CORRECTING FOR SPHERICAL ABERRATION
IN MULTIPHOTON MICROSCOPY
USING A TWO-DIMENSIONAL
SPATIAL LIGHT MODULATOR

by
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ABSTRACT

In the past few decades, there has been an explosion of construction techniques for microscopy systems. These systems can include various types of lasers, including solid state and more recently, fiber lasers. There are many advantages to a fiber laser, including portability, small size, ease of alignment, and low maintenance. In particular, this report details the construction of a 1 µm, 150 fs, all-normal-dispersion (ANDi) fiber laser that is used for microscopic imaging.

In conjunction with the fiber laser, I constructed a two-photon excitation fluorescence (TPEF) microscope that can be used for sub-1-µm-resolution images of scattering media. The key driving specimen for this system is biological systems, but the laser-microscope system can be used to image any scattering media, including high-index materials used in solar cells (for example, CdTe, which has an index of 2.8 at λ ≈ 1150 nm [1]).

After construction of the laser-microscope system, we developed a system using a two-dimensional spatial light modulator (SLM) to correct for spherical aberration that arises at the image plane. To do this, we created a phase mask that induced a variable quartic phase on the spatial profile of the excitation beam.

The SLM system has been tested on a TPEF microscope using specimen scanning techniques. Additionally, I designed and constructed a theta microscope to try to improve axial resolution (≈4 µm from a Yb:KGW laser through a 0.65 NA objective) to match its lateral resolution (≈1 µm) by interfering the foci from two different microscope objectives.
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Angle of paraxial ray ......................................................... $u$
Area ............................................................................... $A$
Axial location of minimum blur ........................................... $\delta_d$
Curvature of lens ................................................................. $C$
Energy ............................................................................. $E$
F-number ........................................................................... $f/#$
Height of paraxial ray .......................................................... $y$
Hermite polynomials ............................................................ $\bar{H}$
Index of refraction ............................................................... $n$
Intensity ............................................................................ $I$
Objective opening angle ....................................................... $\theta$
Optical power ..................................................................... $\phi$
Power ................................................................................ $P$
Pulse width ......................................................................... $\tau$
Radial distance .................................................................. $\rho$
Radius of cladding fiber ..................................................... $b$
Radius of core fiber ............................................................ $a$
Relative core-cladding difference ......................................... $\Delta$
Repetition rate .................................................................... $N$
Resolution .......................................................................... $R$
Seidel coefficient \( S \)
Structural aberration coefficient \( \sigma_l \)
\( V \)-parameter \( V \)
Wavefront aberration coefficient \( W_{ijk} \)
Wavelength \( \lambda \)
<table>
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<tr>
<td>All-normal-dispersion</td>
<td>ANDi</td>
</tr>
<tr>
<td>Boulder Nonlinear Systems</td>
<td>BNS</td>
</tr>
<tr>
<td>Cadmium Telluride</td>
<td>CdTe</td>
</tr>
<tr>
<td>Continuous wave</td>
<td>CW</td>
</tr>
<tr>
<td>Full width half maximum</td>
<td>FWHM</td>
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<tr>
<td>Helium Neon</td>
<td>HeNe</td>
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<td>Multiphoton microscopy</td>
<td>MPM</td>
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<tr>
<td>Near-infrared</td>
<td>NIR</td>
</tr>
<tr>
<td>Photoelastic modulator</td>
<td>PEM</td>
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<tr>
<td>Photomultiplier tube</td>
<td>PMT</td>
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<td>Polarizing Beam Splitter</td>
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<td>Two-photon excitation fluorescence</td>
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<td>Wavelength-division multiplexer</td>
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CHAPTER 1
INTRODUCTION

Throughout history, mankind has striven to understand everything about our world, including the properties of light. Initially, observations were merely qualitative and vague.

1.1 History of optics and microscopy

The first record of a quantitative observation of light’s properties occurred in the first century CE, when Ptolemy described refraction after he saw an oar appearing bent in a pool of water. He then calculated the refraction constants of water and glass to an accurate degree, performing measurements using a simplistic goniometer at the interfaces between air and water, glass and air, and water and glass. Although Ptolemy’s calculated indices of refraction were not completely correct, he had incredible accuracy with such a basic experiment [2].

During the same time, Roman inventors were machining and looking through glass, testing its properties. They experimented with different shapes of clear glass, including samples with thicker glass in the middle than around the edges: essentially, the first lenses. They invented the earliest magnifying glasses this way, observing that if you hold one of these lenses over an object, it would appear larger [3]. These early “microscopes” were made of only one lens and therefore had only one power, up to about 10X magnification. These primitive lenses were first used commercially around the end of the 13th century to be worn as glasses.

The first man credited with combining two lenses in series was Roger Bacon, an English Franciscan monk who studied optical properties of both lenses and mirrors. However, the first compound microscope was constructed by Zaccharias and Hans Janssen in the 16th century. They placed multiple lenses in a tube and observed that the image was magnified much more than a single lens could do. Although these microscopes suffered in image clarity due to high aberrations, the observation motivated more experimentation with the possibility of combining different optics to produce a desired magnification [3].
During the 17th century, Anton van Leeuwenhoek discovered that by carefully manufacturing and polishing his lenses, he could achieve much higher magnification - up to 270X, which was at least a factor of 3 higher than any of his contemporaries. He is credited with the first successful design of a practical microscope, as seen in Figure 1.1. Using this microscope, he was the first man to observe tiny structures that had never been seen before [3].

![Figure 1.1: The first single lens microscope, produced by Anton van Leeuwenhoek. He used a single lens mounted in a small hole in a brass plate, and mounted the specimen on a sharp point. The position and focus could be adjusted by turning the two screws [3].](image)

Van Leeuwenhoek’s microscopes allowed compound microscopes to be further improved to produce more magnification. In the late 17th century, Robert Hooke used the first three-lens configuration to observe the cell in various organisms, both animal and plant [3]. An example of Hooke’s microscope can be seen in Figure 1.2. These compound microscopes aim to improve both resolution and magnification. Resolution refers to the smallest detail of an image that can be observed; essentially, the inherent pixel size of the microscope. Magnification, on the other hand, is the number of times the image is enlarged from the size of the object.
Figure 1.2: Hooke’s original microscope design, the first compound microscope to use three lenses [4].
These historical microscopes all suffered greatly from three primary imaging problems: chromatic aberration, spherical aberration, and achieving a high numerical aperture. Axial chromatic aberration arises from different wavelengths of light focusing at different axial points. This problem is most easily solved using an achromat, first developed by Chester Hall in the 18th century. He observed that by using two lenses of different shapes and indices of refraction, he could force the two outside bands of color - the red and the blue bands - to realign at the image plane [5]. An achromat is a doublet lens that is constructed using two glasses with different dispersions in order to reduce the chromatic focal shift through the lens [6]. A ray trace demonstrating chromatic aberration from a singlet vs. reduced chromatic aberration from an achromat can be seen in Figure 1.3.

![Figure 1.3: Comparison of chromatic aberration from a singlet vs. an achromatic doublet [5].](image)

Spherical aberration arises from each light ray bending at a different angle at different points on the lens. This problem can largely be eliminated using weak lenses in series with each other (known as lens-splitting) that correct for spherical aberration at all but the first lens, as observed by Joseph Jackson Lister in the 19th century [7]. Spherical aberration is discussed in greater detail in Chapter 4. An example diagram of what spherical aberration looks like can be seen in Figure 1.4, and a spot diagram showing a diffraction-limited spot using spherical aberration correction is shown in Figure 1.5.

A high numerical aperture (NA) is advantageous in microscopy because collecting a wider cone of light allows the microscope to have better resolution. Higher focusing angles result in a
Figure 1.4: A diagram demonstrating no spherical aberration (top) vs. spherical aberration (bottom).

Figure 1.5: A spot diagram comparing no spherical aberration correction (left) vs. spherical aberration correction (right). The spherical aberration correction shows that we can achieve a diffraction-limited spot with this correction, as indicated by the airy disk in the image.
tighter focal spot, which is ultimately limited by diffraction and the wavelength of light. Examples of the resultant diffraction-limited focal spot, or Airy pattern, are shown in Figure 1.6 for varying numerical aperture or cone angle.

Resolution is defined empirically by the ability to discriminate between two points. Mathematically, resolution can be determined from the Abbe equation, which gives the diffraction-limited resolution of an optical system:

\[ R = \frac{0.61\lambda}{NA} \]  

where \( \lambda \) is the wavelength of light, and \( NA \) is given by

\[ NA = n \sin (\theta) \]  

where \( n \) is the index of refraction of the immersion medium between the objective and the focal plane and \( \theta \) is half of the objective’s opening angle. Therefore, due to the inverse relationship between resolution and NA, a higher NA will produce a smaller radius, yielding improved resolution due to a smaller "pixel size" in our image.

Figure 1.6: A pictoral example comparing three different numerical apertures [8]. The objective on the left has the lowest NA and therefore the largest airy disk; the objective on the right has the highest NA and therefore the smallest airy disk.
Because NA is dependent on the index of the material being imaged through, immersion objectives, such as oil immersion, allow for higher NA than typical air immersion objectives. For example, the maximum NA for an air immersion objective is 1, whereas for oil immersion it could be as high as 1.3 depending on the index of the oil.

1.2 Modern microscopy

Modern compound microscopes have, for the most part, been corrected for these aberrations: chromatic aberrations are decreased with the use of achromats; spherical aberration is reduced using a series of weak lenses that are machined carefully to have high symmetry; and high NA is achieved with specialized microscope objectives. However, most microscopes have been designed for 2D imaging: samples are taken in slices and observed in vitro. Recent applications require imaging to be done in vivo, so microscopes must be able to image rapidly and in three dimensions without losing image quality, which is the current task researchers face.

In imaging of a biological specimen, there can be a lot of difficulty due to scattering. Scattering media produces non-ballistic photons, as seen in Figure 1.7. Collecting these photons on a two-dimensional detector, such as a CCD camera, will not yield a clear image of the specimen because there is no way to tell where the photons originated. Therefore, scattering can severely limit the image quality. To counteract this, there has been development in imaging of scattering media with single-element devices such as photomultiplier tubes (PMTs) or photodiodes.

The two primary types of microscopy that have emerged to help address the challenge of imaging in scattering media are confocal and nonlinear microscopy. Confocal microscopy is relatively simple: one adds a pinhole at the confocal plane, which rejects the out-of-focus light at the image plane. The confocal plane is at the point of focus from a lens going into the detector, so it only allows the in-focus light to reach the detector, achieving a diffraction-limited, or near-diffraction limited, spot. Using this imaging technique, it is possible to combine the 2D slices taken at varying depths of focus into a reconstructed 3D image. A simple confocal microscope can be seen in Figure 1.8.
Figure 1.7: An example comparing ballistic photons from a collimated source and non-ballistic (diffuse) photons from a scattering source. The ballistic photons can easily be collected with a 2D detector; the non-ballistic photons, however, cannot be collected using a 2D detector because there is no way to map them back to where the originated on the object.

Figure 1.8: A schematic of a simple confocal microscope [9].
Confocal microscopy has a tendency to oversample the specimen as it scans through different z-depths, leading to more photobleaching [10]. Confocal microscopy is limited by linear absorption through the beam path: the fluorophore will absorb some of the beam energy throughout the entire focal volume [11]. Multiphoton microscopy, however, has only nonlinear absorption in the focal volume, so the cone of light going down to the focus is not illuminated. Because of this, multiphoton microscopes are advantageous in imaging scattering media because they can penetrate deeper into the specimen than is possible in a confocal microscope, so more 3D imaging is possible.

Nonlinear microscopy utilizes a variety of nonlinear optical effects. The primary imaging techniques used today include second-harmonic generation (SHG), third-harmonic generation (THG), and two-photon excitation fluorescence (TPEF). An example of these types of imaging can be seen in Figure 1.9. At the focus, the intensity is $\sim 100 \text{ GW/cm}^2$ or higher. In TPEF, this results in two or more NIR photons being absorbed simultaneously, exciting fluorophores in the specimen [12].

![Jablonski diagram comparing TPEF and SHG.](image)

For our imaging, we chose to use both TPEF and THG techniques to test the capability of our imaging devices. The constructed system uses a 1030 nm, 170 fs pulsed fiber laser with a repetition
rate of $\sim$50 MHz and a mode-locked average power of $\sim$120 mW. The laser is used to image through a multiphoton scanning microscope that has the ability to perform nonlinear microscopy measurements, both harmonic generation and multiphoton excitation fluorescence. This provides a reliable system for multiphoton microscopy at a cost of approximately $35,000; commercial scanning microscopes alone sell for upwards of $500,000, and the vast majority of these systems cannot be easily modified for different applications.
CHAPTER 2
FIBER LASER

There are a variety of possible laser types that can be used for microscopy. Some of the most popular in recent history have been solid state lasers, including Yb:KGW (central wavelength 1038 nm, repetition rate 56.5 MHz, average power 2.5 W) and Ti:Sapphire (central wavelength ~800 nm, repetition rate 80 MHz, average power 1.5 W) lasers. Recently, fiber lasers have become a popular option, primarily due to their ease of alignment and maintenance after the initial setup. Fiber lasers also have the appeal of simple passive air cooling, so the gain medium is much easier to keep cool than solid state crystals. Because the gain medium in a fiber is dispersed over a larger volume, it can therefore contact more of the coolant than a laser crystal can [13]. Also, because of their compactness, they can potentially be more portable than either solid state or dye lasers and are therefore more desirable for commercial applications.

Fiber lasers operate based on the phenomenon of total internal reflection (TIR), which can be achieved when the index of refraction of the outer cladding is significantly higher than the index of refraction of the inner core. This can be characterized for the optical fiber by the relative core-cladding index difference $\Delta$:

$$\Delta = \frac{n_1 - n_2}{n_1}$$  \hspace{1cm} (2.1)

with $n_1$ the index of the core and $n_2$ the index of the cladding. A pictorial representation of TIR can be observed in Figure 2.1.

An optical fiber is also characterized by its ability to operate in a single TEM mode. This is described by the $V$ parameter, which determines the number of modes supported by the fiber:

$$V = k_0 a \sqrt{n_1^2 - n_c^2}$$  \hspace{1cm} (2.2)

where $k_0 = 2\pi/\lambda$ with $\lambda$ the wavelength of light, and $a$ the radius of the core [15]. The radius of the outer cladding, $b$, is not critical to the fiber’s ability to operate in a single mode; it is only
critical that this radius is large enough to contain the core fiber.

The field of fiber optics is relatively new – it emerged in the 1950s when researchers added a cladding layer to the glass fiber, which drastically improved the characteristics of the fiber. Initial growth of the field was rapid in the 1960s as fibers were desired for image transmission through a bundle of glass fibers. The next major step in fiber optical development came in the late 1970s when very little loss was observed in the NIR wavelength range, \( \sim 700-1100 \) nm. This spurred other experiments with various nonlinear optical phenomena in optical fibers, including optically induced birefringence, self-phase modulation, and generation of ultrashort pulses. These are some of the fundamental concepts driving the construction of the fiber laser detailed in Section 2.1 below.

2.1 Fiber laser construction

We chose to construct the all-normal-dispersion (ANDi [16]) femtosecond fiber laser primarily to utilize the ability of this fiber laser to self-start into mode-locking. The laser is also incredibly stable, so once constructed, very little maintenance is required to maintain mode-lock [16]. The fiber has a 6 \( \mu \)m core diameter, which allows for single-mode operation at the NIR operating wavelength, \( \sim 1030 \) nm. Fiber lasers propagate the beam through total internal reflection, as seen in Figure 2.1. By using a small-index material for the inner core and a high-index material for the outer cladding, the beam is contained in the core and is easily propagated over a large distance.
In principle, single-mode fiber (SMF) can propagate a beam over a range of km. A simplified schematic of the laser design is shown in Figure 2.2, and the operation is described below.

Figure 2.2: A schematic of the all-normal-dispersion (ANDi) fiber laser design [16]. QWP: quarter-waveplate; PBS: polarizing beam splitter; HWP: half-waveplate; WDM: wavelength division multiplexer.

The core fiber is pumped by a 980-nm diode laser, which supplies the core of the gain fiber with \( \sim 350 \) mW of power. The diode laser is then coupled into the fiber that circulates the cavity by a wavelength-division multiplexer (WDM). A WDM is used to combine the two signals: the beam from the pump laser and the circulating beam in the cavity [17]. In this case, we combined the 980 nm diode laser light with the near infrared light centered at \( \sim 1030 \) nm. A schematic of WDM functionality is shown in Figure 2.3.

Figure 2.3: A schematic of the functionality of a wavelength division multiplexer (WDM). In our case, the WDM was used from right to left to combine the two beams [17].
After the WDM, an ytterbium-doped gain medium is spliced with the main fiber. The spliced region has two cores: the inner core holds the Yb-dopant. Ytterbium has a very simple electronic level structure, and has a small quantum defect, allowing for high efficiencies and low thermal effects in lasers [18]. It also has the ability to have a very high bandwidth: in this laser, we saw a bandwidth of about 20 nm. This allows for very short pulses, observed to be $\sim 170$ fs. [16]. The outer core carries the light from the pump diode, which allows the gain medium in the center to take in energy from the diode laser beam in the outer core, increasing the intensity of the infrared Gaussian beam with every pass through the cavity.

The collimator is comprised of a lens of focal length $f$ placed a distance $f$ away from the end of the fiber. Therefore, as the beam expands leaving the fiber, it is collimated by the lens to be output to the part of the cavity not comprised of fiber. Typically, the lens used in the collimators is an asphere so that we keep spherical aberration to a minimum in the laser cavity.

The cavity contains two quarter-waveplates and one half-waveplate. These waveplates are all zero-order, which means that the total retardation is the desired retardation without any excess [19]. Zero-order waveplates are advantageous because they are more constant with respect to temperature and wavelength fluctuations, so our laser will be able to maintain mode-lock even if we have some fluctuations in temperature and/or center wavelength. Mode-locked control with these waveplates will be discussed more in-depth in Section 2.2.

A polarizing beam splitter (PBS) is used as the output coupler for the laser. We have a mix of S- and P-polarization circulating the cavity: the P-polarization will pass through the PBS and continue to circulate in the cavity, while the S-polarization will be reflected and output. The polarization of the circulating light is determined by the orientation of the waveplates, as discussed in Section 2.2 below.

A quartz birefringent filter is used to control the center wavelength of our laser. It is a linear retarder that is placed at its Brewster angle, then rotated about its axis to achieve varying wave retardation effects at the center wavelength of the laser [20]. Because the filter is placed immediately after the PBS, it experiences minimal losses: the P-polarization is able to pass through the filter
without reflection loss, whereas S-polarization would be lost in reflection. This filter has a center wavelength of 1030 nm with a bandwidth of 10 nm [16].

Finally, an isolator is used to ensure light travels in one direction. This uses the Faraday effect: the isolator rotates the plane of polarization, causing the optical circulator to prevent the light that enters the cavity to exit out the same port. This happens because the outgoing beam has a different polarization than the incoming beam [21]. A picture of the completed laser is shown in Figure 2.4.

![Quartz filter](image)

Figure 2.4: Picture of the operational ANDi laser. The output of the laser is at the PBS, which is the small cube near the center of the photo. QWP: quarter-waveplate; PBS: polarizing beam splitter; HWP: half-waveplate.

### 2.2 Mode-locking

Once this laser is successfully lasing in continuous wave (CW) operation, the next step is to mode-lock the laser. We found the mode-locking regime through a simple empirical process, described here. Mode-locking is achieved through a series of waveplates added to the cavity, including two zero-order 1/4-waveplates, a 1/2-waveplate, and a quartz birefringent filter. The quartz birefringent filter will have optimum power transmission when it is aligned at its Brewster angle. This is the first plate added to the cavity: the Brewster angle can be optimized by measuring the power transmission as a function of the angle of incidence. We observed that maximum power
can be achieved at a few different places through a full $360^\circ$ rotation. Once the power is maximized, the 1/4 and 1/2-waveplates can be added and aligned with the laser beam.

Because there is no preset orientation for the waveplates, mode-locking was achieved initially through trial and error: we used a photodiode connected to a spectrometer to monitor the output of the laser, then rotated each of the waveplates individually to observe their effect on the spectrum. The quartz plate is used to shift the center wavelength to the desired position ($\sim 1030$ nm). Once this is aligned, the 1/4 and 1/2-waveplates are rotated relative to each other until a square, broad spectrum is observed. Our goal was to see a high bandwidth arise when the waveplates were oriented correctly because a wide wavelength spectrum corresponds to a narrow temporal pulse. An example of the spectrum observed during this initial trial run can be seen in Figure 2.5.

![Figure 2.5: Spectrum of the ANDi laser, observed with $\lambda/2$ at $281^\circ$, $\lambda/4$ at $15^\circ$, and the quartz filter at $300^\circ$. We were unable to mode-lock without the second $\lambda/4$-plate in the cavity.](image)

We mode-locked the laser using a spectrometer and power meter simultaneously. The spectrometer shows when we reach a high enough bandwidth to achieve a short temporal pulse, while the power meter shows that we have reached a high enough power to generate ultrashort pulses. The final repetition rate of this laser is 45 MHz with a stable pulse train, and in mode-locked operation, the average power is $\sim 120$ mW, corresponding to a single pulse energy of $\sim 3$ nJ [16].

After this had been completed, we set the waveplates to the optimal configuration from this first trial run. We then used a photodiode and an oscilloscope to do fine adjustments until we observed a characteristic mode-locked output on the oscilloscope: in the mode-locked regime, we see a train
of short pulses on the oscilloscope as seen in Figure 2.6.

![Image of the pulse train output of the ANDi fiber laser just after mode-locking. The repetition rate of the laser is 50 MHz, corresponding to a 20 ns pulse separation.]

This mode-locking was initially very unstable: it would appear on the screen for a minute, then drop. In order to make it more stable, the waveplates were fine-tuned until the pulse train was more stable on the oscilloscope. Once mode-locking is achieved, it is very easy to regain if lost: all we had to do was re-optimize the power through minute adjustments of the waveplates.

2.3 Future applications of the fiber laser

In total, we completed the construction of two of these fiber lasers. One is now in operation in Dr. Sarkar’s lab for use in imaging biological specimens, and the other is being used at NREL.
CHAPTER 3

TWO-PHOTON EXCITATION FLUORESCENCE MICROSCOPE

There is a variety of nonlinear processes that can be used for imaging, including second-harmonic generation (SHG), third-harmonic generation (THG), and two-photon excitation fluorescence (TPEF). SHG and THG are nonresonant nonlinear phenomena and are electronic perturbations of the molecular ground state. These signal wavelengths are dependent on the laser wavelength: for example, in a KGW laser, SHG will be ∼520 nm, while THG will be ∼350 nm. TPEF, on the other hand, is a resonant phenomenon: it can only occur if the incident laser energy is a multiple of the energy gap between the ground and excited states of the material (in our case, the energy states of the fluorophore) [22]. The signal wavelength of TPEF is dependent on the fluorophore, but is usually somewhere between the green (∼530 nm) and the orange (∼600 nm). A Jablonski diagram comparing these different imaging techniques can be seen in Figure 3.1.

Figure 3.1: A Jablonski diagram comparing TPEF, SHG, THG, and CARS (coherent anti-Stokes Raman scattering) [12].

TPEF is an imaging technique that can be implemented for samples that fluoresce in the visible spectrum. Some of the samples used in our lab include biological samples injected with a fluorophore and solar cells used to build solar panels. There are many advantages of TPEF over normal fluorescence with ultraviolet light (∼400 nm). Physically, the ultraviolet light will cause the spec-
imen to photobleach over time, making it no longer useful. NIR light will heat up the sample due to quantum defects at the focus, but because we are scanning the focus through the specimen, we generally do not harm the specimen with the NIR light. A Jablonski diagram demonstrating the difference between single-photon excitation and TPEF is shown in Figure 3.2.

![Jablonski Diagram](image)

Figure 3.2: A Jablonski diagram comparing single-photon excitation to TPEF [23].

We can estimate the average intensity for both NIR and ultraviolet light by

\[
I = \frac{P}{A} \tag{3.1}
\]

where \( I \) is the average intensity, \( P \) is the average power, and \( A \) is the cross-sectional area of the focal volume in the axial direction. We find this area by

\[
A \approx \pi R_{\text{axial}}^2 \tag{3.2}
\]

where \( R_{\text{axial}} \) is defined by

\[
R_{\text{axial}} = \frac{1.67 \lambda}{\text{NA}^2} \tag{3.3}
\]

with \( \lambda \) the wavelength of light and \( \text{NA} \) the numerical aperture of the objective. Then, for NIR, we find the intensity with \( \lambda = 1030 \text{ nm} \), \( \text{NA} = 0.75 \), and \( P \sim 1 \text{ mW} \), which results in

\[
I_{\text{NIR,avg}} \sim 13400 \text{ W/cm}^2 \tag{3.4}
\]
Similarly, we find the ultraviolet intensity with $\lambda = 400$ nm, NA = 0.75, and $P \sim 1 \mu W$, which results in

$$I_{\text{UV,avg}} \sim 20 \text{ W/cm}^2$$

(3.5)

This is surprising – we would expect to see an ultraviolet intensity that is much higher than our infrared intensity because we see fluorescence through the entire focal volume (Figure 3.3). However, the problem lies in linear vs. nonlinear absorption. Ultraviolet light illuminates the entire cone of light down to the focus due to linear absorption through the entire volume. This in turn causes the image to be blurry because you collect photons from the entire excitation volume, not just the focal volume. This can be eliminated using confocal microscopy; however, we chose to use NIR light: it has very low linear absorption through the specimen, and only has significant nonlinear absorption at the focus, so we can absorb two photons simultaneously only in the focal volume and therefore only image at the focus. The difference in excitation volume between ultraviolet and NIR light can be seen in Figure 3.3.

Figure 3.3: A pictoral comparison of single-photon excitation from ultraviolet light and TPEF from NIR light [24].
TPEF is typically performed at a peak intensity of \( \sim 100 \text{ GW/cm}^2 \). We do a simple estimate to see how the detected TPEF scales with repetition rate, pulse duration, energy, and focal area. Instantaneously, the detector sees

\[
I^2 = \frac{E^2}{A^2 \tau^2}
\]  

(3.6)

with \( I \) the intensity of light, \( E \) the energy per pulse, \( A \) the approximate area of the focal volume, and \( \tau \) the width of each pulse in time. We assume a roughly square pulse and integrate \( I^2 \) over time to see what the detector measures:

\[
\text{detector} \propto \int I^2 N d\tau
\]  

(3.7)

\( (N \) the repetition rate of the laser) which results in

\[
\text{detector} \propto \frac{E^2 N}{A^2 \tau}
\]  

(3.8)

So, the detected signal depends directly on the number of counts, which is directly proportional to the repetition rate, and inversely on the pulse width. For our fiber laser (\( \lambda = 1030 \text{ nm} \)), we have a pulse width around 200 fs, a repetition rate on the order of 50 MHz, and an average pulse energy of \( \sim 3 \text{ nJ/pulse} \). For an objective with an NA of 0.75, we get an intensity of \( I_{\text{NIR,peak}} \sim 250 \text{ GW/cm}^2 \), which is sufficient for TPEF generation.

### 3.1 Microscope construction

The TPEF microscope uses a series of highly reflective mirrors to relay the output from the fiber laser to the scanning mirrors. The mirrors are coated with silver, which has an average reflectance of \( > 97.5\% \) at our operating wavelength of 1030 nm [25]. Highly reflective mirrors are critical in this system because we require minimal losses (\( > 50\% \) throughput) from the laser to the objective. These are also first surface mirrors: the silver coating is not covered by a thick glass, so there are not aberrations or dispersion picked up from each mirror as a result of beam propagation through a layer of glass.

Our two scanning mirrors, made by Cambridge Technology (Model 6210H), are also coated with silver, like the turning mirrors. They are each able to move in one orthogonal axis direc-
tion, so we have two degrees of freedom from the scan mirrors alone. Scanning mirrors are an advantageous design over using a translation stage to move the specimen itself for imaging primarily because mirror scanning is much faster than specimen scanning, resulting in reduced imaging times. There is also a lower chance of agitation of the specimen during the high-speed imaging process using scan mirrors [26]. In the case of taking data \textit{in vivo} for a biological specimen, it is important to not agitate the specimen because movement of the sample causes additional undesired blur in the resulting image. Mirror scanning allows us to avoid this distortion by capturing an image in a time that can be shorter than specimen movement.

After the scan mirrors, we have a pair of Thorlabs LSM05-BB scan lenses to image the beam from the mirrors to the microscope objective. These lenses are custom-designed to operate in the near-infrared, and they focus light to a diffraction-limited focus even when the light is incident at an angle other than normal to the lens surface. In the tangential ($x$) direction, the spot size does not exceed 40 $\mu$m until the angle is greater than 6 degrees off-axis; in the sagittal ($y$) direction, the spot size does not exceed 35 $\mu$m until the angle is greater than 7 degrees off-axis. The lenses are therefore advantageous to the microscope design because the scan mirrors will cause the laser beam to hit the lenses at varying angles, which could cause off-axis aberrations, including coma and astigmatism, to be present in the image. However, these lenses are optimized to minimize these aberrations.

The scan lenses each have a focal length of 110 mm. They are placed in a $4f$-relay configuration in order to relay the image from the scan mirrors to the entrance pupil of the microscope objective: the scanning mirrors are a distance $f$ (110 mm) away from the first lens, the lenses are separated by $2f$ (220 mm), and the objective is placed so that the entrance pupil of the objective is a distance $f$ (110 mm) away from the second lens. The reason for this relay system is to minimize vignetting, the loss of intensity of incident light around the edges of the image due to a reduction in effective lens opening for off-axis light.

After the scan lenses, a Thorlabs DMLP900T dichroic mirror is used to force the beams of light to go the direction we want: it transmits 932-1300 nm, and reflects 400-872 nm.
setup, the 1030 nm light from the laser passes directly through the filter, whereas the fluorescence (green, $\sim$500 nm) from the specimen is reflected by the filter, which we can then collect in a photomultiplier tube (PMT). A simplified schematic of the microscope is shown in Figure 3.4, a detailed schematic is shown in Figure 3.5, and a picture of the completed microscope follows in Figure 3.6.

![Schematic diagram of the microscope system](image)

Figure 3.4: A simplified schematic of the final TPEF microscope system. The dichroic after scan lens 2 has been removed for simplicity.

Initial alignment of the microscope was done using a Helium Neon (HeNe) laser. This laser operates as a continuous wave laser at 633 nm, and because it is in the red range, it will not have exactly the same alignment as the fiber laser. However, it is useful for an initial alignment because it operates in the visible range, making a rough alignment significantly easier than it would be with a laser beam in the NIR. In addition, the scan mirrors were replaced with a normal turning mirror for the initial alignment to ensure that the beam is initially aligned at $90^\circ$.

3.2 Experimental results

Some of the easiest biological samples that can be imaged with fluorescence are onion cells. Onion cells have a very characteristic hexagonal shape, and they have large nuclei that can be
seen easily under a microscope. An example of an onion cell imaged using TPEF can be seen in Figure 3.7.

Lens tissue colored with a fluorophore can also yield clear images for testing microscope design. The tissue has many crosslinking fibers that can be seen in 3D imaging because some are in focus at a certain depth, while others focus at a different depth. An example of a lens tissue scan can be seen in Figure 3.8.

In Figure 3.7 and Figure 3.8, the scans were taken using a specimen-scanning system with a 2 µm step size per pixel. Beam scanning was not fully developed under this thesis, so specimen
scanning was used to test the microscope. The dwell time at each pixel was 20 ms, so an average 200x200 pixel scan took about 15 minutes. The laser used was a Yb:KGW laser at 1038 nm with a repetition rate of 56.5 MHz, and the average power at the specimen was \(\sim 10 \text{ mW}\). The NA of the objective was 0.75. Each pixel was collected on a single-element PMT detector, and a BG39 filter was used in the COHNA collection objective to pass the TPEF wavelength and filter out the laser light [27].

This system was constructed in part for the imaging of Cadmium Telluride (CdTe), a material used in solar cells. CdTe has an index of refraction of 2.8 at a wavelength of \(\sim 1150 \text{ nm}\), so it is
Figure 3.7: Raw data of a TPEF scan of an onion cell. The fluorophore used for this image was an orange highlighter. The scale bar on the right shows the relative intensity from photon counting.

difficult to image due to a high index change from air to the material. This is discussed in further detail in Chapter 4.
Figure 3.8: Raw data of a TPEF scan of a piece of lens tissue. The fluorophore used for this image was an orange highlighter. The scale bar on the right shows the relative intensity from photon counting.
Spherical aberration arises from a plane wave passing through any spherical surface, such as a lens or mirror. This occurs because the curvature of the lens, for example, is higher at the edges than in the center, so the rays bend more at the edges than in the center. Mathematically, this can be observed through Snell’s Law:

$$n_i \sin (\theta_i) = n_r \sin (\theta_r)$$  \hspace{1cm} (4.1)

where $n_i$ is the index of the incident material, $\theta_i$ is the angle of incidence, $n_r$ is the index of the refractive material, and $\theta_r$ is the angle of refraction. At low angles, Snell’s law is approximately linear:

$$n \sin (\theta_{\text{small}}) \approx n \theta_{\text{small}}$$  \hspace{1cm} (4.2)

So, at low angles, we will not pick up much spherical aberration. However, as the lens power increases, these angles increase and Snell’s law behaves nonlinearly, so we can have increasing spherical aberration through optical elements in a system. Additional spherical aberration can be accumulated when a converging or diverging beam passes through an interface where there is a change in refractive index between the materials at the interface [28]. These aberrations can be reduced using lens-splitting (also described in Section 1.1, which involves using a series of weak lenses to achieve the same overall optical power as you would get from a single lens of a higher power. This is achieved in some sense using achromats – one of the lenses is positive and the other is negative, so generally, this corrects for some spherical aberration internally. However, we typically do not have an aberration-free beam incident on the microscope objective for imaging.

4.1 Modeling aberrations in high-index materials

In imaging of high-index materials, there is a significant amount of spherical aberration enhanced by the interface of the specimen. Because our laser beam hitting the entrance pupil of
the objective may not be perfect, there is already some inherent spherical aberration at the image plane. A converging or diverging beam that is focused into a high-index material can accumulate significant spherical aberration, which degrades the resolution of the image. We want to be able to reduce the effects of spherical aberration at the image plane, which requires that we correct for the aberrations before the lens hits the objective.

Spherical aberration is inherently a fourth-order Seidel aberration. Seidel aberrations describe all of the aberrations that can be picked up in an optical system. They are defined by

\[ W = \sum_{i,j,k} W_{ijk} \bar{H}^i \rho^j \cos^k \phi \]  

(4.3)

where the \( W_{ijk} \) coefficient can be positive or negative and has units of length; \( \bar{H} \) are the Hermite polynomials; \( \rho \) is the radial distance; and \( \phi \) is the power of the optical element. Spherical aberration is defined as \( W_{040} \), and can be found by

\[ W_{040} = \frac{S_1}{8} = -A^2 y \Delta \left\{ \frac{u}{n} \right\} / 8 \]  

(4.4)

where \( W_{040} \) is the wavefront aberration coefficient; \( S_1 \) is the Seidel coefficient; \( A = n(u + y C) \) with \( n \) the index of refraction, \( u \) the angle of the ray, \( y \) the height of the ray, and \( C \) the curvature of the lens; and \( \Delta \) defined as \( \Delta \left\{ \frac{u}{n} \right\} = \frac{u_2}{n_2} - \frac{u_1}{n_1} \). We can also define the thin lens form of \( W_{040} \) by

\[ W_{040} = \frac{1}{32} y^4 \phi^3 \sigma_l \]  

(4.5)

where \( y \) is the height of the ray, \( \phi \) is the power of the optical element, and \( \sigma_l \) is the structural aberration coefficient [29].

It is also worth noting that spherical aberration can be partially offset by a second order aberration called defocus. Empirically, defocus and spherical aberration are related by

\[ W_D = -\frac{3}{2} W_{040} \]  

(4.6)

Achieving this relation will give us minimum blur in the image. Then, the axial location of the minimum blur waist with respect to the paraxial plane is given by

\[ \delta_d = 8 \left( \frac{f}{\#} \right)^2 ( - W_D ) = 8 \left( \frac{f}{\#} \right)^2 \left( - \frac{3}{2} W_{040} \right) \]  

(4.7)
where $\delta_d$ has units of length [30].

Ultimately, a surface phase map can be written in order to correct for spherical aberration at a certain depth in a material. The Zernike coefficients, which are related to the wavefront aberration coefficients, are necessary to make the mask for the SLM. The fourth coefficient is the primary factor for spherical aberration [31].

To determine the value of the Zernike coefficient, a sample file of the surface being imaged is created in ZEMAX, an optical design software used to model and simulate optical systems before constructing them in the lab. By adding a “Zernike standard phase” surface, we are able to model what the mask needs to look like in ZEMAX, then extract the data as a text file and use Mathematica to convert the text to a bitmap. A sample of the image of the mask for the correction of spherical aberration through a flat surface standard glass as seen in ZEMAX is seen in Figure 4.1.

![Surface Phase Map](image)

Figure 4.1: The mask generated using ZEMAX for the correction of spherical aberration in BK7 glass, a standard glass used in cover slips. The units in the scale bar are in radians.

To model how well the phase map corrects for spherical aberration, the optical path difference can be plotted. Ideally, we would see $W$ stay as close to zero as possible. Because spherical aberration is fourth-order, we see a fourth-order polynomial in the plot, Figure 4.2. This is an optical path difference plot, which, for low aberrations, should be as flat as possible.
Figure 4.2: A plot of the optical path difference from the spherical aberration correction simulation. This is the difference between the edge of an ideal spherical wave and the actual wave, where the aberrations are highest.

4.2 Testing spherical aberration masks

To compensate for spherical aberration, we use a two-dimensional spatial-light modulator (SLM) to shape the laser wavefront before it hits the microscope objective [32]. The SLM is from Boulder Nonlinear Systems (BNS), and is part of their XY Nematic Series, model P512-1064, which allows for phase and/or amplitude programming. The face of the SLM is 7.68x7.68 mm, and it is coated for a wavelength of 1064 nm, which yields an RMS reflected wavefront distortion of $\lambda/6$ at this wavelength [33]. The laser wavefront shape is programmed to the SLM as a mask using Blink, a program developed by BNS, which takes a bitmap file in the software and writes the shape of the bitmap onto the face of the SLM [33].

The SLM is 512x512 pixels, and it can take up to $2\pi$ phase, so all of the masks we generated include a mod-$2\pi$ phase wrapping. This phase wrapping can be seen on the flat edges of the mask: for example, in Figure 4.3, there are 5 rings between the center and the edge of the mask: hence,
10\pi phase wrapping. The SLM is used in reflection, acting as a mirror in our system. We tested masks with both a positive phase and a negative phase. Examples of both masks can be seen in Figure 4.3 and Figure 4.4.

Figure 4.3: An SLM mask for adding a +10\pi phase shift to the incident beam.

Figure 4.4: An SLM mask for adding a -5\pi phase shift to the incident beam.

This prototype SLM setup has been tested using a Yb:KGW laser, which operates at 1038 nm in the near-infrared, similar to the fiber laser. In our system, we reduced the power of the laser
using neutral density filters to $\sim 20$ mW, measured just before the SLM, to ensure that we did not damage the SLM.

There are two telescopes used in this experimental setup. The first telescope expands the beam to fill the face of the SLM, so we had to expand the beam diameter from 2 mm to $\sim 7.5$ mm. We did this using focal lengths 5 cm and 15 cm separated by 20 cm. The second telescope expands the beam after the SLM to fill the back of the microscope objective. We want to fill the back of the objective because it results in the smallest spot size at the image plane. We also use this telescope as an image-relay system: We need to image the face of the SLM to the back of the objective to achieve the spherical aberration correction at the image plane. To do this, we separate the two lenses in the telescope by the sum of their focal lengths, and each lens is its focal length away from the object or the image plane. This is another example of a $4f$ relay system: we used lenses with focal lengths of 400 mm and 500 mm. The 400 mm lens was placed 40 cm after the SLM, the 500 mm lens was placed 90 cm after the first lens, and the microscope objective was 50 cm after the second lens. A schematic of this system is shown in Figure 4.5.

![Figure 4.5: A simplified schematic of the SLM setup with the Yb:KGW laser. The lens pair is used as a 4f-relay system to image the face of the SLM to the back of the objective, and as a beam expander to make the beam completely fill the microscope objective to achieve the best imaging resolution.](image.png)

Both of the telescopes above use achromats to avoid adding chromatic aberrations into the system, as described in section 1.1 above. A schematic of the full experimental setup is shown in Figure 4.6.
Figure 4.6: Schematic of the full experimental setup for the spherical aberration measurements.
The microscope used to test the prototype SLM setup also uses TPEF, but instead of scan mirrors, the slide being imaged is on a translation stage. Although this is an easy way to scan through a specimen, for instances where high scan speeds are desired, there is a limit on how fast the stage can be moved to avoid distortion in imaging. This is the primary motivation for moving to the scan mirrors, as described in Section 3.1 above.

To test the mask needed to correct for spherical aberration, we made a series of masks ranging from \(-50\pi\) to \(+50\pi\), then tested the spherical aberration correction using THG through two coverslips separated by air. The coverslips are each \(\sim 170 \mu m\) thick. THG for the KGW laser is at \(\sim 346\) nm, so we used a 350 nm interference filter that allows only this wavelength of light to pass through. Ideally, we would see a delta function every time we pass through an interface: we would see a spike of THG as the index of refraction changes from 1 to 1.5, and vice versa. However, for a Gaussian beam, the smallest full-width half-maximum (FWHM) we can observe is the Rayleigh range of the laser, determined by \(R_{axial}\) as shown in Section 5.1 below. For the KGW laser and a 0.75 NA objective, we discovered that our narrowest possible FWHM=3.08 \(\mu m\).

Our control was set using the SLM as a mirror with no phase written to any of the pixels. Then, we ran THG scans with masks of varying phase to determine empirically where the biggest spherical aberration correction was observed. This was determined in two ways: a higher intensity and a smaller FWHM. Our best scan for intensity was observed using a mask with \(-4\pi\) phase shift, as seen in Figure 4.7.

As noted before, spherical aberration can be balanced with defocus to create a better image. This can be seen in Figure 4.7: the peaks are shifted to the left with the spherical mask. We could shift the peaks back to overlap with the uncorrected peaks using a mask with defocus overlayed with the spherical aberration correction mask.

We observed the FWHM values by normalizing each plot and comparing the width at 50% of the maximum. The narrowest peak was observed for the first interface with the \(-5\pi\) mask and for the second interface with the \(-3\pi\) mask. In both cases, a beam with full spherical aberration correction would have FWHM=3.08 \(\mu m\). With our values of 4.13 \(\mu m\) for the first peak and 4.31
Figure 4.7: A THG scan demonstrating spherical aberration correction through a glass coverslip. We see three peaks because through the scan, we pass through three interfaces: the first interface of the first coverslip, the far interface of the first coverslip, and the near interface of the second coverslip.

\( \mu m \) for the second peak, we are very close to full spherical aberration correction, but could possibly program the mask to a more specific phase to achieve an even tighter focus at the image plane. The normalized FWHM plots can be seen in Figure 4.8 and Figure 4.9.

Table 4.1 shows the other FWHM values from the two peaks. The lowest value for each peak is indicated in bold.

### 4.3 Applications of spherical aberration correction

These results are promising: because we have been able to demonstrate spherical aberration correction at a depth of over 100 \( \mu m \) in the material, it seems to be possible to generate better imaging at depths greater than could be done before. In addition, this implies that we can employ similar techniques to correct for other aberrations, such as coma and astigmatism, when we start to try off-axis imaging.
Figure 4.8: A normalized plot of the THG scan to compare FWHM values of the first peak. The mask-corrected peak has a FWHM of $4.13 \pm 0.5 \, \mu m$, which is close to the theoretical value for full correction, $3.08 \, \mu m$. To achieve this theoretical value, we would need to write a more specific phase to each mask.

Figure 4.9: A normalized plot of the THG scan to compare FWHM values of the second peak. The mask-corrected peak has a FWHM of $4.31 \pm 0.5 \, \mu m$, which is close to the theoretical value for full correction, $3.08 \, \mu m$. To achieve this theoretical value, we would need to write a more specific phase to each mask.
Table 4.1: FWHM values for the THG sectioning measurements. The minimum observed value for each peak is indicated in bold.

<table>
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<th>Phase mask</th>
<th>First peak FWHM</th>
<th>Second peak FWHM</th>
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<tbody>
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<td>5.76 $\mu$m</td>
</tr>
</tbody>
</table>
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

As stated in Section 4.1, it is possible to partially compensate for spherical aberration using defocus. This would also be done using the SLM: in future research, we may be able to overlay two masks to correct for both spherical aberration and to shift the focus using defocus, allowing us to achieve better images inside of materials than we can by correcting for spherical aberration alone.

5.1 Theta microscope design

A further application of spherical aberration correction is the increased improvement of 3D imaging techniques. One problem that arises in 3D imaging is that there is good lateral resolution for a small spot size, but poor axial resolution because it is dependent on the Rayleigh range of the laser beam. For example, for a 0.65 NA objective with the KGW laser, lateral resolution can be determined by

\[ R_{\text{lateral}} = \frac{0.61 \lambda}{\text{NA}} = \frac{(0.61)(1038\text{nm})}{0.65} = 974\text{nm} \]  

(5.1)

while axial resolution is determined by

\[ R_{\text{axial}} = \frac{1.67 \lambda}{\text{NA}^2} = \frac{(1.67)(1038\text{nm})}{0.65^2} = 4102\text{nm} \]  

(5.2)

So we have about a factor of 4 difference between axial and lateral resolution. One technique to correct for this is to use the lateral resolution of two different laser beams and interfere them so that there is a smaller spot size in both dimensions. This concept can be seen in Figure 5.1.

Construction of this type of microscope requires two different arms to be aligned to both spatially and temporally overlap. For spatial overlap, we require that the incoming beam have only S-polarization: that way, when the beams cross orthogonally, they will still be able to interfere because their polarization is in the same direction. We are able to accomplish this using a 1/2-
waveplate at the beginning of the microscope. For temporal overlap, we require that the two arms be the same path length (a path length difference of less than 30 µm) so that when the pulse splits at the 50/50 beam splitter, the same pulses will come together at the same time and interfere. A schematic of the experimental setup can be seen in Figure 5.2, and a picture of the first-generation microscope can be seen in Figure 5.3. Beam 1 is the static arm that sets the path length, and Beam 2 has a translation stage to allow precise path length tuning.

This laser was aligned to pinholes through the system, then fine-tuned using a CCD camera at the overlapping focal points of the two objectives. An example of the beam overlap can be seen in Figure 5.4.

The medium used for the testing phase is highlighter fluid diluted by deionized water. This TPEF is bright enough to be seen by eye. The overlapping spots can be seen in Figure 5.5.

In order to cause constructive and destructive interference at the image plane, a photoelastic modulator (PEM) is proposed to be used. A PEM is a variable waveplate that can oscillate at 42 kHz between no change in polarization and a full wave change. For our purposes, we want to oscillate between no change and a 1/2-waveplate to induce oscillating constructive and destructive
interference. To observe this oscillating signal, we need to use a lock-in amplifier: the oscillations will be small relative to the background signal, so we have to lock-in on the oscillations using the modulation frequency of the PEM as a reference.

So far, this part of the project has not been completed. Future testing is required using a photodiode to detect TPEF and display using a lock-in amplifier.
Figure 5.3: A picture of the first generation theta microscope.
Figure 5.4: The overlapping TPEF as seen by objective 1. Beam 1 appears as a spot and beam 2 as a line because we are collecting through objective 1. We can observe a similar image collecting through objective 2 with the beams reversed.

Figure 5.5: The overlapping TPEF can be seen by eye.
REFERENCES CITED


