorescence channel). These signals were then sent to an FPGA card (Xilinx, Inc., San Jose, California, USA) and digitized through an analog to digital converter built in the card.

### 2.3.4 Horizontal scan synchronization

In order to ensure each line of the video capture began at the proper time, a synchronization signal must be generated. A small subsystem was implemented to generate this signal (Figure 2.4). In brief, a laser is reflected off the same polygon facet as that which reflects light into the eye. A photodiode is placed in the path of this light source, which then emits a short pulse as the laser crosses the photodiode. This triggers the start of a new line. The photodiode can be placed anywhere within the extent of the scan, and the synchronization can be delayed in software to capture the imaging field of view.
Figure 2.4 - Horizontal synchronization subsystem. A red laser is collimated and directed at a vertical angle toward the polygon scanner. The polygon scanner sweeps the light in an arc, and at a particular angle the light is intercepted by a photodiode, which captures the laser light and converts it to an electrical synchronization signal. As the scanner continues spinning, the next facet repeats the process, triggering another new line. Dashed lines correspond to the extent of the scan.

2.4 Summary

This chapter covers the design decisions, optical design, and implementation of a two-photon AOSLO for imaging mice. The major change in this system compared to past AOSLO systems built at Rochester was the change from a resonant scanner to a polygon scanner, which required modification of the optical design. This system was built and used for the initial implementation of AOFLIO, which is described in chapter 4.
Chapter 3

Measuring the fluorescence lifetime
3.1 History and overview of fluorescence lifetime measurement

The term fluorescence was coined in 1852 by George Stokes, who is famous for formulating the Stokes shift (Stokes 1852). Around the same time, Edward Becquerel sought to study this newly described phenomenon in a comprehensive way (Berezin and Achilefu 2010). He developed the first instrumentation to measure the phosphorescence lifetime. Phosphorescence is a process similar to fluorescence in that energy is absorbed by the substance and then emitted as light. The major difference is that phosphorescence decays much more slowly than fluorescence because the electron enters a long-lived quantum state prior to decaying back to the ground state. At the time, scientists believed fluorescence to be an instantaneous process, so they studied the comparatively long-lived phosphorescence.

The technological innovation which first allowed the fluorescence decay to be studied in 1921 was the Kerr cell (Berezin and Achilefu 2010). A Kerr cell consists of two orthogonal polarizers with a liquid that is sensitive to an electric field between. When no electric field is applied, the molecules are randomly oriented, making the liquid optically isotropic, and the crossed polarizers prevent light from passing through the Kerr cell. When an electric field is applied, the liquid becomes birefringent, changing the polarization of the light and allowing it to pass through the cell. By quickly applying and then removing the electric field, very short pulses of light could be generated. This concept of producing a very short light pulse (ideally much shorter than the decay time of the fluorophore) is the basis for measuring the fluorescence lifetime in the time domain.
In order to measure short fluorescence lifetimes, not only does the pulse of excitation light have to be very short, but the detection scheme must be able to measure the decay with adequate time resolution. The first technique used for measuring the fluorescence lifetime in the time domain was using time-gated detection (Bennett 1960). The basic idea of this method is to turn on the detector only for a very short time in order to capture a portion of the decay. For the next pulse of excitation, that time gate is shifted to capture a different portion of the decay. This is repeated until the entirety of the decay is captured. This method suffers from systematic errors due to noise that can be picked up by the detector and low sensitivity. In addition, because it only captures a limited time band of the fluorescence, it necessarily discards a significant portion of the fluorescence signal. However, it can still be used in some cases where the signal is very high and the fluorescence decay must be collected rapidly (Lakowicz 2006).

Another way to measure the fluorescence lifetime is using the frequency domain technique. Frequency domain measurement of the fluorescence lifetime is accomplished by modulating the intensity of the excitation light at a rate comparable to the reciprocal of the lifetime (ex. For a 10 ns decay, the intensity could be modulated at 100 MHz). The fluorescence would also oscillate at the same frequency, but the amplitude and phase shift will be dependent on the fluorescence lifetime of the sample. Frequency domain measurements can be used to rapidly acquire a fluorescence lifetime image in wide field instruments, however they have problems with accurately measuring the lifetime if the fluorescence signal is too low to resolve the frequency modulation, as can be the case in two-photon adaptive optics ophthalmoscopy. Additionally, in a scanning instrument such
as an AOSLO, the interplay of the raster scan and a frequency modulated intensity make it difficult to measure the phase shift of the fluorescence. Due to these limitations, I chose to forego frequency domain measurement techniques and time-gated measurement in favor of time-correlated single photon counting (TCSPC).

TCSPC was first demonstrated in the 1960’s, and is now widely used for fluorescence lifetime measurements due to its high sensitivity and time resolution (Meiling and Stary 1963). Below is described the basic method for measuring the fluorescence lifetime using TCSPC, however there are many subtleties involved in the technique which will be described in the coming sections. A block diagram of the implementation in the two-photon AOSLO systems is also shown in Figure 3.1.

1. Fluorescence is excited by a pulse of light, and the emitted fluorescence photons are captured by a single photon detector.

2. The detector emits an electronic pulse when a photon arrives. The arrival time of this pulse is then compared to a synchronization pulse, indicating the time at which the light pulse was emitted.

3. Knowing the time difference between the excitation pulse and the arrival of the fluorescence photon, the fluorescence photon can be placed at the proper time in the fluorescence decay.

4. Steps 1-3 are repeated many times until a histogram of photon arrival times is constructed.

5. The histogram can be fit using a nonlinear least squares fit to extract time constants.
Figure 3.1 – Overview of the method for TCSPC implementation in a two-photon AOSLO. A laser pulse travels through the AOSLO system and excites fluorescence, which is then detected by the single photon detector and transferred to the TCSPC module. Concurrently, a small portion of the pulse is detected by a photodiode and sent directly to the TCSPC module as a reference pulse. The arrival time of the signal from the fluorescence photon is compared to the reference pulse to place the photon in the correct time bin. This is repeated many times to build a histogram, which is then fit with exponential functions.

\[ I(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \]

\[ \tau_{mean} = a_1 \tau_1 + a_2 \tau_2 \]
3.2 TCSPC instrumentation

3.2.1 The light source

The light source is a critical choice for TCSPC. A high repetition rate, short-pulsed laser is the preferred choice for most applications. A high repetition rate is preferred because traditional TCSPC requires that much less than 1 photon is detected for every light pulse. Therefore, a faster repetition rate allows for a higher photon count rate. Short pulses are required because it simplifies the removal of the light source’s impact on the fluorescence decay. Very short pulses (i.e. femtosecond lasers) are so brief that they can effectively be considered a delta function (a 70 fs light pulse is ~1000 times shorter than the typical instrument response function (IRF) in a TCSPC measurement).

The two-photon AOSLO described in chapter 2 is already well-suited for TCSPC detection because it uses an ultrafast laser to excite fluorescence. This laser (Mai Tai XF-1 DS; Newport Spectra-Physics) emits pulses of ~70 fs, with an 80 MHz repetition rate. Therefore, it fulfills both main criteria for a light source for TCSPC detection. With a pulse every 12.5 ns, there are 8 pulses for every pixel in the image (each pixel is 100 ns in the time dimension).

3.2.2 The detector

Two types of photon counting detectors are widely used in TCSPC – the microchannel plate photomultiplier tube (MCP PMT) and the hybrid PMT. The most important TCSPC-specific characteristic of a detector (characteristics such as quantum
efficiency, spectral range, and package size are all still important) is the transit time spread (TTS). The TTS refers to the distribution of transit times that a photon can take through the detector. A high TTS reduces the time resolution of the detector because it increases the uncertainty in when the photon arrived at the detector. A low TTS becomes important when attempting to measure short fluorescence lifetimes (i.e. the TTS should be significantly shorter than the shortest lifetime to be measured). The shortest fluorescence lifetime I have measured \textit{in vivo} is \(~150\) ps; therefore, the TTS should be much lower than this. An additional consideration is afterpulsing. Afterpulsing is caused by ionization of residual gas molecules by the electrons inside a PMT (Application note, The HPM-100-40 Hybrid Detector, Becker and Hickl GmbH), and manifests in the IRF as a small bump following the main peak. Afterpulsing can increase the background noise in TCSPC measurements and should therefore be minimized.

MCP PMT’s work differently from a typical PMT. Instead of having multiple dynodes that amplify the initial photoelectron, they have narrow channels to amplify the signal (Lakowicz 2006). As a result, they have very low TTS (~25 ps) because the electrons all travel nearly the same path. The downside to these detectors is that they do not amplify the signal nearly as much as a standard PMT, causing them to perform linearly over a more limited range. They can also be more expensive than other detectors, and only need to be used when trying to measure incredibly short fluorescence lifetimes.

Hybrid PMT’s combine the advantages of a PMT with those of an avalanche photodiode. In these detectors, a photocathode ejects electrons, which are then accelerated by a strong electrical field into an avalanche diode (Figure 3.2). These
detectors produce most of their gain in one step, resulting in a low TTS (~50 ps). In addition, they have very low afterpulsing because only single electrons are traveling in the detector prior to interaction with the avalanche diode. Finally, these detectors have a slightly higher quantum efficiency than a standard analog PMT. This is the type of detector used throughout this thesis.

**Figure 3.2** - Hybrid photomultiplier tube. In a hybrid PMT, a photocathode emits photoelectrons which are then accelerated into an avalanche diode by a strong electric field. Because most of the gain is produced in one step, the transit time spread is low for these detectors. Modified from HPM-100-40 application note, Becker & Hickl GmbH.

### 3.2.3 The TCSPC device

The goal of the TCSPC device is to measure and place each fluorescence photon in the proper time bin of the decay. By repeatedly doing this, a histogram of photon arrival times is created that can be fit with an exponential decay model to extract lifetime parameters.

There are a number of key components to the TCSPC device to complete this task (Figure 3.3). After being converted to an electrical pulse, the photon signal first
encounters a constant fraction discriminator (CFD) to accurately determine the arrival time. Complicating this process is the fact that the pulses from individual photons do not always have the same amplitude, preventing a threshold-based detection scheme from properly determining the arrival time (Figure 3.4). This uncertainty from the pulse amplitude can be minimized by using a CFD. The CFD works by dividing the pulse into two parts. It then inverts one part and delays the other part by half of the pulse width and then adds them together. One can then use the zero crossing point as the arrival time, significantly reducing the influence of the pulse amplitude. By using a CFD instead of leading edge discrimination, the timing jitter from this step can be reduced from 1 ns to ~50 ps (Birch and Imhof 1999). A CFD is also used on the synchronization signal to minimize timing jitter for the reference pulse.

**Figure 3.3** - TCSPC flow chart. The sample is excited and fluorescence is emitted. A CFD determines the emission photon arrival time and another CFD determines the synchronization pulse arrival time. A TAC begins charging when the emission photon arrives, and stops when the synchronization pulse arrives. This pulse is sent to an ADC which digitizes the signal. The photon is then placed in the proper time bin of a histogram. Modified from (Lakowicz 2006).
**Figure 3.4** - Threshold discrimination. a) Leading edge discrimination introduces timing jitter because of the variable pulse height originating from individual photons. b) Constant fraction discrimination avoids this problem by dividing the pulse into two parts. A fraction of the pulse is inverted and the other part delayed, then the two are recombined. The zero crossing point is then used as the arrival time, minimizing timing jitter. Modified from (Lakowicz 2006).

Following determination of the precise arrival time for both the fluorescence photon and the synchronization pulse, the signals are directed to a time-to-amplitude converter (TAC). The TAC generates a voltage proportional to the time between the excitation pulse and the fluorescence photon by charging a capacitor. Typically, the TAC is the rate-limiting component in the TCSPC device, because it has to reset every time it discharges. In order to minimize the effect of this reset time (also known as the dead time), the TAC is typically operated in reverse mode, which means that it starts charging when a photon pulse arrives and discharges when the next synchronization pulse arrives (Bowman, Berglund et al. 1993). By operating the TAC in reverse mode, it does not have
to discharge after every synchronization pulse (for this work, every 12.5 ns). Instead, it only has to reset after each fluorescence photon, which is significantly less frequent.

After the TAC, the photon has now been converted to a voltage which corresponds to the arrival time of the photon. The last component is the analog-to-digital converter (ADC), which digitizes the signal for the computer to place in the correct time bin of the histogram. This process then repeats until acquisition is complete.

### 3.2.4 Generation of the synchronization pulse

To properly place a fluorescence photon in the histogram, the time it arrives must be compared to the time at which the fluorescence was excited. This is typically done in one of two ways.

1. In many modern ultrafast pulsed lasers, there is an electronic output which outputs a pulse train that is synchronized with the optical pulses that it outputs. In this case, the electronic output of the laser is simply connected to the synchronization port in the TCSPC device.

2. If the laser does not have an electronic output, or the signal is not of the proper amplitude, a small portion of the laser pulse can be directed to a fast photodiode. This photodiode then outputs an electrical pulse for each optical pulse and is connected to the TCSPC device.

Method #2 is utilized in this thesis, because the laser synchronization output does not have a large enough amplitude signal to be used by the TCSPC card.
3.3 Implementation of TCSPC in a two-photon AOSLO

I implemented TCSPC in the two-photon AOSLO for mouse, described in Chapter 2, and later in a two-photon AOSLO for primate imaging (Sharma, Schwarz et al. 2016). The hardware implementation in an existing AOSLO is straightforward. The changes made to the system are shown in Figure 3.5. The existing femtosecond laser for two-photon excitation works well for TCSPC due to its high repetition rate and short pulses. The hybrid PMT detector can either replace the existing detector or a flip mirror can be used to direct light to the hybrid PMT (HPM-100-40; Becker & Hickl, Berlin, Germany). I chose to use the flip mirror approach so that it was easy to swap between TCSPC collection and typical analog detection for other experiments. The TCSPC card (SPC-160; Becker & Hickl) is a PCI card and therefore is connected to an open PCI port on the acquisition computer. Finally, a portion of the excitation light is directed to a photodiode for the synchronization signal.
Figure 3.5 - System diagram with TCSPC. To implement TCSPC in the AOSLO, several changes are made. The PMT detector is swapped out for a hybrid PMT with low timing jitter, and a portion of light is directed straight from the laser to a photodiode. The TCSPC card is connected as shown. Hardware changes to incorporate TCSPC into the base AOSLO system are outlined in red.
3.4 Fitting TCSPC data

3.4.1 Fitting the exponential decay

Once the data has been collected with the TCSPC technique, it will look something like the histogram in Figure 3.6. To determine the fluorescence lifetime(s) present in the decay, the data must be fit. The fluorescence from a collection of identical molecules is known to decay exponentially, in the form of:

\[ F(t) = F_0 e^{-t/\tau} \]

Where \( F_0 \) is the initial fluorescence at time \( t = 0 \) and \( \tau \) is the fluorescence lifetime. If there is more than one type of molecule fluorescing or multiple configurations of the molecule (ex. the molecule is bound to an enzyme, changing its structure), the fluorescence decay can be described by a sum of exponentials with different contributions:

\[ F(t) = F_0 \sum_i a_i e^{-t/\tau_i} \]

Where now \( a_i \) corresponds to the relative contribution of each fluorescence lifetime component to the overall decay, and \( \tau_i \) are the fluorescence lifetimes. The fit requires a higher signal-to-noise ratio (SNR) and often improves only marginally as one continues to add terms. For this thesis, a double exponential fit was frequently used. With a double exponential fit, the mean fluorescence lifetime, \( \tau_m \), can be calculated by:

\[ \tau_m = a_1 \tau_1 + a_2 \tau_2 \]
Because the full exponential decay is fit with these two terms, $a_1 + a_2 = 1$.

**Figure 3.6** - Data fit. Each red dot represents the number of photons present in that time channel. The data is fit using a nonlinear least squares fitting process. The lifetime parameters can then be extracted.

### 3.4.2 Nonlinear least squares fitting

In a real data set, the histogram data is fit to a sum of exponentials as described in the previous section. When the lifetime components are unknown, as is often the case in biological samples, both the relative contributions and the lifetime values themselves must be determined. The most common method of fitting this data is to use a nonlinear least squares fit. This method requires a large photon count so that the uncertainty has a Gaussian distribution, but it allows multi-exponential decays to be resolved. Least squares analysis is appropriate when the following assumptions are met (Johnson 1994):

1. The experimental uncertainty only exists in the dependent variable (in this case, the photon count).
2. The uncertainty in the dependent variable has a Gaussian distribution centered on the correct value.

3. There are no systematic errors in either the dependent or independent variable.

4. The fitting function is the correct mathematical model (for TCSPC data this is an exponential decay model).

5. The data points are independent observations.

6. There is a sufficient number of data points such that the parameters are overdetermined (i.e. more data points than the number of parameters being fit). In practice, there must be significantly more data points than the minimum in order to reduce uncertainty in the fit.

TCSPC data generally satisfy these 6 assumptions; therefore, least squares analysis is appropriate. In least squares analysis, the goal is to determine parameter values which provide the best fit between the data, \( N(t_i) \), and the model, \( N_c(t_i) \). This is done by minimizing the goodness of fit parameter, given by (Lakowicz 2006):

\[
\chi^2 = \sum_{i=1}^{n} \frac{1}{\sigma_i^2} [N(t_i) - N_c(t_i)]^2 = \sum_{i=1}^{n} \frac{[N(t_i) - N_c(t_i)]^2}{N(t_i)}
\]

where \( n \) is the number of time channels and \( \sigma_i \) is the standard deviation in the \( i \)th time channel. Because the photon counts follow a Poisson distribution, the standard deviation is equal to the square root of the number of photon counts (\( \sigma_i = \sqrt{N(t_i)} \)). \( \chi^2 \) is not normalized based on the number of data points (time channels); therefore the reduced \( \chi^2 \), or \( \chi_R^2 \), is typically used. The reduced \( \chi^2 \) is simply
where \( p \) is the number of floating parameters. The \( \chi_R^2 \) is typically close to 1 when the model fits the data well. If it is significantly higher than 1, then the model likely needs to be modified (i.e. add exponential terms) or there are systematic errors unaccounted for (IRF not calibrated properly, scatter within the system, etc.).

### 3.4.3 IRF determination

The IRF of a TCSPC system can be determined by measuring the photons originating from an instantaneous process initiated by the same light source as the fluorescence to be measured. Common ways of measuring the IRF include detecting a reflection in place of the fluorescence sample or a material which emits photons via second harmonic generation (SHG). In the AOSLO described in Chapter 2, reflected light from the excitation laser is prevented from reaching the detector by a dichroic mirror just outside the eye. SHG has half the wavelength of the excitation light, and is therefore reflected to the detector by this dichroic mirror. I used SHG generated from urea crystals (Sigma Aldrich Corp., St. Louis, Missouri, USA), which are known to produce high SHG signal (Kurtz and Perry 1968). The urea crystals were placed on a microscope slide and placed at the focus of the model eye. From this, I found that the IRF of the system was \( \sim 80 \) ps (Figure 3.7). I then used this IRF to de-convolve the data from the fluorescence lifetime measurements prior to curve fitting.
Figure 3.7 – SHG signal from urea crystals. By measuring the decay of an instantaneous process such as SHG, the IRF of the system can be determined. The urea signal was fit with a single exponential and the IRF was ~80 ps.

3.4.4 TCSPC calibration

As with any measurement system, it was necessary to calibrate the TCSPC system to ensure accuracy. For the calibration, the fluorescence decay of Rhodamine B (Sigma Aldrich Corp.) dissolved in methanol was measured. When fit to a single exponential decay curve, the measured lifetime of Rhodamine B was $2.54 \pm 0.25$ ns (mean \pm standard deviation), consistent with the results of a multi-laboratory study designed to establish fluorescence lifetime standards, which found a fluorescence lifetime of $2.5 \pm 0.1$ ns (Boens, Qin et al. 2007).

3.4.5 Phasor representation

TCSPC data can be analyzed by fitting the fluorescence decay at each pixel to an exponential function, as described in section 3.4.1, but this is not the only way to analyze fluorescence lifetime data. Another is the phasor approach. This approach transforms the
TCSPC histogram to a vector, which is then represented on a polar plot (Digman, Caiolfa et al. 2008). This technique can help to visualize fluorescence lifetime data, because molecules with different fluorescence lifetimes will separate on the phasor plot (Figure 3.8). Additionally, the phasor plots can be used to identify the number of fluorescent species in a fluorescence lifetime image – two fluorescent species will form a line on the phasor plot, while three will form a triangle. Phasor plots were not utilized heavily in the analysis of data in this thesis, but an exploratory dive was taken in analyzing a subset of the data in chapter 5.

![Figure 3.8](image)

**Figure 3.8** – Illustration of phasor plot principles. Fluorescence exhibiting a fast decay will lie on the half circle nearer to the x-axis. Fluorescence exhibiting a slow decay will lie on the half circle nearer to the y-axis. Fluorescence which is a combination of a fast and slow decay will be along a line connecting the two pure species, with the position on the line related to the relative contributions from the fast and slow component. Modified from “New SPCImage Version Combines Time-Domain Analysis with Phasor Plot” application note, Becker & Hickl GmbH.
3.5 Summary

This chapter provided an introduction to the principles and techniques for measuring the fluorescence lifetime. The TCSPC method is the superior choice for implementing AOFLIO in the two-photon AOSLOs due to its sensitivity and ease of implementation with the existing system hardware. The next several chapters will describe the first results with the AOFLIO technique.
Chapter 4

Two-photon fluorescence lifetime imaging of exogenous fluorophores in living mice
4.1 Introduction

Fluorescence lifetime imaging of the retina can detect changes early in disease progression (Schweitzer, Hammer et al. 2004, Schweitzer, Deutsch et al. 2015). By combining fluorescence lifetime imaging with adaptive optics two-photon ophthalmoscopy, it is now possible to interrogate fluorophores involved in retinal function at a cellular scale. This may allow for new measures of cell health which can monitor changes to the retina on a fine scale.

Deployment of new fluorescent sensors that correlate with cellular parameters is of great interest, particularly in mouse models, where the sensor can be used to measure changes compared to normal when a specific manipulation has been made. Of particular interest to this work is a sensor called Peredox, which changes its fluorescence lifetime depending on the ratio of NAD$^+$: NADH in the cytosol of the cell, making it a useful sensor for evaluating the redox state of the cell. The fluorescence lifetime can be translated to an NAD$^+$: NADH ratio using a dose-response curve (Mongeon, Venkatachalam et al. 2016). For the first study, the fluorescence lifetime of Peredox-labeled inner retinal cells was measured in normal mouse retina. The goal of this study was to establish a baseline by which measurements made in potentially unhealthy cells could be compared.

For the initial implementation of AOFLIO, mice were the species chosen due to the availability of mice (either transgenic models or through intravitreal injection) which express bright fluorophores in specific cells of the retina. This provides two advantages:
high two-photon fluorescence signal and known fluorescence lifetimes of the chosen fluorophores. Because the fluorescence decay must be resolved, fluorescence lifetime measurements require more captured photons than an intensity measurement. The two-photon absorption is proportional to the numerical aperture to the fourth power (Denk, Strickler et al. 1990). Therefore, the higher numerical aperture of the mouse eye when compared to the primate eye (0.49 vs. 0.25) can provide a much higher fluorescence signal. The use of extrinsic fluorophores with high quantum efficiency further enhances the signal, making this preparation an ideal first step for AOFLIO. Prior to measuring a sensor with an unknown fluorescence lifetime (at least in in vivo retinal neurons), studies with two fluorophores of known fluorescence lifetime (EGFP and sodium fluorescein) were conducted. Unlike with Peredox, there was no expectation that these fluorophores would experience a change in fluorescence lifetime based on intracellular parameters. The purpose of these initial studies was to validate the AOFLIO measurements prior to use with a fluorescent sensor.

4.2 Methods

4.2.1 Animal preparation

Adult Thy1-EGFP (n = 3) and C57BL/6J (n = 2) mice from 1-4 months of age were used in this study (The Jackson Laboratory, Bar Harbor, Maine, USA; stock #007788 and #000664, respectively). Thy1-EGFP mice are bred on a C57BL/6J background. For the Peredox study, mice were intravitreally injected with a viral vector, AAV2/2-CAG-Peredox, obtained from the Cepko lab at Harvard University. EGFP- or
Peredox-labeled inner retinal cells, primarily ganglion cells, were identified using low resolution fundus imaging (Micron IV; Phoenix Research Labs, Pleasanton, California, USA or Spectralis HRA + OCT; Heidelberg Engineering, Heidelberg, Germany). Mice were anesthetized with Ketamine/Xylazine injection (100 mg/kg and 50 mg/kg, respectively). For high resolution in vivo imaging, their pupils were dilated with a drop each of tropicamide (1%; Akorn, Inc., Lake Forest, Illinois, USA) and phenylephrine (2.5%; Akorn, Inc.) and mice were placed in a bite bar mount with a heating pad and further anesthetized with isoflurane gas (1-2%) supplemented with 98% oxygen. The details of this imaging protocol have been described fully in an earlier publication (Geng, Dubra et al. 2012). Both C57BL/6J mice, but only 1 of the Thy1-EGFP mice were injected intraperitoneally with 0.1 mL of sodium fluorescein (10%, diluted with saline at a three-to-one ratio, for a final concentration of 3.3%; Akorn, Inc.). During imaging, each mouse was placed with its pupil at the exit pupil of the system. All experiments were approved by the University Committee on Animal Resources at the University of Rochester.

4.2.2 Image acquisition

The optic nerve head and vasculature present in the wide field fundus images were used to navigate to the location of interest in the AOSLO. Fluorescently-labeled cells which showed strong fluorescence and were near distinct vascular patterns were chosen for imaging. Two-photon fluorescence was excited with 910 nm light for EGFP and fluorescein, and 750 nm light for Peredox measurements. Power of the two-photon
excitation source was 7 mW or less measured at the mouse pupil. The excitation source for two-photon fluorescence was co-focused with the reflectance light source. The sources were focused to the uppermost vascular layer, just above the ganglion cell layer (Kornfield and Newman 2014). The two-photon excitation source focus was then changed slightly to bring the ganglion cells into focus. All imaged locations were within 15° of the optic nerve head. Images were acquired at a frame rate of 16 Hz. Due to breathing motion of the animal, there was noticeable motion from frame to frame prior to stabilization. A high contrast reflectance video was collected for motion estimation. For the EGFP and fluorescein imaging, but not the Peredox study, an image-based tracking algorithm (Yang, Zhang et al. 2014) was used to optically stabilize the image by locking onto the reflectance signal focused on the vascular layer in the inner retina.

Fluorescence lifetime data was acquired using a time-correlated single photon counting (TCSPC) module (SPC-160; Becker and Hickl). A small portion of the energy in the pulsed excitation laser was sent to a fast photodiode (PHD-400; Becker and Hickl), which served as the synchronization signal for the TCSPC module. Photons were allocated in one of either 256 or 1024 time bins, depending on their arrival time with respect to the synchronization pulse. Images were acquired with 140 x 180 pixel resolution using SPCM software in First In First Out (FIFO) imaging mode (Becker and Hickl). In this mode, the TCSPC module transmits the photon data to the computer where it is then assigned to the correct pixel and time bin, allowing for larger data sets to be gathered than is possible with on-board storage (Becker 2014). Each image was acquired over 180 seconds.
4.2.3 Image analysis

Commercial software (SPCImage; Becker and Hickl) was used to analyze the fluorescence decay at each pixel. Images of vasculature labeled by sodium fluorescein were fit to a model with a single exponential decay (Figure 4.1(a)), chosen due to negligible improvement in goodness of fit with additional terms:

\[ F(t) = F_0 e^{-t/\tau} \]

where \( F(t) \) is the fluorescence intensity at time \( t \) after the excitation pulse, \( F_0 \) is the initial fluorescence intensity, and \( \tau \) is the fluorescence lifetime. Vessels were manually outlined in SPCImage software and the lifetime calculated for each pixel within a vessel. The average lifetime of all pixels in the vessel was then assigned as the lifetime for that vessel, and the overall average lifetime was a weighted average of the lifetimes of all vessels.

Cells labeled with EGFP were fit with a double exponential decay model (Figure 4.1(b)), as found in a previous study (Hess, Sheets et al. 2003):

\[ F(t) = F_0 \times (a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}) \]

where \( a_1 \) and \( a_2 \) are the contributions of the fast and slow lifetimes (\( a_1 + a_2 = 1 \)), and \( \tau_1 \) and \( \tau_2 \) are the corresponding lifetimes. Regions of interest were drawn around the cells and lifetimes for each pixel were calculated in SPCImage software. Lifetime values \( \tau_1 \) and \( \tau_2 \) were averaged in Matlab (Mathworks, Inc., Natick, Massachusetts, USA) across
all pixels in the region of interest for each image. The mean fluorescence lifetime, $\tau_m$, was calculated by:

$$\tau_m = a_1 \tau_1 + a_2 \tau_2$$

In all displayed images, 5x5 pixels were binned around the pixel being calculated in order to increase the SNR of calculated lifetimes. The fluorescence lifetime for Peredox was measured in the same way as EGFP, since it also exhibits a double exponential decay (Mongeon, Venkatachalam et al. 2016). In all analysis, an incomplete exponential decay model was used to account for long-lasting fluorescence. With this fitting, the model considers that the fluorescence decay may not reach 0 before the next pulse arrives.

Figure 4.1 – Example histograms and fits for (a) fluorescein in vasculature, and (b) an EGFP-labeled cell. The red open circles correspond to the number of photons collected for each time bin. The blue dashed line shows the double-exponential fit to the data. The black solid line is the IRF of the system, which is de-convolved from the data. This fit was performed at each pixel within the image which met the threshold criteria (see section 4.3.1).
4.2.4 Statistical Analysis

A Wilcoxon rank sum test was used to test for differences in the fluorescence lifetime of fluorescein between animals, and for differences in $\tau_1$, $\tau_2$, and $\tau_m$ of EGFP between animals. Analysis of variance (ANOVA) was used to compare Peredox fluorescence lifetime values between mice and in the same mouse on different days. A significance value of $<0.05$ was used for all tests. The statistical analysis was completed in Matlab (Mathworks, Inc.).

4.3 Results

4.3.1 Determination of photon threshold for fluorescence lifetime calculation

A photon threshold was set for fluorescence lifetime calculation in AOFLIO images in order to ensure adequate SNR. Pixels which had less than the required number of photons were displayed in black. Photon threshold for lifetime calculation was based mainly on two factors: number of time channels acquired and number of exponentials fit to the decay. For a single exponential decay, a simulation study found that, for a 256 time channel histogram, ~500 photons are required to achieve $<5\%$ error in lifetime calculation using SPCImage (Walsh, Sharick et al. 2016). I have found that the accuracy of the fit is most heavily influenced by the number of photons in the time channel with maximal number of photons (more photons results in a higher signal-to-noise ratio in each channel). Therefore, since I used 1024 time channels for the images of sodium fluorescein in vasculature, I decided to scale the threshold fourfold to 2000 photons. In
general, the fluorescein fluorescence was very bright and resulted in far more photons than the threshold required.

For the images of EGFP, I used 256 time channels. The study referenced above found that approximately 4185 photons were needed to achieve a cumulative error (sum of the errors of $\tau_1$, $\tau_2$, $a_1$, and $a_2$) $<25\%$ for a similar fluorophore (high $a_1$ and $\tau_2 - \tau_1 > \sim 2$ns in their case. Because of the low efficiency of two-photon fluorescence imaging through the pupil of the living mouse eye, I relaxed this constraint down to 2000 photons without substantially reducing the accuracy of the lifetime fit (Walsh, Sharick et al. 2016).

In order to collect the required number of photons in each pixel, I binned the decays from neighboring pixels rather than increase the image acquisition time. An analysis of the necessary binning factor is illustrated in Figure 4.2. A very low threshold of photons to calculate the lifetime was set in Figure 4.2 in order to show the inaccuracy of the fluorescence lifetime calculation when too few photons are present in the decay. With no binning (Figure 4.2(b)), there were too few photons in each pixel to resolve a double exponential decay, resulting in an underestimation of the cell’s fluorescence lifetime and a large uncertainty. By binning each pixel with its 8 nearest neighbors (3 x 3 binning, Figure 4.2(c)), the distribution of calculated lifetimes narrowed, and the mean lifetime increased to within the expected range. However, again due to the number of photons in each pixel, the fitting routine had challenges calculating $\tau_1$ and $\tau_2$, underestimating the former and overestimating the latter. By going to the 5 x 5 pixel bin (Figure 4.2(d)), the distribution of lifetimes calculated within the cell further narrowed.
The brightest pixel in the image now had over 5000 photons, greater than the 4185 photons found necessary to achieve <25% error (Walsh, Sharick et al. 2016). As can be seen in Figure 4.2(b), (c), and (d), binning does reduce the spatial resolution of the lifetime image. However, there is still subcellular resolution in the lifetime image, and there is no binning in the intensity image, therefore maintaining its resolution.

**Figure 4.2** - Binning analysis of the EGFP-labeled cell from Figure 4.4. Panel (a) shows the two-photon fluorescence intensity image of the cell. Panels (b), (c), and (d) show fluorescence lifetime images of the cell with no binning, 3 x 3 binning, and 5 x 5 binning, respectively. A very low threshold of photons was set in order to show the inaccuracy of the fluorescence lifetime calculation when too few photons are present in the decay. The black outline in panels (b), (c) and (d) indicates the boundaries of the cell, with pixels inside the outline containing greater than 2000 photons with 5 x 5 binning. Panel (e) shows histograms of the lifetime calculated at each pixel with the different binning factors shown in (b), (c), and (d). As the binning factor increases, the lifetime fit converges on the expected lifetime of EGFP. Scale bar is 5 µm.
4.3.2 Two-photon fluorescence lifetime imaging of sodium fluorescein in retinal vasculature

Prior to administration of sodium fluorescein, the background fluorescence level was very low (<0.02 photons/pixel/s) due to the excitation wavelength and emission filters chosen. Following injection, sodium fluorescein was present in retinal vasculature as seen in the two-photon fluorescence image shown in Figure 4.3(a). A subfield of the two-photon fluorescence image was collected using TCSPC (Figure 4.3(b)) and the fluorescence lifetime calculated for each pixel in the subfield (Figure 4.3(c)). Vessels were then segmented manually and an average fluorescence lifetime calculated for the vessel. The mean fluorescence lifetime measured over 80 vessels was 3.21 ± 0.06 ns (Table 4.1). There was no significant difference in fluorescence lifetime measured across 2 mice (51 vessels in one mouse and 39 vessels in another; p = 0.57) or in repeated measurements in the same mouse (35 vessels in the first session and 16 in the second; p = 0.14).

Figure 4.3 - Images of a capillary bed in the mouse inner retina. Panel (a) is a two-photon fluorescence intensity image acquired with a standard, analog PMT. Panel (b) is an image of a sub-portion of the location from panel (a) acquired with the single photon counting detector and the TCSPC system. The pixel density in this image is reduced
compared with panel (a), however the structure of the capillaries is still visible. Panel (c) is a fluorescence lifetime image of the same location as panel (b). The fluorescence lifetime image is mostly uniform because the fluorescence lifetime is robust against intensity variations. Scale bar is 25 µm.

Table 4.1 - Fluorescence lifetime values for EGFP and sodium fluorescein measured with AOFLIO

<table>
<thead>
<tr>
<th></th>
<th>Number of mice</th>
<th>Number of measurements</th>
<th>$\tau_1 \pm SD$ (ns)</th>
<th>$\tau_2 \pm SD$ (ns)</th>
<th>$\tau_m \pm SD$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>2</td>
<td>80 vessels</td>
<td>N/A$^a$</td>
<td>N/A$^a$</td>
<td>3.21 ± 0.06</td>
</tr>
<tr>
<td>EGFP</td>
<td>3</td>
<td>7 cells</td>
<td>1.43 ± 0.28</td>
<td>3.01 ± 0.37</td>
<td>2.28 ± 0.99</td>
</tr>
</tbody>
</table>

$^a$Fluorescein data was fit with a single exponential model.

4.3.3 Two-photon fluorescence lifetime imaging of EGFP-labeled ganglion cells

Thy1-EGFP mice express EGFP in a sparse (<10%) subset of retinal ganglion cells (Figure 4.4(a)) (Barnstable and Dräger 1984, Feng, Mellor et al. 2000). Expression and location within the mouse retina were confirmed with low resolution fundus imaging, which was also used for navigation in the small field of view AOSLO instrument. Cell somas provided the highest fluorescence signal and were often identifiable in a single frame. The SNR of a typical cell soma compared to the background noise, including autofluorescence, was ~50. The fluorescence emitted from cell somas exhibited a double-exponential decay, with the two components $\tau_1 = 1.43 \pm 0.28$ ns, and $\tau_2 = 3.01 \pm 0.37$ ns (Table 4.1). The mean lifetime was 2.28 ± 0.99 ns, and was consistent throughout the soma of the cell (Figure 4.4(c)). There was no significant difference in $\tau_1$, $\tau_2$, or $\tau_m$ across 7 cells in 3 mice ($p = 0.7$, 0.8, 0.4, respectively). The fluorescence signal from axons was much lower, and they became visible only after summing a large number of
frames. Fluorescence emitted from dendrites and axons did not yield the required number of photons to calculate lifetime.

Figure 4.4 - Images of an EGFP-labeled ganglion cell in a Thy1-EGFP mouse retina. Panel (a) shows sparse EGFP labeling of cells and axons in the mouse retina. The blue box denotes the region of AOFLIO imaging which is shown in panel (b). Panel (b) is a two-photon fluorescence intensity image of a ganglion cell soma. The same fluorescent axon can be seen in panels (a) and (b). Panel (c) is a fluorescence lifetime image of the same location. The lifetime was consistent throughout the soma of the cell, despite the intensity of fluorescence changing throughout. The fluorescence lifetime was not calculated in the axon because it did not reach threshold criteria. Scale bar is 25 µm.

4.3.4 Simultaneous two-photon fluorescence lifetime imaging of EGFP-labeled ganglion cells and sodium fluorescein

A Thy1-EGFP mouse was first placed in the system and a ganglion cell imaged to measure the fluorescence lifetime. The mouse was then injected with sodium fluorescein. In the resulting fluorescence image, fluorescence originated from both the EGFP in the ganglion cell and the sodium fluorescein in the surrounding capillary bed (Figure 4.5(c)). The cell was indistinguishable in the fluorescence intensity image, and could not be spectrally filtered because EGFP and sodium fluorescein have nearly identical emission spectra (Figure 4.5(b)) (Spiess, Bestvater et al. 2005, Lakowicz 2006). Because of their
different fluorescence lifetimes, I was able to identify the cell using fluorescence lifetime imaging (Figure 4.5(d)). The cell was most easily identifiable by looking at the contributions from the fast and slow portions of the fluorescence lifetime when $\tau_1$ and $\tau_2$ were set to the known lifetimes (from previous measurements in sections 4.3.2 and 4.3.3) of EGFP and sodium fluorescein, respectively. Because the long lifetime component of EGFP was similar to the lifetime of fluorescein fluorescence, I considered a two-component fit. The mean lifetime for each pixel was then displayed, and showed a lower value in the region of the cell. By multiplying the intensity image ($I(x, y)$) by the contribution from the fast ($a_1(x, y)$) or slow ($a_2(x, y)$) lifetime at each pixel two images can be obtained. The image containing the fast lifetime component results in the cell becoming visible (Figure 4.5(e)), whereas the image containing the slow lifetime component improves the contrast of the vessel, due to reduced contribution from the cell (Figure 4.5(f)).
Figure 4.5 - Images of an EGFP-labeled cell surrounded by a bed of capillaries with fluorescein. Panel (a) shows the wide field image of the ganglion cell and its axon connecting to the optic nerve. Panel (b) shows the overlapping emission spectra for EGFP (purple dashed line) and fluorescein (red solid line), acquired from Chroma Technology Corp. Panel (c) shows the two-photon fluorescence intensity image; the cell is indistinguishable from the vessels. Panel (d) shows the fluorescence lifetime image. The EGFP fluorescence from the cell exhibits a lower fluorescence lifetime than the fluorescein, revealing the location of the cell. Panels (e) and (f) are intensity images which have been scaled by $a_1(x, y)$ (the contribution of the fast lifetime component) or $a_2(x, y)$ (the slow lifetime component), respectively. The fast lifetime component corresponds to EGFP fluorescence, causing the cell to appear in (e), while the slow lifetime component corresponds to the fluorescein, suppressing the cell in (f) and improving the vessel contrast. Scale bar is 100 µm for panel (a) and 15 µm for panels (c) – (f).

4.3.5 Two-photon fluorescence lifetime imaging of Peredox-labeled inner retinal cells

The mice intravitreally injected with Peredox exhibited variable expression of the fluorophore. The fluorescence lifetime of Peredox was measured using AOFLIO in four mice that showed sufficient expression in the wide field instrument. Two of these mice
were also imaged one week later to determine the stability of the measurements in normal mice. In the six imaging sessions (four mice on day one and two of the four mice on day eight), the average value recorded for $\tau_m$ was $1.70 \pm 0.26$ ns. The average $\tau_m$ value for each individual mouse on both imaging days are shown in Figure 4.6. I found a significant difference when comparing the fluorescence lifetime between different mice (ANOVA, $p = 0.005$), but not when comparing the same mouse across imaging days (ANOVA, $p = 0.21$). The average values for individual fluorescence lifetime parameters were $\tau_1 = 0.89 \pm 0.36$ ns, $\tau_2 = 3.52 \pm 0.62$ ns, and $\alpha_1 = 64 \pm 7\%$.

**Figure 4.6** – $\tau_m$ values of Peredox in four normal, wild type mice. The fluorescence lifetime of Peredox was measured in labeled inner retinal cells in four mice. Two of the mice were imaged one week later to determine the stability of the measurements. Measurements were significantly different between mice ($p = 0.005$), but not in the same mouse 1 week later ($p = 0.21$).
4.4 Discussion

The mouse eye provides an ideal testbed for AOFLIO. Previous reports of FLIO have mostly focused on the human eye, but due to the low numerical aperture of the eye, high absorption of blue light by the crystalline lens, and lack of adaptive optics, it has been challenging to isolate fluorescence from single cells in the retina, and correction factors have had to be made for fluorescence from the crystalline lens (Schweitzer 2005, Klemm, Schweitzer et al. 2015). The advantage in focusing power provided by the high numerical aperture of the mouse eye, as well as the use of adaptive optics to optimize the point spread function, allows individual cells to be imaged. Based on previously reported axial resolution in a mouse AOSLO, I estimate the axial resolution of the system to be ~18 µm (Geng, Dubra et al. 2012). The thickness of the nerve fiber layer in the wild type mouse was found to be 19 µm on average (Ferguson, Dominguez Ii et al. 2013). As light is focused in the ganglion cell layer, fluorescence contributions are reduced from the lens or other retinal layers interfering with the fluorescence signal.

Adaptive optics image quality in the mouse can vary drastically between imaging sessions. Because the fluorescence lifetime is less affected by these parameters (for example, it is largely independent of fluorescence intensity or fluorophore concentration (Berezin and Achilefu 2010)), it may be less dependent on image quality than measures which depend on the optical quality (ex. Image-based metrics), an important factor for longitudinal imaging studies. In Figure 4.3(b), a slight motion blur can be seen in the vessel. The number of photons outside of the vessel, which may be due to the motion blur
or out of focus fluorescence from another vessel layer, is far fewer (~3x greater in the vessel), but the lifetime calculated is very similar. In addition, fluorescence lifetime imaging can provide quantitative measurements, rather than relying on relative changes as is the case in many ratiometric fluorescence techniques. In this study, I found no differences in fluorescence lifetime across cells (in the case of EGFP) or vessels (in the case of fluorescein), despite differences in image quality between images.

The fluorescence lifetime of EGFP that I measured in vivo was comparable to that previously measured in vitro by other groups (Pepperkok, Squire et al. 1999, Hess, Sheets et al. 2003). The lifetime of fluorescein that I measured was lower than the ~4 ns reported by other groups (French, So et al. 1997, Magde, Wong et al. 2002). I speculate that this is due to the high concentration of sodium fluorescein used in this study, which can result in self-quenching, which occurs when the energy of an excited state molecule is transferred to a non-fluorescent trap during the process of resonance energy transfer (Lakowicz, Malicka et al. 2003). This is a well-known property of fluorescein which can cause reduced quantum yield and fluorescence lifetime.

The fluorescence lifetime of Peredox that I measured in inner retinal cells is similar to that measured in neurons in mouse hippocampal brain slices (1.70 ± 0.26 ns vs ~1.75 ns; (Mongeon, Venkatachalam et al. 2016)). It is much lower than the fluorescence lifetime measured in astrocytes in brain slices (~2.1 ns; (Mongeon, Venkatachalam et al. 2016)). This would suggest that the cells we measured have NADH levels more similar to neurons than astrocytes in the brain. Previous work has shown that NADH levels increase when neurons undergo stimulation (Díaz-García, Mongeon et al. 2017). Therefore, any
future measurements using Peredox in retinal neurons should consider the likely increase in Peredox fluorescence lifetime as a result of visual stimulation. It may be possible to identify on- and off-ganglion cells via opposite direction changes in fluorescence lifetime upon visual stimulation.

As compared with two-photon fluorescence imaging, where images with greater than 500 pixels in each linear dimension can be collected in the system, resolution is lost when acquiring fluorescence lifetime images. A consequence of the fast scanning rates (resulting in pixel dwell times of ~25 ns for a 500 pixel linear dimension), is that the AOFLIO images must be downsampled. This is because the acquisition software did not allow for shorter than a 100 ns pixel clock when generated internally, and if I provide my own external pixel clock, it alone saturates the TCSPC card data transfer to the computer, preventing photon data from being transferred and sometimes crashing the acquisition program. This downsampling results in images with only 140 x 180 pixel resolution. At this pixel resolution, the pixel size is approximately the same size as the diffraction-limited spot size, meaning that I am sampling below the Nyquist limit in this implementation. By reducing the number of pixels acquired, there is a higher probability of a photon being detected in each pixel, leading to a higher number of photons per pixel. This allows for a higher SNR for the lifetime decay calculated for each pixel. In addition, spatial binning of the data improves the accuracy of the lifetime calculation, but reduces the spatial resolution of the lifetime image by introducing blur. Alternatively, it is possible to increase the number of photons in each pixel by lengthening the image
acquisition time. However, I chose to limit the acquisition to 180 seconds due to concerns about photodamage from increased exposure time.

AOFLIO is well-suited to image fluorophores typically encountered in retinal imaging; their lifetimes range from ~0.5 ns (free NADH) to ~4 ns reported previously for measurements of fluorescein (Magde, Wong et al. 2002). With an IRF of only ~70 ps, a lifetime of 0.5 ns can be easily measured. The upper bound of measurement is 12.5 ns, which corresponds to the period of the pulsed laser used for fluorescence excitation. It is important to note that the true upper bound of measurement is probably much lower, as the fluorescence has only decayed to its 1/e value at the time calculated to be the fluorescence lifetime – therefore, a fluorescence lifetime near the pulse period of the laser would result in contributions to the fluorescence from previous excitation pulses. To mitigate this effect, I used an incomplete exponential decay model (Leung, Yeh et al. 2011).

As demonstrated, I showed it is possible to distinguish fluorophores in the retina with similar spectra via their fluorescence lifetime. This has great potential as the number of spectrally separable channels in visible fluorescence is limited by the spectral specificity and overlap of a fluorophore with its spectral neighbors. This is particularly important for imaging the outer retina, where both NADH and fluorescent retinoids reside. These molecules have similar excitation and emission spectra, making it challenging to separate them with traditional fluorescence imaging (Chen, Tsina et al. 2005). The ability to separate fluorescence contributions from these fluorophores by their lifetime could help mitigate confounding factors in studies of the visual cycle involving
retinoid fluorescence (Sharma, Schwarz et al. 2016) or prospective studies of photoreceptor health. As with intensity-based imaging methods, differentiating fluorophores based on lifetime requires that the emitted fluorescence intensities be of similar order of magnitude, otherwise the signal from the stronger fluorophore would overwhelm any signal from the weaker fluorophore.

In this chapter, I combined two-photon adaptive optics scanning light ophthalmoscopy with FLIO. In comparison to existing literature (Schweitzer, Hammer et al. 2004, Dysli, Dysli et al. 2014, Schweitzer, Deutsch et al. 2015, Dysli, Wolf et al. 2016, Dysli, Berger et al. 2017), I demonstrated two advantages. By using adaptive optics, I was able to correct the aberrations induced by the eye and provide a tighter focus both laterally and axially, allowing single cells to be resolved. In addition, the use of two-photon fluorescence provides a clear path to translation to the primate eye, which blocks nearly all light below 400 nm (Dillon, Zheng et al. 2000). This capability will allow AOFLIO of fluorophores involved in cellular processes that cannot be excited with single photon excitation (Hunter, Masella et al. 2011, Sharma, Williams et al. 2016).

Conversely, single photon FLIO using a modified clinical device is able to image a 30° field of view, providing it with the ability to rapidly assess large-scale changes in the retina.

The ability to measure fluorescence lifetime on a cellular scale in the living eye provides a sensitive measure of cell health. This technique could be used to evaluate vision restoration therapies such as stem cells or antioxidant therapy. By measuring the fluorescence lifetime, it may be possible not only to determine whether cells are alive, but
also whether they are functioning normally. This could be done using fluorescently labeled molecules or by measuring the fluorescence lifetime of intrinsic fluorophores such as NADH. AOFLIO with intrinsic fluorescence is likely to require longer acquisition times due to lower concentrations and reduced fluorescence efficiency of these fluorophores.

4.5 Conclusion

In this first implementation of AOFLIO, I demonstrated its functionality in imaging several extrinsic fluorophores in the retina. By utilizing exogenous fluorophores that can be inserted in the mouse retina, I was able to increase the specificity by targeting specific molecules. I showed that AOFLIO can be used to differentiate fluorophores that are spectrally similar due to a difference in fluorescence lifetime – an advantage that may help to differentiate endogenous fluorophores such as NADH and all-trans-retinol in future studies. Finally, I established the baseline measurements for an NADH sensor in inner retinal cells, opening the possibility of using this sensor to investigate changes in cellular metabolism that these cells may undergo upon visual stimulation or due to an unhealthy state.
Chapter 5

Two-photon fluorescence lifetime imaging of photoreceptors in macaque
5.1 Introduction

The non-human primate is a suitable animal model in which to implement two-photon AOFLIO. In addition to its anatomical similarities (fovea, similar rod: cone ratio, anterior optics), the macaque eye shares the limitation of the human eye that prevents ultraviolet light from reaching the retina (a challenge that does not exist for imaging the mouse retina). This necessitates the use of two-photon excitation to access fluorophores such as all-trans-retinol and NADH in the retina.

Fluorescence lifetime ophthalmoscopy has been used previously to study autofluorescence of the retina (in these cases the human retina), with several key limitations (Schweitzer, Hammer et al. 2004, Schweitzer, Deutsch et al. 2015, Dysli, Wolf et al. 2016, Dysli, Berger et al. 2017). All of these studies were performed using a modified scanning laser ophthalmoscope; as such, they were not able to achieve cellular resolution in their fluorescence lifetime measurements. Additionally, the fluorescence excitation source used was centered at either 448 nm or 473 nm. At these excitation wavelengths, all-trans-retinol and NADH can’t be efficiently excited. Instead, the fluorophores likely dominating the fluorescence signal in these studies include FAD and lipofuscin. To target all-trans-retinol and NADH, two-photon excitation can be used to bypass the transmission challenge present in the primate eye.

Adaptive optics scanning light ophthalmoscopy has been combined with two-photon fluorescence imaging to visualize a multitude of cell types in the living macaque retina (Sharma, Williams et al. 2016). In addition, this technique has been used to
monitor the retinoid cycle in vivo (Sharma, Schwarz et al. 2016). It has been shown that the fluorescence kinetics of rods and cones are different, with the fluorescence from cones reaching a maximum much faster than that of rods. The dominant time-varying fluorophore in the outer retina is thought to be all-trans-retinol, which is produced by reduction of all-trans-retinal following photoactivation of rhodopsin (Kaplan 1985, Chen, Tsina et al. 2005). As rods and cones show different fluorescence time dynamics in response to visual stimulation, I sought to determine whether or not these cell classes could be distinguished also by their fluorescence lifetime. In this chapter, I demonstrate AOFLIO on a cellular scale in the living macaque, and show that rods and cones have different lifetimes.

5.2 Methods

5.2.1 Animal preparation

Three male macaque monkeys (macaca fascicularis), ranging in age from 4-9 years old, were used in this study. Animals were handled according to the protocols prescribed and approved by the University of Rochester’s committee for animal research and in accordance with the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vision Research. Macaques were anesthetized with ketamine (10–20 mg/kg) and midazolam (0.25 mg/kg) and secured in a stereotaxic device in ventral recumbency. After intubation, anesthesia was maintained by inhalation of isoflurane (1-5%). To reduce eye movements, paralysis was induced with Rocuronium Bromide (200-500 μg/kg/hour), administered for up to a 6-hour period. During paralysis, respiration
was maintained by a ventilator. Mydriasis and cycloplegia were induced with 1 to 2 drops each of phenylephrine hydrochloride (2.5%) and tropicamide (1%). A rigid gas permeable contact lens, coated with Genteal (Alcon, Fort Worth, TX, USA), was placed on the cornea to maintain hydration and correct base refractive error. The eye was held open with a lid speculum, and the animal’s pupil was aligned to the exit pupil of the AOSLO.

### 5.2.2 System

An AOSLO designed for collection of two-photon fluorescence from the living non-human primate retina was modified for two-photon fluorescence lifetime imaging. This system was built by Dr. Robin Sharma as part of his doctoral thesis work and the full details can be found there (Sharma 2015). The system is largely similar to the AOSLO described in chapter 2 of this thesis, with several important differences.

The primate AOSLO has an additional PMT which captures reflectance signal from the two-photon excitation source. For this study, two-photon fluorescence was excited at 730 nm. The additional PMT allows the user to precisely determine and adjust the focus of the two-photon excitation source by observing the reflectance video at that wavelength.

A dichroic mirror (FF665-Di02; Semrock Inc., Rochester, New York, USA) diverts fluorescence from 400 nm – 665 nm to the single photon counting detector (HPM-100-40; Becker and Hickl, Berlin, Germany). Three filters, two with transmission from 400 nm – 680 nm (ET680SP-2P8; Chroma Technology Corporation, Bellows Falls,
Vermont, USA) and one which transmits from 400 nm – 550 nm (E550sp-2p, Chroma Technology Corporation), were used for fluorescence lifetime imaging.

The primate AOSLO uses a resonant scanner as the fast, horizontal scanner. As described in section 2.2.3, the mouse AOSLO uses a polygon scanner to provide a linear scan. The acquisition software in the primate AOSLO (developed in-house) performs real-time de-sinusoiding, which corrects for the sinusoidal scan in the reflectance video. Because commercial software is used to capture the AOFLIO image, there is not yet a solution to de-sinusoid the AOFLIO image. Therefore, features on the left and right edges of the image appear elongated in the horizontal direction. Images were cropped to remove the warped edges.

5.2.3 Image acquisition

Due to breathing of the animal, there was noticeable motion between frames prior to image stabilization. An image-based tracking algorithm operating in closed loop stabilizes the image by locking onto the 790 nm reflectance video focused at the photoreceptor layer (Yang, Zhang et al. 2014). The algorithm then controls a 2-axis fast steering mirror (S-334.2SL; Physik Instrumente, Karlsruhe, Germany) to optically stabilize the video. As a result, there is minimal motion in the fluorescence lifetime data, allowing averaging over many frames without post-registration.

Fluorescence lifetime data was acquired using a TCSPC module (SPC-160; Becker and Hickl). A small portion of the energy in the pulsed excitation laser was sent to a fast photodiode (PHD-400; Becker and Hickl), which was used as the
synchronization signal for the TCSPC module. Fluorescence photons were assigned to one of 256 time bins, depending on their arrival time with respect to the synchronization pulse. Images were acquired with 250 x 300 pixels using SPCM software (Becker and Hickl). A fluorescence intensity image was generated by summing all photons captured at a pixel. Each image was acquired over 150 - 180 seconds, beginning immediately upon opening of the two-photon laser shutter. The field of view used was 1.1° x 1.3°. The power of the two-photon excitation source at 730 nm was 7 mW, measured at the cornea. 19 locations in 3 monkeys from 2° - 22° were imaged and analyzed.

5.2.4 Image analysis

Although cones were masked at all eccentricities, rod data was not analyzed for less than 5°. Images were cropped to remove from the analysis the edges which were warped due to the sinusoidal scan of the resonant scanner. A threshold of 100 photons in the maximum channel was set to ensure sufficient signal-to-noise in the lifetime fit. Using a binning factor of 2, this threshold was achieved at all pixels except those which were very dark (e.g. photoreceptors underlying a large vessel). Pixels below the threshold were not analyzed.

Fluorescence lifetime data at each pixel were fit to a double exponential decay model in SPCImage (Becker and Hickl).

\[ F(t) = F_0 \times (a_1 \exp \frac{-t}{\tau_1} + a_2 \exp \frac{-t}{\tau_2}) \]
where $F_0$ is the initial fluorescence intensity, $F(t)$ is the fluorescence intensity at time $t$ after the excitation pulse, $a_1$ and $a_2$ are the contributions of the fast and slow lifetimes, and $\tau_1$ and $\tau_2$ are the corresponding lifetimes. The mean fluorescence lifetime, $\tau_m$, was:

$$\tau_m = a_1 \tau_1 + a_2 \tau_2$$

The quality of the fit was determined using the $\chi^2_R$ parameter. A $\chi^2_R$ value closer to 1 corresponds to a better fit by the model to the data. Fluorescence parameters $a_1$, $\tau_1$, $\tau_2$, $\tau_m$ and intensity for all pixels were exported from SPCImage and sent to custom software for cone masking analysis. In addition, phasor plots were generated using custom software programmed in Matlab (Mathworks, Inc., Natick, Massachusetts, USA).

### 5.2.5 Cone masking analysis

Cones were masked using custom software developed in Matlab (Mathworks, Inc.). Using the fluorescence intensity image, cones were identified and masked by placing an adjustable ellipse marking the perimeter of each cone. The mask was then applied to the corresponding fluorescence lifetime image. The radii of the ellipses masking each cone were shrunk by 20% to remove any pixels with fluorescence contribution from rods due to binning and user error when placing cone masks. The selected cone lifetime parameter was then output for each pixel in the masked image. The mask was then expanded by 20% from its original size and inverted such that only pixels from rod-populated regions were included, then output in a separate file. 20% was chosen as the optimal value for shrinking or expanding the mask as a balance between keeping as many pixels as possible, while not including pixels which may be assigned incorrectly as
a cone or rod. I found that the mean cone lifetime increased in a sample image (due to a reduction in rod pixels being incorrectly assigned as cones) when the radius was shrunk 20% before leveling off with further radius reduction (Figure 5.1). For each image location, all pixels determined to be cones were averaged to determine $\tau_m$ for that location, and all pixels from rods were averaged to obtain $\tau_m$ for rods at that location. This was repeated to determine the average $a_1, \tau_1$, and $\tau_2$ values for cones and rods. I did not analyze $a_2$ separately because $a_1$ and $a_2$ were constrained by the equation $a_1 + a_2 = 1$.

Figure 5.1 – By shrinking (for cone analysis) or expanding (for rod analysis) the mask placed around each cone in the AOFLIO image, most errors in size or placement of the mask, and the effects of pixel binning, can be negated in the analysis of rod and cone fluorescence lifetimes. A 20% change in radius was chosen as the optimal value because the change in $\tau_m$ plateaus at around this value. A further change in mask size removes an unnecessarily large number of pixels from the analysis. Figure prepared by Sarah Walters.
5.2.6 Statistical analysis

Statistical differences were determined using a three factor (photoreceptor type, eccentricity, and monkey) analysis of variance (ANOVA) in Matlab (Mathworks, Inc.). A p-value of less than 0.05 was used as the threshold for significance.

5.3 Results

5.3.1 Determination of AOFLIO fitting parameters

AOFLIO fitting parameters vary depending on the fluorophore composition of the tissue being imaged. I determined the best parameters to fit the data for this study of two-photon fluorescence of macaque photoreceptors using 730 nm excitation. Visual inspection of the AOFLIO images showed contrast between the rods and cones due to a longer fluorescence lifetime in the cones (Figure 5.2(b)). By plotting the data on a phasor plot (Figure 5.2(e)), it was clear there was a difference in lifetime between cones and rods. The pixels contained within the blue circle are shown in Figure 5.2(f), corresponding mostly to cones. The pixels contained within the orange oval are shown in Figure 5.2(g), corresponding mostly to rods. Because the data forms a narrow elliptical cluster on the phasor plot, a double exponential fit is appropriate (Digman, Caiolfa et al. 2008). In addition, I analyzed the fit in the time domain by comparing a double (Figure 5.2(c)) and triple (Figure 5.2(d)) exponential fit at the same pixel. I found only a minor improvement in fit quality and a slight change in the measured lifetime by adding a third
component to the fit. For the full image shown in Figure 5.2(b), the double exponential fit resulted in a $\chi^2$ of $1.15 \pm 0.14$ (mean ± standard deviation) and $\tau_m$ of $176 \pm 60$ ps, while the triple exponential fit gave a $\chi^2$ of $1.07 \pm 0.15$ and $\tau_m$ of $167 \pm 52$ ps. For the same image, the amplitude of the 3\textsuperscript{rd} component in the triple exponential fit ($a_3$) was only $2.46 \pm 1.62$ %. Similar characteristics were found in the rest of the data in this study. For all these reasons, I chose to use a double exponential fit to simplify the analysis and interpretation of the data.
**Figure 5.2** – Determination of AOFLIO fitting parameters. a) Two-photon fluorescence image of photoreceptors in the macaque. b) AOFLIO image acquired concurrently. c) and d) Double and triple exponential fits of the pixel shown with the red arrow. Adding a third exponential only marginally improves the fit. e) Phasor plot including all pixels from the AOFLIO image in panel b). f) AOFLIO image only showing pixels from inside the blue circle in panel e). This area of the phasor plot corresponds mostly to cones. g) AOFLIO image showing only pixels from inside the orange oval in panel e). This area of the phasor plot corresponds mostly to rods. Scale bar is 25 µm. Figure prepared with assistance from Khang Huynh.
5.3.2 Fluorescence lifetime measurements were consistent between monkeys

I imaged the photoreceptor mosaic in 3 monkeys using 730 nm excitation, allowing me to resolve cones at all locations imaged. I found no significant difference (p > 0.05) in any of the lifetime parameters I analyzed (a1, τ1, τ2, τm) when comparing different monkeys (Figure 5.3). There were no interaction effects between the three factors (monkey, eccentricity, photoreceptor type) considered in the ANOVA. Therefore, I averaged the data from the 3 monkeys for the remainder of the analysis.

![Figure 5.3](image)

**Figure 5.3** - Mean fluorescence lifetime measured in cones and rods for the 3 monkeys imaged. I found no significant differences in any of the lifetime parameters analyzed between the 3 monkeys. Error bars correspond to the standard deviation of the mean lifetime measurements in each monkey.

5.3.3 Cones exhibit longer fluorescence lifetime than rods

As described in section 5.2.5, cones were masked in each image using the fluorescence intensity image. Cones displayed a significantly longer mean fluorescence
lifetime than rods \( (p < 0.01) \). In most cases, cones were easily discernible in the fluorescence lifetime image by their longer fluorescence lifetime (Figure 5.4). In all locations imaged, the cones had a longer mean fluorescence lifetime than the surrounding rods. When the mask was randomized (same number of “cones”, but randomly placed in the image), the difference disappeared \( (p = 0.56; \text{paired sample t-test}) \), indicating that this difference was not an artifact of the masking itself.

**Figure 5.4** - AOFLIO images of photoreceptors excited at 730 nm. Panels a), b), and c) show fluorescence intensity images of photoreceptors at 7, 10, and 20 degrees eccentricity. Panels d), e) and f) show the corresponding fluorescence lifetime images. Cones are distinguishable from rods by their longer fluorescence lifetime. Scale bar is 25 \( \mu \text{m} \).

In order to further understand where the differences in the mean fluorescence lifetime between rods and cones originated, I looked at the individual components \( \alpha_1, \tau_1, \)
and $\tau_2$. The results shown in Table 5.1 are the mean ± standard error. I found that $\tau_1$ and $\tau_2$ were both longer for cones, and that $a_1$ was lower for cones. All of these parameters contribute to a longer mean fluorescence lifetime for cones when compared to rods.

**Table 5.1** – Fluorescence lifetime components compared between rods and cones. Cones exhibit longer $\tau_1$ and $\tau_2$, while rods have a higher $a_1$. Together, these parameters drive a difference in the mean lifetime $\tau_m$, which is longer for cones than it is for rods. *Indicates significant difference with $p < 0.01$.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (ps)*</th>
<th>$\tau_2$ (ps)*</th>
<th>$a_1$ (ps)*</th>
<th>$\tau_m$ (ps)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cones</td>
<td>129 ± 3</td>
<td>2060 ± 21</td>
<td>92.8 ± 0.4</td>
<td>265 ± 11</td>
</tr>
<tr>
<td>Rods</td>
<td>114 ± 4</td>
<td>1880 ± 24</td>
<td>95.4 ± 0.3</td>
<td>195 ± 9</td>
</tr>
</tbody>
</table>

**5.3.4 The short lifetime component increases toward the periphery, leading to a reduction in the mean lifetime**

The mean fluorescence lifetime $\tau_m$ of cones and rods decreased further away from the fovea (Figure 5.5(a); $p < 0.01$). There was a sharp fall-off in the cone lifetime from $2^\circ$ - $5^\circ$, however, there was still a significant difference in mean lifetime if all data from $<5^\circ$ was removed ($p = 0.01$). I found no significant difference in $\tau_1$ ($p = 0.05$) or $\tau_2$ ($p = 0.64$) as a function of eccentricity. The amplitude of $a_1$ increased further away from the fovea (Figure 5.5(b); $p < 0.01$), with a sharp rise from $2^\circ$ - $5^\circ$. This rise in $a_1$ coupled with no significant changes in $\tau_1$ or $\tau_2$ suggest that the change in the mean lifetime with eccentricity is due to a change in the contributions of the short and long lifetime components, not a change in the lifetimes themselves.
5.3.5 The fluorescence lifetime of photoreceptors altered by light exposure

Using the AOFLIO technique, I imaged two locations with existing retinal damage. The first location had been exposed to 34 J/cm\(^2\) of 460 nm light, causing disruption to the photoreceptor mosaic and retinal pigment epithelium (Figure 5.6(a, b)). The second location had multiple exposures of pulsed, 730 nm light, causing a subset of cones in the field to appear dark in the fluorescence image (Figure 5.6(d, e)). The cones in the damaged regions were masked and the measured fluorescence lifetime of cones and rods inside the damaged regions were compared to eccentricity-matched locations of normal, healthy photoreceptors. In both locations of light damage, the mean fluorescence lifetime of rods was reduced when compared to the normal tissue (Figure 5.6(c, f)). In location 1, some cones were missing, and the remaining cones had a mean fluorescence
lifetime lower than the healthy tissue. In location 2, the fluorescence lifetime of cones was similar to that of the healthy tissue.

Figure 5.6 - (a) Fluorescence intensity and (b) mean fluorescence lifetime images of the location damaged by exposure to 460 nm light. The region exposed to 460 nm light is contained in the white box. (c) The average $\tau_m$ of all pixels inside cones and all pixels in rods in both the damaged region and an eccentricity-matched control region. The measured lifetime in both cones and rods is reduced. (d) Fluorescence intensity and (e) mean fluorescence lifetime images of the location with multiple exposures to pulsed, near-infrared light. The altered region is contained in the white box. (f) The average $\tau_m$ of all pixels inside cones and all pixels in rods in both the damaged region and 3 eccentricity-matched control regions. The measured lifetime in rods is reduced. Scale bar is 25 μm. Error bars indicate the standard deviation of the mean lifetime in each location ((c) and (f)) and the propagation of error for the 3 eccentricity-matched normal locations in (f).
5.4 Discussion and conclusion

In this study, I present the first report of AOFLIO to image autofluorescence on a cellular scale. With the transverse and axial resolution achievable by adaptive optics, I can more confidently ascribe fluorescence lifetime measurements to particular cell classes. I found that fluorescence originating from cones had a longer fluorescence lifetime than fluorescence coming from rods. Although there was some variability across eccentricities, the lifetime was consistently longer in cones within an image. In the future, this may simplify cone identification and provide a sensitive measure for damage to photoreceptors.

The measured fluorescence lifetime is influenced by several different factors which could be different between rods and cones. One possibility is that there is a difference in relative fluorescence contribution. For example, cones could have a larger relative contribution from all-trans-retinol, while rods have a higher contribution from NADH. Pure all-trans retinol has a fluorescence lifetime of 3 – 3.9 ns in different solvents (Chihara, Takemura et al. 1979). To my knowledge, it is unknown how the fluorescence lifetime is modified when retinol is bound to a protein such as retinol binding protein, which transports retinol from the outer segments to the retinal pigment epithelium (Liou, Bridges et al. 1982). The fluorescence lifetime of NADH is known to depend on its conformation and binding state, with free NADH in solution having a lifetime of ~450 ps (Vishwasrao, Heikal et al. 2005, Yaseen, Sutin et al. 2017), while NADH bound to the mitochondrial enzyme malate dehydrogenase exhibits a lifetime of
~800 ps (Vishwasrao, Heikal et al. 2005). The known fluorescence lifetime of both fluorophores is much longer than the short lifetime component $\tau_1$ (129 ps for cones, 114 ps for rods) that I observed. One possible explanation for this short lifetime component could be due to all-trans retinal, which has a very short fluorescence lifetime in solution with ethanol, although the fluorescence signal is weak (Dysli, Wolf et al. 2016). FAD is also known to have a very short fluorescence lifetime of 40-130 ps in its protein-bound form (Nakashima, Yoshihara et al. 1980). It is excited efficiently at 730 nm and the emission is also within the band captured by my detector (Huang, Heikal et al. 2002); therefore, it could be influencing the measured fluorescence lifetime and may provide a partial explanation for the short lifetime component I report. The unknown nature of the fluorescence lifetime components and relative concentrations of the different molecules endogenous to the photoreceptors makes it difficult to confidently ascribe the lifetime measurements to a particular fluorophore. Furthermore, it is likely due to contributions from multiple fluorophores.

There are a number of important differences between rods and cones which could indirectly lead to a difference in fluorescence lifetime. Rods and cones have similar dark currents (Nikonov, Kholodenko et al. 2006), and therefore likely similar ATP expenditure in the dark (Okawa, Sampath et al. 2008). In bright light, rod metabolic demand is reduced by ~75% (Okawa, Sampath et al. 2008). Cones consume much more ATP in bright light than rods (Wong-Riley 2010). The conditions used for AOFLIO constitute a bright light scenario, with cone photopigments being bleached almost instantly by the imaging beam, although rods are not fully bleached (Sharma, Schwarz et al. 2016). This
could manifest in the fluorescence signal as a decrease in relative NADH concentration in cones due to higher metabolic demand.

In addition to differences in metabolism between rods and cones, the role of retinol must be considered. Cones are known to recover photopigment after being bleached even in the absence of RPE, while rods do not (Wang and Kefalov 2009). Additionally, cone photopigment recovers much faster from a bleach than in rods and cones typically do not saturate even in very bright light, while rods saturate at low light levels (Penn and Hagins 1972, Schnapf, Nunn et al. 1990). Both of these differences suggest a preferential regeneration of photopigment in cones by an alternate visual cycle (Wang and Kefalov 2011). At this point it is unclear how the alternate cone cycle affects the two-photon fluorescence signal, particularly because cones are bleached by the imaging light but rods are not. One possible avenue for addressing this question would be to compare fluorescence lifetime signatures from photoreceptors in unfixed, *ex vivo* retina with and without RPE to determine how the presence or lack of the canonical visual cycle affects the fluorescence lifetime of rods and cones.

There exist significant challenges in the acquisition of fluorescence lifetime data using two-photon excitation. Reliable optical stabilization or post-capture image registration is required to avoid image blur and fluorescence lifetime data being assigned to the incorrect pixel. Until this hurdle is overcome, a significant advantage of fluorescence lifetime imaging (the reduced reliance on good image quality) cannot be realized. The current optical stabilization paradigm allows for reliable capture of fluorescence lifetime data when the reflectance video of the photoreceptors provides
good contrast. This is typically the case in normal retina, but is not always true in unhealthy retina (Scoles, Sulai et al. 2014). Currently, the fluorescence lifetime software does not have the capability to correct for the sinusoidal scan of the resonant scanner. In this work, my solution is to crop the image down to the nearly linear central portion of the scan. Future improvements including correction of the sinusoidal scan will allow for a larger effective field of view by eliminating the need to crop the images. In its current form, two-photon AOFLIO requires long acquisition times with high incident optical power in order to generate fluorescence lifetime images with high fidelity. Exposure to 7mW of 730 nm light for 180s is known to damage S cones (Schwarz, Sharma et al. 2017); therefore, reducing the incident optical power and/or reducing exposure times is critical. Now that I have found a difference in lifetime between rods and cones, it may be possible to significantly reduce necessary exposure times and optical power by pooling data from all pixels within a cone and fitting only one decay curve to all photons within the cone. Although this precludes the possibility of acquiring subcellular information, it would provide one avenue for implementing this technique in humans.

Interpretation of fluorescence lifetime data can also be challenging. In this study, I have chosen to limit the analysis to a two-exponential fit. However, it is very likely that there are more than 2 fluorescent species contributing to the fluorescence signal, even if their contributions are small. In addition, there is a dearth of knowledge of the lifetime of fluorophores in the living retina. Because the fluorescence lifetime of many molecules is affected by their environment as well as enzyme-binding, it can be challenging to extrapolate lifetime values from the studies which established fluorescence lifetimes of
many of these molecules in solvents (Chihara, Takemura et al. 1979, Lakowicz, Szmacinski et al. 1992). Measurements of the fluorescence lifetime in ex vivo retina have been made (Cubeddu, Taroni et al. 1999, Peters, Hammer et al. 2011, Schweitzer, Gaillard et al. 2012, Miura, Huettmann et al. 2013), although even in these cases it can be challenging to compare fluorescence lifetime values due to known effects of viscosity (which can prolong lifetime values in fixed tissue) and differences in ex vivo preparation. Furthermore, NADH levels drop quickly in isolated tissue, likely reducing their contribution to the ex vivo signal in comparison to their contribution in vivo (Obi-Tabot, Hanrahan et al. 1993, Palczewska, Golczak et al. 2014). In ex vivo measurements with fixed tissue in my lab, there was found a general increase in the measured fluorescence lifetime, along with a disappearance of the differences in lifetime between cones and rods using 730 nm excitation (Figure 5.7). These results highlight the need for in vivo fluorescence lifetime imaging on a cellular scale, but also underscore the challenges in interpreting in vivo fluorescence lifetime data.

![Figure 5.7](image)  

**Figure 5.7** – Images of fixed, ex vivo primate photoreceptors. a) Two-photon fluorescence intensity image of cones and rods and b) fluorescence lifetime image collected concurrently. Unlike in vivo fluorescence lifetime measurements of photoreceptors, there is no clear difference in fluorescence lifetime between rods and cones.
Fluorescence lifetime imaging has great potential as a diagnostic technique due to its ability to detect early cell dysfunction (Skala, Riching et al. 2007). Applied to the retina, this could allow researchers to explore the progression of models of retinal disease and the effects of novel treatments on disease progression. Two-photon AOFLIO is a promising modality for investigating key retinal fluorophores inaccessible with single-photon fluorescence on a cellular scale, allowing for more confident attribution of particular fluorescence lifetime signatures to specific cell classes and to see early changes in individual cells.
Chapter 6

Future directions
6.1 Context

This thesis has consisted of two main engineering developments. During the course of this thesis, I designed and built the first dedicated two-photon AOSLO for imaging the mouse eye. In addition, I adapted the fluorescence lifetime imaging technique for use in high resolution in vivo retinal imaging in order to capture the first AOFLIO images. Both of these techniques are still in their infancy, and as such, there are many improvements that can and will be made to them in the future.

In addition, there were several scientific studies conducted using the above techniques, which begin to demonstrate their efficacy and show the avenues of study they open. The mouse provides a fantastic model for inserting exogenous fluorophores, both for the purposes of validation, which is necessary for a new technique, but also as markers of function. In this thesis, I used the AOFLIO technique to measure the fluorescence lifetime of Peredox in inner retinal cells of normal mice (Chapter 4), with the hope that this will serve as a baseline for future studies of metabolic changes using this vector. In collaboration with Sarah Walters, I also deployed the fluorescence lifetime imaging hardware in the two-photon AOSLO for imaging the macaque retina, and used the technique to study differences in fluorescence lifetime between rods and cones. This thesis has only scratched the surface of what high resolution fluorescence lifetime imaging can be used for in the retina. In this chapter, I will discuss some of the exciting
new studies this technique has unlocked, as well as some suggestions for pathways to pursue in the future.

6.2 Engineering Improvements

Building an AOSLO for mouse imaging brings its own unique set of challenges stemming from the small size of the eye and the imaging needs. During the course of this thesis, I attempted to address as many of these challenges as possible, but some remain unresolved. Additionally, there are a number of improvements that could be made to the AOSLO system to increase its utility.

6.2.1 Final telescope lens

In both this system and a previously built AOSLO for mouse imaging, there is a lens in the final telescope before the mouse eye (see Figure 2.2). The rationale for this lens is as follows: the performance of an all reflective system relies heavily on the angle of incidence on the final mirror before the mouse eye. This angle should be small in order to minimize astigmatism at the retinal plane, but it must be large enough to accommodate ray clearance. Due to these antagonistic constraints, the decision to include a lens as the final optical element was made, which minimizes aberrations and does not cause ray clearance issues.

The inclusion of this lens creates its own set of challenges, however. Although I used a lens with an anti-reflective coating optimized for my imaging wavelength, the dim reflection from the lens surface would still overwhelm the reflection from the retina at
particular focus positions. This was most problematic in the inner retina, where the retinal reflectance is low. The solution that I typically used when this became a problem was to tilt the lens so that the reflection was not captured by the detector. Of course, this negates the initial purpose for using the lens in the system, because the lens tilt degrades system performance. In a recent analysis in Code V, measuring the effect on the optical performance when tilting the final lens, revealed that the wavefront error increased by 8x when a 5° tilt was introduced, and 1.7x with a 2° tilt. Therefore, tilting the lens is probably not the best solution to this problem.

6.2.2 Horizontal scan synchronization signal

The synchronization of the horizontal scan is discussed in section 2.3.4. The synchronization relies on placing a photodiode in the path of a light source reflected off the polygon scanner as it spins. The photodiode outputs a pulse when the light scans across, triggering the start of a new line in the video capture. Initially, this method of synchronization had some instability, which was determined to be due to a slight jitter in the photodiode response that was present at the scanner’s highest speed (27,000 RPM). This jitter can be seen in Figure 6.1. It disappeared when the scanner was operated at a lower speed (18,000 RPM). If the higher speed was desired to produce a faster video frame rate, a new photodiode with less timing jitter could be implemented.
Figure 6.1 - Jitter in horizontal scan synchronization. a) The waviness in the vertical grids is due to a jitter present when the polygon scanner is operated at its maximum speed. b) When the scanner is run more slowly, the jitter goes away.

6.2.3 Adaptive optics correction in the mouse eye

In the mouse retina, as in the monkey retina, the strongest reflection comes from the photoreceptor layer. Therefore, the light source is focused at this layer to produce the sharpest possible spots on the wavefront sensor. The challenge occurs when imaging the inner retina. In order to image the inner retina, I offset the focus of the wavefront sensing source from my imaging source. The goal is for the wavefront sensing source to be focused at the photoreceptor layer, while the imaging source is focused in the inner retina. In practice, this is something that must be optimized in the model eye prior to imaging, and it can be challenging to offset the light source the correct amount to achieve optimal focus for both light sources when a mouse is placed in the system. This can result in blurred spots on the wavefront sensor, leading to a sub-optimal adaptive optics correction.
Additionally, when one wants to image through the full thickness of the retina in one imaging session, it is necessary to change the focus of the wavefront source to keep it focused on the photoreceptors, while using the deformable mirror to change the focus of the imaging source. Therefore, there is a requirement for a high dynamic range for the wavefront sensing source. In the current system, the dynamic range of the translation stage is not large enough to section through the retina. To overcome this limitation, either the translation stage could be replaced with a longer range translation stage, or the collimating lens can be replaced with a shorter focal length lens.

### 6.2.4 Fluorescence excitation wavelengths

The AOSLO system was originally designed to be able to excite two-photon fluorescence in two bands – one centered at 730 nm and the other at 900 nm (see section 2.2.1 for detailed explanation). The wavefront sensing source and reflectance imaging source were at 850 nm and 790 nm, respectively. The current setup is shown in Figure 6.2(a). This setup was chosen at least in part because I knew that these wavelengths worked well for their respective purposes and that light sources were obtainable. However, this design decision had the unfortunate side effect of preventing this middle band for use in excitation of two-photon fluorescence. At the time of design, there were no fluorophores of interest for the research that required excitation in this band. Once I began working with the Peredox vector (section 4.3.5), this band became very important, as the optimal two-photon excitation for Peredox is at 800 nm (Mongeon, Venkatachalam et al. 2016).
To open the full band of excitation the ultrafast laser is capable of, both the wavefront sensing source and the reflectance source would need to be replaced. In addition, the dichroic at which the two-photon light source enters the AOSLO would have to be replaced. New filters for the wavefront sensing and reflectance detectors would also need to be purchased. A new detector with different spectral sensitivity for the wavefront sensor channel would be required. A new detector with different spectral sensitivity for the reflectance channel would be beneficial, but not required. A new setup opening this full band would look something like that shown in Figure 6.2(b). These changes would be fairly easy to make; the prohibitive factor is cost.

**Figure 6.2** – Source and dichroic mirror setup for mouse two-photon AOSLO. a) Current setup. The 790 nm and 850 nm sources prevent two-photon excitation in this band, which is the optimal band for excitation of some fluorophores of interest. b) Proposed new setup. By moving the reflectance source to 680 nm and the wavefront sensing source to 1000 nm, the entire band of the two-photon excitation source can be utilized. Changes outlined in orange.
6.2.5 Implementation of reflectance imaging with two-photon excitation source

Currently, the two-photon mouse AOSLO is capable of reflectance imaging at 790 nm. This uses a separate reflectance source from the two-photon excitation source, allowing the sources to be focused at different retinal layers. This is beneficial for mouse imaging, because I typically want to focus the reflectance source at a vessel layer in the inner retina for registration purposes. If a reflectance channel for the two-photon excitation source was implemented, this video could be used to precisely focus the two-photon excitation. In the two-photon AOSLO for primate imaging, this strategy has allowed them to find the best focus for photoreceptors and more. In addition, if this detector were placed on a motorized three-dimensional translation stage, offset detection strategies could be used to attempt to improve contrast in the reflectance image.

In theory, this should be a simple change to the system. An additional dichroic mirror would be placed in the detection path, separating reflected light at 730 (or 900) nm from the reflected light at 790 nm, and a confocal detector would be placed at the retinal conjugate. The difficulty lies in fitting this detector onto an already crowded optical table. One possible solution would be to orient the detector vertically and use a flat mirror to direct the reflected light upward into the detector.

6.3 Scientific studies

The successful implementation of AOFLIO will allow for future studies to learn more about the fluorophore content of the retina and how the fluorescence signatures change
under varying conditions. There are countless directions this technology could be taken in the future, but I will outline several that I believe are both impactful and are possible now or in the near future.

### 6.3.1 AOFLIO in humans

Rochester has one of the best AOSLO instruments in the world for imaging humans (Zhang, Yang et al. 2015). Among its many capabilities is single-photon fluorescence imaging in the visible range (Yang, Song et al. 2015). Single-photon fluorescence imaging at this wavelength can be used to target important retinal fluorophores such as lipofuscin. Other groups have measured the fluorescence lifetime of the retina using visible wavelengths (see section 1.8), but they do not have cellular resolution. By implementing AOFLIO in a human AOSLO, changes to the fluorescence lifetime of lipofuscin and other fluorophores could be studied on a cellular scale in human disease. This may give a sensitive measure of the changes that occur to these fluorophores in disease, perhaps leading to additional understanding of the disease and/or a new diagnostic measure.

Further down the line, two-photon AOFLIO could be implemented in humans. My colleague Dr. Christina Schwarz has assessed the safety and feasibility of two-photon ophthalmoscopy for use in humans (Schwarz, Sharma et al. 2016). No damage or loss of function was found for light levels that enabled functional imaging, although a reduction in infrared autofluorescence was seen. This is a phenomenon that has been seen previously in AOSLO instruments (Masella, Williams et al. 2014), but has not been
found to be harmful (Schwarz, Sharma et al. 2016). A challenge for two-photon AOFLIO in humans is the low fluorescence signal at safe excitation light levels. Strategies such as summing all the photons acquired in the field of view, or masking cones using the reflectance image and then summing all photons from cones, then separately all photons from rods, could address this challenge. Although this would limit the lateral resolution of the measurement, it could still be used to compare the fluorescence lifetime of different cell classes, or track changes in the fluorescence lifetime in a patient with progressing disease.

Both of these avenues of implementing AOFLIO in humans are currently being pursued.

### 6.3.2 AOFLIO of inner retina

Measuring the fluorescence lifetime of inner retinal cells is an enticing prospect because it could allow for the study of cellular metabolism in the living retina. Similar studies have been done in the brain, in which the authors were able to measure the fluorescence lifetime of neurons and how it changes under brief periods of anoxia (Yaseen, Sakadzic et al. 2013). AOFLIO could also be used to investigate potential changes to the fluorescence lifetime of the inner retina in response to glaucoma-like symptoms in an optic nerve crush model of mice.

Two-photon fluorescence ophthalmoscopy has been used to image inner retinal cells (Sharma, Williams et al. 2016), but the low fluorescence signal in the inner retina has so far made it difficult to measure the fluorescence lifetime. As with AOFLIO in
humans, there are a couple ways to address this problem. The first is to sum the fluorescence signal over many pixels – the entire field of view, a mask containing all identified ganglion cells, etc. This would enable a fluorescence lifetime measurement to be made, but would limit the spatial resolution of the measurement. The second strategy would be to attempt to increase the fluorescence signal. Dr. Christina Schwarz and Sarah Walters are currently working on compressing the excitation pulse width using a multiphoton intrapulse interference phase scan device, to enhance the fluorescence signal. This work may lead to a higher fluorescence signal in the inner retina, allowing the fluorescence lifetime to be measured on a cell-by-cell basis.

### 6.3.3 AOFLIO measurements after visual stimulation

Previously, my lab has found that the fluorescence intensity with 730 nm two-photon excitation increases following visual stimulation (Sharma, Schwarz et al. 2016, Sharma, Schwarz et al. 2017). It is believed that this is a result of the production of all-trans-retinol. By taking measurements pre- and post-stimulation, it could be determined that the change in fluorescence lifetime is mostly due to increased contribution from retinol, which will help in determining the \textit{in vivo} fluorescence lifetime of retinol. Another interesting possibility is to compare pre- and post-stimulation measurements between rods and cones to see if the differences in fluorescence lifetime between rods and cones are a result of increased retinol production in cones following stimulation. In areas of photoreceptor degradation, the post-stimulation fluorescence lifetime
measurements may further differentiate healthy photoreceptors from those that are damaged, due to reduced production of retinol in damaged areas.

The two-photon mouse and monkey instruments both have the capability to deliver full-field stimuli to the retina. The major difference would be a change in wavelength of stimulation due to differences in wavelength sensitivity between the animals. To capture post-stimulation measurements of the fluorescence lifetime, multiple measurements would have to be added to increase the SNR.

6.3.4 Fluorescence lifetime sensors for vision restoration applications

As part of the National Eye Institute’s audacious goals initiative, my lab has been working on developing imaging techniques to evaluate vision restoration efforts. The Peredox sensor (which was used in section 4.3.5) is one example of a fluorescence lifetime-based sensor that could be used to measure the health of retinal cells. Another sensor that could be used is called SweetieTS, which has a fluorescence lifetime change depending on the glucose concentration (Díaz-García, Mongeon et al. 2017). Either of these sensors could be inserted into cells of interest in order to monitor cell health during vision restoration efforts. The lab is beginning to insert these sensors into mouse retinas and explore their applications.

One method for vision restoration is to insert stem cells into a degenerated retina (Wright, Phillips et al. 2014). The health of these cells can then be monitored with a fluorescence-lifetime based sensor once they are inserted into the retina. This would be
advantageous in comparison to a non-functional fluorescent marker (e.g. EGFP), because one could monitor not just whether the cells are alive or not, but monitor the cell health. For example, if a stem cell labeled with SweetieTS exhibited a gradually reducing fluorescence lifetime and then died, one could conclude that it may have died due to inadequate supply of glucose.

6.4 Conclusions

There were two main goals for this thesis. The first major undertaking was to design and build a new AOSLO for two-photon fluorescence imaging of the mouse retina (chapter 2). This design had several innovations when compared to previous systems built in Rochester. It was the first mouse system designed from the beginning with the intention of using two-photon fluorescence as the main imaging modality. Additionally, it was the first system in Rochester to use a polygon scanner, and therefore requiring no digital de-sinusoiding.

The second goal was to implement fluorescence lifetime imaging in an AOSLO for the first time (chapter 3). This technique holds great promise for enhancing our understanding of the fluorescence originating from the retina by analyzing the fluorescence lifetime signatures of different retinal layers and even individual cells.

These two goals intersected in chapter 4, where I undertook the first major study using the two-photon AOSLO for mouse and implement fluorescence lifetime imaging. In this study, I used exogenous fluorophores to label particular features in the mouse retina and test the fluorescence lifetime technique. Following validation, I showed the
capability to image a fluorescent sensor of cellular metabolism. In future studies, AOFLIO can be leveraged in combination with this sensor or others to study functional measures in the mouse retina under intact conditions and over time in the same animal.

In chapter 5, I translated the AOFLIO technique to the two-photon AOSLO for primate imaging and studied the fluorescence lifetime of the photoreceptors. This study established AOFLIO as a viable technique for imaging autofluorescence in the retina, and the baseline fluorescence lifetime characteristics of photoreceptors across the primate retina were determined.

As with all technology development, AOFLIO opens new avenues of scientific study. At this point we are poised to deploy fluorescence lifetime imaging to learn more about how the retina functions in health and disease. Future implementations of AOFLIO in humans may provide a sensitive measure of unhealthy cells prior to irreversible damage, and it is my hope that this technique will become a valuable tool in the ever-growing suite of adaptive optics imaging methods.
References

"The HPM-100-40 Hybrid Detector." Application Note.


