APPLICATIONS OF CONFOCAL MICROSCOPY

A Thesis

by

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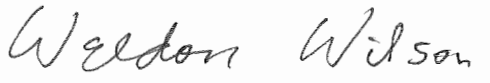

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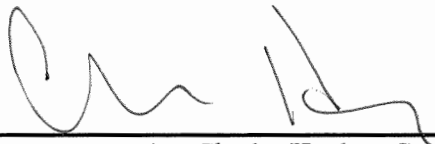

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ABSTRACT

Fluorescent microscopes have long been used in the study of life sciences to image biological specimen. However, they lack the ability to image thick samples and have low resolution. Over the years there have been many improvements that have been made to fluorescent microscopes; one of them being the induction of confocal optics.

Confocal microscopy uses the same basic principles from fluorescence microscopy but uses simple optical techniques to image deeper into a specimen and produce shaper images. The inclusion of laser scanning ramped up image acquisition times and Confocal Laser Scanning Microscopy (CLSM) proved itself to be a vital tool in the medical and life science labs. CLSM's main strength lies in its optical sectioning technique that allows for the procurement of thin optical slices to construct 3D images. Optical sectioning comes from the use of a spatial filter to block any unfocused light that originates outside of the microscopes focal plane. A draw back, is that the spatial filter requires regular maintenance to preserve its sensitive alignment.

A unique approach to the removal of the spatial filter and still maintaining confocal parameters implements the use of pump and probe pulses of light. The pump and probe serve to modify the absorption characteristics of the fluorescent molecules in a process known as transient absorption. Subsequent to the alteration of the absorption properties is an alteration to the fluorescent emittance as well. The traits that come with pump-probe spectroscopy present parameters that are equivalent to that of the confocal

microscope. Thus, removing the need for a spatial filter and simplifying the overall design of the microscope.

In this paper is a detailed description of an approach to build a confocal laser scanning microscope. Using common optical components and 3D printed mounts, the goal was to be as cost effective as possible while maintaining the resolution standards for a functioning confocal microscope. Following the completion of the microscope is an attempt to utilize transient absorption with pump-probe spectroscopy. While typical pump-probe systems apply one or more pulsed laser sources I demonstrate the methodology of using a single continuous source and an optical chopper to induce transient absorption.

DEDICATION

Dedicated to my parents.

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Lastly, I would like to give my greatest thanks to my parents for their never-ending love and support.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by committee members Dr. Hughes, Dr. Wilson, and Dr. Mattison of the Engineering and Physics department.

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NOMENCLATURE

AOM	Acousto-Optic Modulator
FWHM	Full Width at Half Maximum
APD	Avalanche Photo Diode
CLSM	Confocal Laser Scanning Microscopy
NA	Numerical Aperture
PSF	Point Spread Function
TAM	Transient Absorption Microscopy

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CHAPTER I

INTRODUCTION

First developed in 1955 by M. Minsky, confocal microscopy provides axial sectioning to traditional light microscopy. While effective, initial designs did not find widespread adoption into the biological research community until the advent of CLSM in the 1980s [1]. It was this new development for imaging biological specimen that brought confocal microscopy into the lime light. Making use of already familiar fluorescent microscopy techniques, CLSM showcased its ability to effectively and efficiently generate high quality images in a shorter amount of time than that of conventional fluorescent microscopy. It soon became clear that this was the more advantageous way to image specimen. It didn't take long for researchers to extensively study and improve upon CLSM making it a staple in biological research. Its popularity can be seen from the number of systems that are in production today and how it is still being extensively studied and modified to fit the different needs of research groups.

Confocal microscopy was developed to overcome drawbacks from conventional fluorescence microscopy. One of the major limitations was its inability image thick specimen. Fluorescent microscopes would illuminate a wide spot on the specimen and the emitted fluorescent light would then be collected by a photo detector and produce an image. While one would get an image, it would be hazy and with low resolution. Confocal microscopes could produce images with sharper detail and higher contrast by illuminating one point on the specimen at a time and blocking the unfocused light from the specimen that's not in the microscopes focal plane. The images gathered represent

thin slices of the specimen allowing for one to build a collection of optical slices and form 3-D images. The invention of the laser was added to confocal microscopes making them faster and more convenient to use.

History of Confocal Microscopy

Possibly the earliest confocal system can be traced back to 1939 when Hans Goldmann used a slit lamp combined with a photographic system to capture entire optical sections onto film [2]. The first confocal scanning system didn't come around until 1955 and was developed by Marvin Minsky during his fellowship at Harvard University [3]. Minsky's design utilized a stage scanning system that scanned the stage in a raster pattern rather than the illumination beam. An advantage of stage scanning is that one is always scanning along the optical axis thus avoiding lens aberrations. Another reason for Minsky's design was to avoid the challenge of maintaining sensitive alignment of the optics.

Minsky then scanned a specimen point-by-point and by doing this he was able to avoid most of the unwanted light that was excited from the undesired focal plane. Another part of the design included an aperture placed in front of a photo detector that would filter out even more unwanted light. However, scan times were slow and stage scanning would vibrate the specimen and produce unwanted artifacts in the images.

There was another approach, developed by Petran, who made use of a Nipkow spinning disk in the image plane that could illuminate many spots at once [4]. The then emitted fluorescent light was directed and focused to individual confocal apertures. This design was in essence many confocal systems acting together in parallel.

However, only a small fraction of the illumination beam is used and the intensity of the emitted light would be reduced so much that only the brightest parts of fluorescence could be detected [5]. As well, there was also the challenge of realigning the optical components after each use.

Fluorescence Microscopy

If a photon of light is of sufficient energy and is incident on molecule, the molecule will absorb the photon. As excited electrons relax back to the ground state, certain molecules may emit a photon of longer wavelength. This is a process known as fluorescence. Fluorescence microscopy exploits this process by using an illumination source of a specific wavelength and energy to excite fluorescent dyes (fluorophores) that have been placed in the specimen. Samples may be stained or tagged with fluorophores making the key targets the most visible. In addition, multiple fluorophores that require different excitation wavelengths can be placed in the specimen and by either using multiple illumination sources or by switching sources, one can collect emissions simultaneously or sequentially, respectively.

Prior to the invention of confocal microscopy, traditional fluorescent microscopy would simply illuminate a wide portion on the specimen by using an objective lens and then collect the emitted light. The parts of the specimen labelled with fluorophores would be located on the focal plane of the microscope and would be the brightest. However, the excitation light will cause fluorescence emission above and below the focal plane. Much of this light is collected by the objective lens and contributes to the

out of focus blur and makes distinguishing fine detail almost impossible [6]. Most researchers got around this by restricting their studies to flatter and thinner specimen.

Most fluorescent systems use a dichroic mirror to direct the excitation light and the fluorescent light. A dichroic mirror, also known as a dichromatic mirror, allows light of a shorter wavelength to be reflected while longer wavelength light will be allowed to pass through. Whatever the excitation light's wavelength, a dichroic mirror is chosen such that that light will be reflected and directed towards the microscope objective and into the sample. The then emitted fluorescence light of a longer wavelength will travel back through the objective and will pass through the dichroic mirror and into the photodetector. Figure 1 shows the basic setup of a fluorescence microscope.

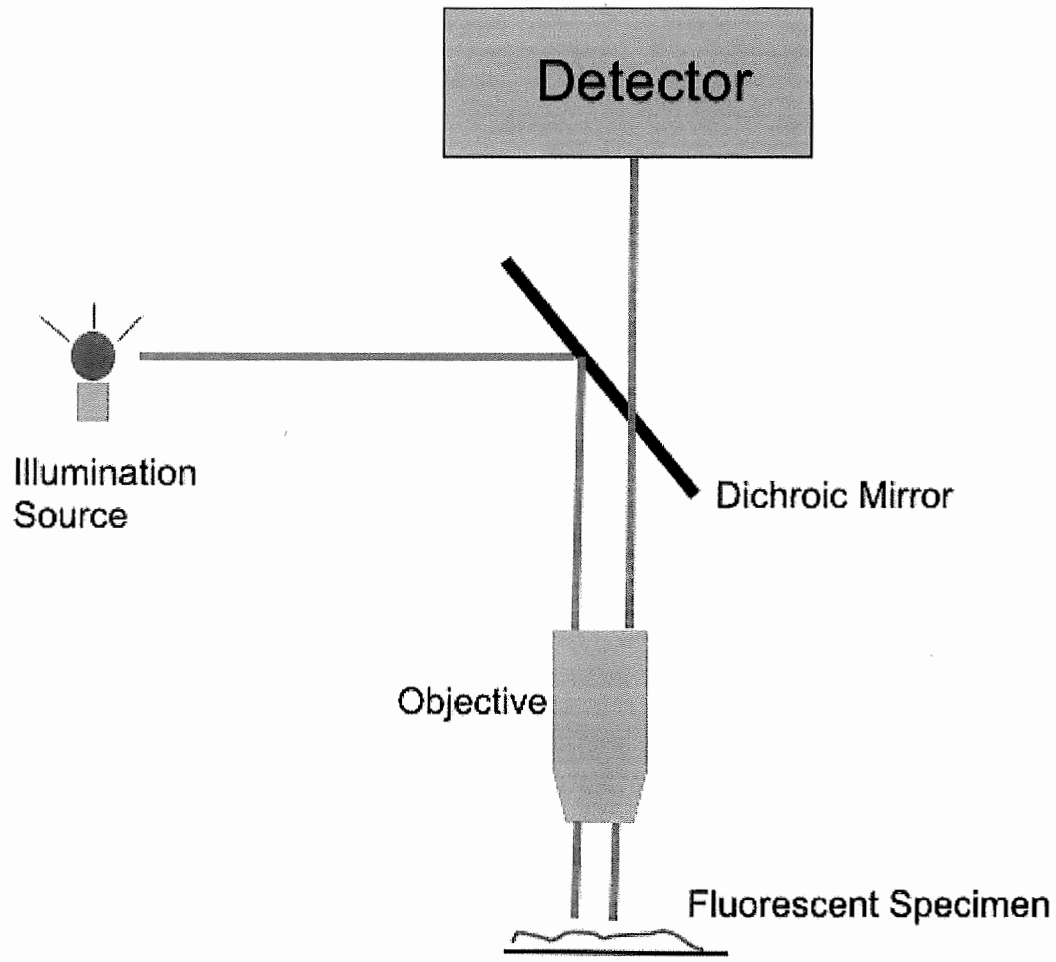

Figure 1. A diagram of a simplified fluorescent microscope. The blue ray represents the illumination or excitation light. The blue ray is directed towards the objective where it is focused onto the specimen. The emitted red ray is captured by the objective and passes the dichroic mirror and onto a photodetector.

Confocal Optics

As the name suggests, confocal microscopy makes use of confocal optics to diminish the effect of out of focus light. The term confocal comes from the optical design where the light used to illuminate a specimen is focused to a spot that is optically conjugate to a point of light focused in a similar way at an aperture in front of a

photodetector. The design takes light from the focal point of one lens and focuses it to the focal point of the second lens. Thus, one has conjugate foci.

The goal is to block out as much unfocused light as possible. A fluorescent confocal microscope uses confocal optics to minimize the amount of fluorescent light and only illuminates a single spot of the specimen (like a fluorescent microscope) using the first focal point of the confocal system. Still though, some light is scattered and some is absorbed into other areas of the specimen and causes unwanted fluorescence. To avoid this unwanted light a small aperture, usually a pinhole, is placed at the focal point of the opposite lens in the system. Thus, any fluorescence that is not from the first focal point will be blocked by the pinhole. This is known as optical sectioning [7]. The light is then incident upon a photodetector where an image clear of haze can be formed. Figure 2 shows a simplified version of confocal lenses and an aperture. The red rays represent light illuminated from the focal plane of the first lens while the blue rays originate from some other plane. By placing the aperture at the focal point of the second lens it is clear that the red rays will pass through. The blue rays, so long as they don't originate at the first focal point, are blocked by the aperture.

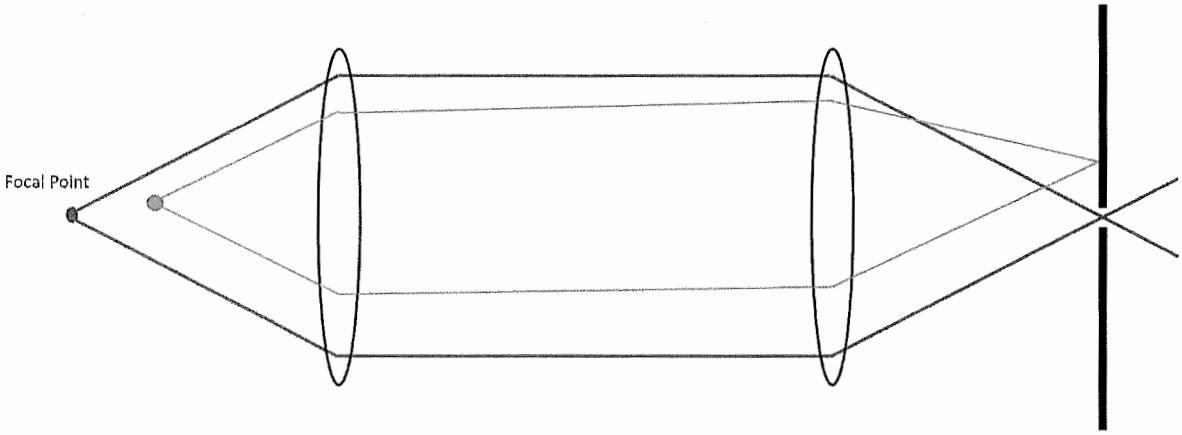

Figure 2. Conjugate focal points. Red rays originating from the first lens' focal point pass through the aperture. Blue rays originating from elsewhere are blocked from passing the aperture.

The size of pinhole defines how strong the optical sectioning capability is and the optical sectioning is what allows for 3-D image construction. One might think the best option would be to make the pinhole as small as possible. However, the smaller the pinhole the less light gets in and the weaker the signal is. To get around this one could increase the intensity of the excitation light. Although, high intensities can be harmful and damage the specimen. As well, in the case of fluorophores, high intensity light can degrade them and cause them to not fluoresce. It has also been shown that there is a limit to the improvement in resolution that can be made from decreasing the size of the pinhole.

Confocal Laser Scanning Microscopy

CLSM was a product of the technological advances that came during the mid to late 1980s. Advances with electronics, electro-mechanics, optics, and lasers were able to improve almost every aspect of confocal microscopy.

Confocal systems are classified by the way they scan specimen. Early instruments were typified by Minsky's design and used a stage scanning device. The optics were kept stationary and the stage was the only moving part. Stage scanning systems could at best produce scanning speeds up to 1 frame per second. To get clean images required a lot of time and great patience.

Once lasers became more accessible confocal systems adopted the method of scanning the beam rather than the stage. Scanning the beam was not a new idea and most systems were composed of two scanning mirrors that could scan in orthogonal directions relative to the beam path. The mirrors would be driven by galvanometers that would be controlled by a computer. Figure 3 shows a simple setup for a confocal laser scanning microscope. The design is similar to a typical fluorescence microscope. The illumination light is reflected from a dichroic mirror but instead of heading directly towards the objective, it is scanned first by two mirrors and then passes through the objective and onto the specimen. The laser light excites the fluorophores and the specimen fluoresces. The emitted light is de-scanned through the scanning mirrors and passes through the dichroic mirror. Where it is then focused through a pinhole and into a detector.

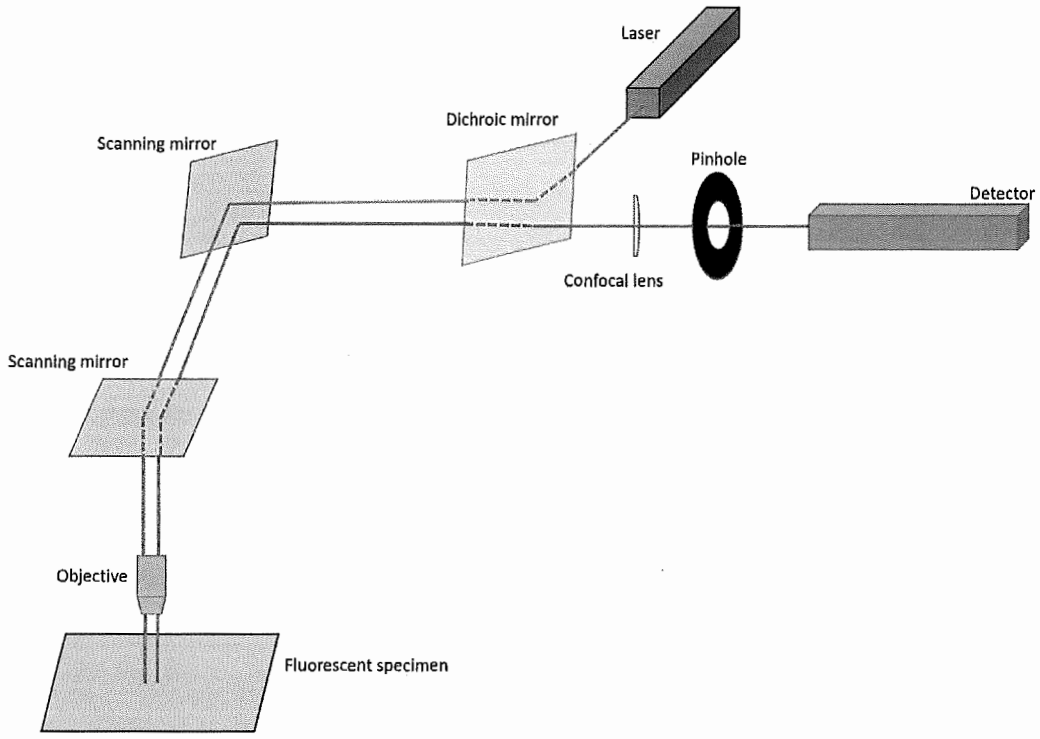

Figure 3. A schematic diagram showing the basic components and layout of a confocal laser scanning microscope.

Usually there will be a long wavelength filter before the confocal lens to stop any excitation light that may have made it through. By scanning the beam, early confocal systems could produce image acquisition speeds ranging from 1 frame/s up to 30 frames/second[1]. Modern systems can produce video rate acquisition speeds.

In a confocal laser scanning microscope, only one point of the specimen is illuminated at any given time. The microscope scans the specimen point by point, line by line, and then builds an image digitally on a computer, pixel by pixel [8]. The picture that is captured by the microscope is a thin planar region of the specimen (optical sectioning). By moving the focal plane in the axial dimension using either stage

scanning or tunable lenses, the computer can image optical slices and then amass a 3-D rendition of the area of the sample under review.

Transient Absorption Spectroscopy

Here I present the theory behind a different imaging technique, Transient Absorption Microscopy (TAM). An alternative imaging technique that eliminates the need for a confocal spatial filter while still providing similar resolution that may be achieved with a confocal microscope.

As mentioned above, CLSM makes use of a confocal objective lens combined with a pinhole (the spatial filter) to eliminate out of focus light originating from regions outside of the microscope objective's focal plane. This is the optical sectioning process. As effective as CLSM is, TAM can eliminate the need for a spatial filter, leading to a reduced overall cost and complexity of the system by eradicating the sensitive process of optically aligning a micron size pinhole, all while producing equivalent images to CLSM.

The physical process behind TAM is transient absorption. In transient absorption, a portion of the electrons in a molecule are excited to some higher state by an excitation or "pump" pulse. The pump pulse will then change the absorptive properties of the molecule. Then a probe pulse is sent through the sample with some time delay relative to the pump [9]. Figure 4 displays a simplified version of the process. By measuring the probe signal through the amount of emitted light, with the pump both on and off, one can find the change in transient absorption. The difference between the two is the transient absorption signal and contains information about the dynamic processes that occur

during electron excitation and relaxation, making pump-probe microscopy extremely useful in studying the fundamental processes of biology, chemistry, and condensed matter [10].

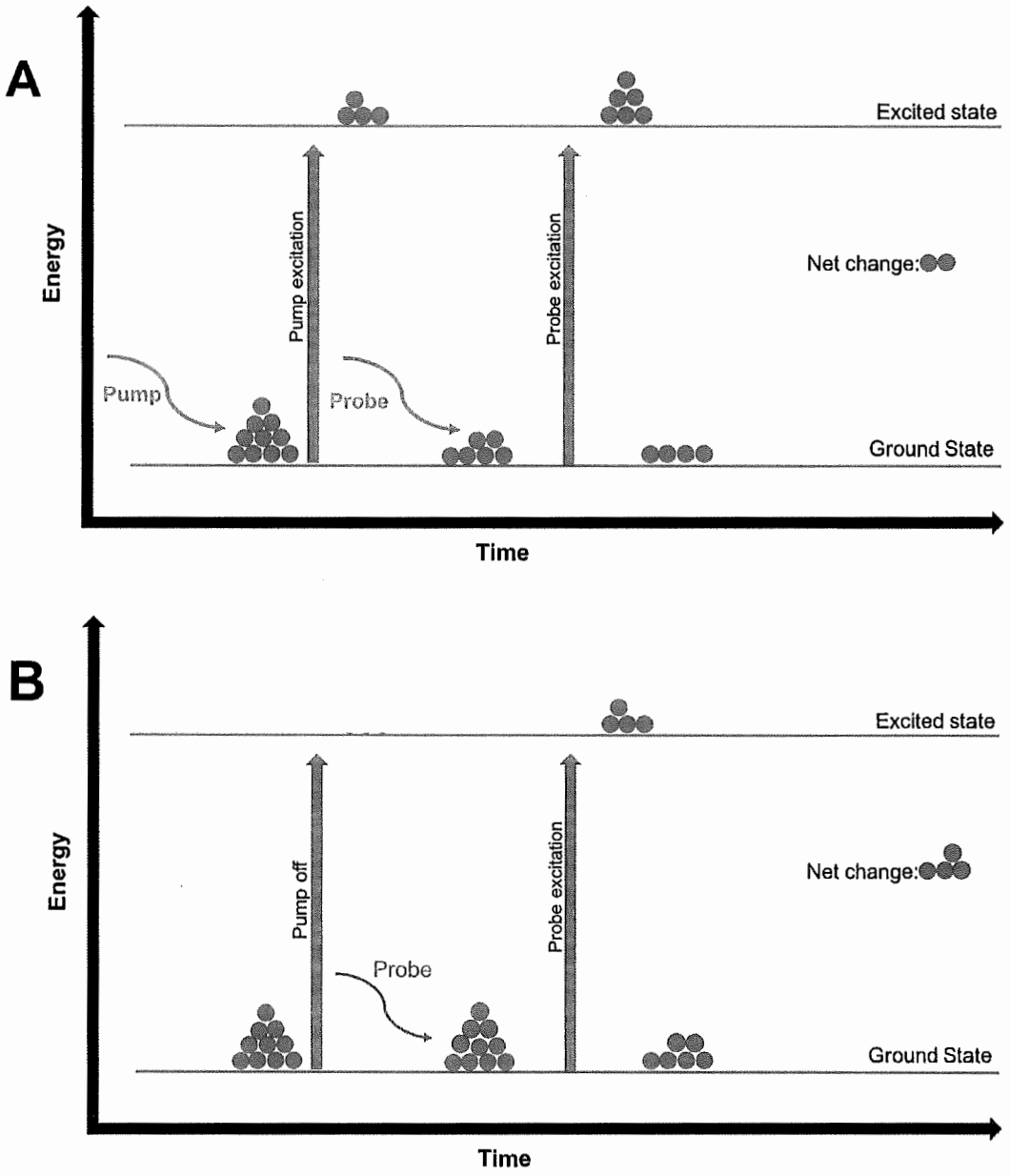

Figure 4. A schematic of an idealized two energy level diagram describing the transient absorption process. (A) The pump pulse drives some of the electrons to an excited state. The partially electron depleted ground state is probed and has less electrons to excite. (B) The pump is off and only the probe excites the electrons in the ground.

A common approach to TAM is to use one pulsed laser, separate the beam into two paths, modulate the amplitude of both beams and then recombine the two beams onto the sample [11]. The probe repeatedly samples the population of the ground state electrons after the pump has altered the absorption state of the molecule. Depending on the modulation frequency of the two beams and how they spatially overlap, optically resolved images can be formed.

However, it has been shown that if the pump and probe are both modulated to the same frequency and the time delay between the two is set to zero, then the change in the absorption is maximized [9]. This then leads to parameters that are equivalent to that of a confocal system. The interaction between the pump and probe produces a transient absorption signal that appears as the second harmonic of the amplitude modulation frequency that can be observed on an oscilloscope. This modification leads to a simplification in overall system design; however, this simplification comes at the cost of a mild decrease in signal-to-noise ratio and requirement for efficient chopping of the beam. There is no more need for the sensitive alignment of two beam paths and one optical element can be used to modulate the beam.

CHAPTER II

METHODS AND THEORY

Here I will discuss the procedures, materials, as well as some of the theory that goes along with the construction of the confocal laser-scanning microscope.

Construction of the source beam

TO-can light sources are excellent sources of low-cost single mode coherent lasers. Unfortunately, due to the properties of their design, TO-can sources produce elliptical Gaussian beams. For this system, we chose a TO-can single mode laser diode (L520P50) that has a measured wavelength of 520nm and a maximum power output of 50mW. Since laser diodes are highly divergent in the transverse plane from the beam path, the first optical component the beam comes in contact with is an aspherical lens for collimation.

The importance of Gaussian beams arises from their many unique characteristics as indicated by the following features. When collimated, Gaussian beams can be spatially confined and transported in free space without any angular spread. This is, of course, an idealized situation, yet it is possible to confine the light into a spatially localized beam that comes as close as possible to non-divergent. Once circularized, the beam power is locally concentrated cylindrically around the beam axis. In the transverse plane the intensity distribution is a symmetric Gaussian function centered on the beam axis. Another important feature is that the beam remains Gaussian when passing through simple optical components.

A Gaussian beam intensity with a power P is described by the function

$$I(\rho,z) = \frac{2P}{\pi W^2(z)} exp\left[-\frac{2\rho^2}{W^2(z)}\right]$$

Where z is the axial direction, ρ= $[x^2 + y^2]^{\frac{1}{2}}$, and $W(z)$ is the measure of the beam width. This Gaussian function has its peak on the z-axis at $\rho = 0$ and decreases as ρ increases as shown graphically in figure 5 below.

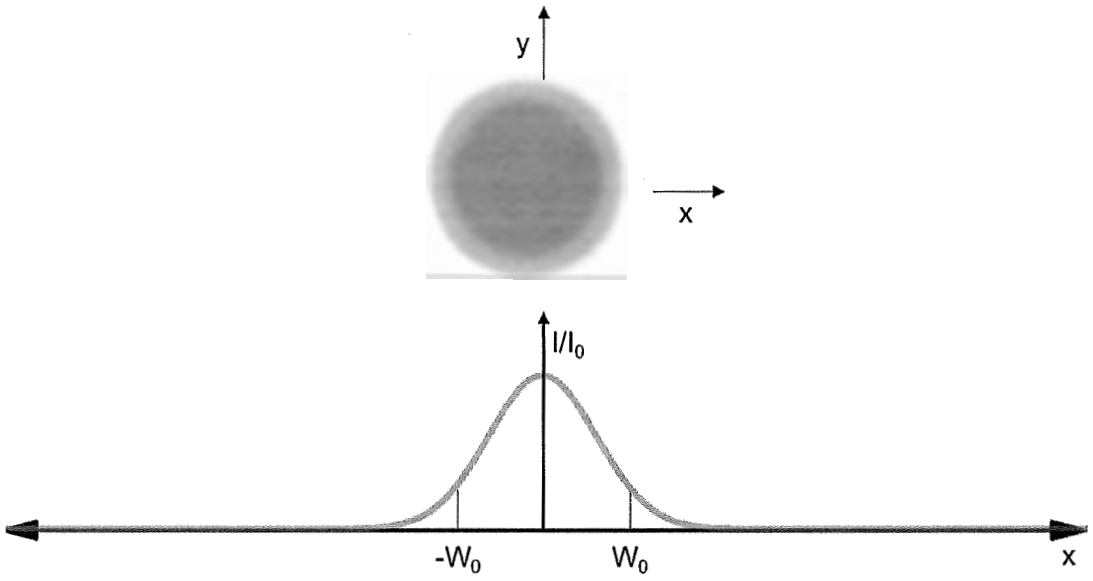

Figure 5. A graph of the intensity function for a Gaussian beam.

Along any transverse plane the intensity of the beam has its peak value on the beam axis itself and decreases by the factor $\frac{1}{e^2}$ at the radial distance $\rho = W(z)$. The majority of the power lies within a circle of radius of $W(z)$ and hence $W(z)$ is referred to as the beam radius/width. Given by

$$W(z) = W_0\left[1 + \left(\frac{z}{z_0}\right)^2\right]^{\frac{1}{2}}$$

One can see that it is at its minimum value W_0 when at the plane $z = 0$ and hence W_0 is known as the beam waist and plays a role in the depth of focus of the beam. $2W_0$ is known as the spot size. Having a minimum width at $z = 0$, the beam has its best focus at the plane $z = 0$ and moderately becomes out of focus in either direction. The depth of focus is modeled by

$$2z_0 = \frac{2\pi W_0^2}{\lambda}$$

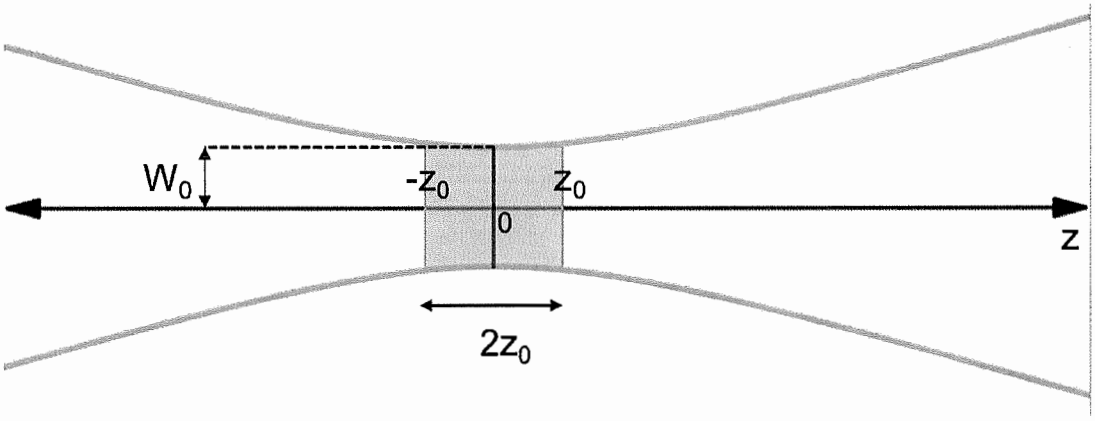

Figure 6. Graph detailing the depth of focus of and waist size for a focused Gaussian beam.

It is easily seen that the depth of focus is proportional to the area of the beam at its waist and inversely proportional to its wavelength. z_0 is referred to as the Rayleigh length which determines the distance the beam can propagate without diverging significantly.

$$z_0 = \frac{\pi W_0^2}{\lambda}$$

To have a well collimated beam there must be a large Rayleigh length. [12]

Once the beam is collimated, it is still elliptical in shape. This elliptical shape would produce unwanted aberrations in our confocal microscopy system; therefore, we needed to shape the beam into a nearly symmetric Gaussian beam prior to the introduction into the microscope. There are three primary methods of shaping an elliptical Gaussian beam into a symmetrical one; an anamorphic prism pair, the use of two cylindrical lenses, or a spatial filter.

The beam is then directed towards a fiber launching stage. Working with the elliptical beam we were able to launch the beam into our fiber and measured 33% coupling efficiency. Coupling efficiency may be improved by the introduction of two cylindrical lenses placed in the beam path after the a-spherical lens to circularize the beam to assist with coupling the maximum amount of power from the laser into a fiber optic cable. However, 33% coupling efficiency is more than sufficient for our application.

It is important to consider the shape of the source beam that is incident on the specimen as it plays a key role in what is known as the Point Spread Function (PSF) which contains information about the resolution of the system. A simple definition most commonly used for resolution defines it as the minimum distance at which points can be distinguished as individuals. In a perfect optical system, the resolution depends only on three factors; wavelength of the beam, the numerical aperture (NA) of the lens, and the refractive index of the media between the objective and sample. [13]

The advantage of a confocal microscope is its ability to image and record optical slices. Confocal microscopy achieves this by imaging the smallest point allowed by the

optics onto the sample. However, due to the diffractive nature of light, a focused point on a specimen is really a distribution, or spread, of light with varying intensities. To obtain the best image quality, one wants to image a symmetric diffraction limited spot onto the specimen. The size and shape of this spot in three dimensions is what ultimately determines the resolution of the system. Although, most sources are not spot shaped and thus need to be modified, typically with some circular aperture. For our system, we chose to use a fiber optic cable with collimators.

The fiber being used is a single mode patch fiber (P1-460B-FC-1) that guarantees a fixed Gaussian intensity profile of the beam at its output. At the input of the fiber is a fixed focus collimator (F280FC-A) working in reverse, to focus the beam into the fiber. At the output of the fiber is an identical collimator that also circularizes the beam to produce a symmetrical Gaussian distribution with a 3mm beam diameter. This is now the beam that will be used as our source for the microscope.

Once a source has been created a diffraction limited spot is imaged by the objective onto the specimen. The sample then fluoresces and only a fraction of the emitted light is collected and focused again to the confocal pinhole. The pinhole serves to block unfocused light and as well to create another diffraction limited spot to be imaged onto the photodetector.

The distribution, or spreading, of the light is what characterizes the imaging properties of the system and is described by the PSF. Below is a figure detailing the properties of the PSF in the lateral dimensions. The brightest central spot is known the Airy disc and the concentric outer rings of successively decreasing maximum and

minimum intensities are known as the airy patterns [14]. The airy patterns describe the lateral intensity distribution as a function of distance from the optical axis, shown in figure 7 below [13].

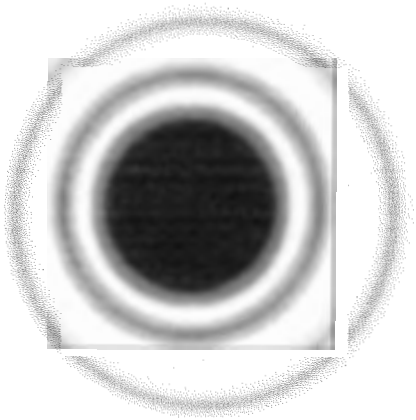

Figure 7. The centralized airy disc and its surrounding concentric airy patterns. Note how the airy patterns decrease in intensity the further they are from the airy disc.

The theoretical lateral resolution (also known as spot size) is determined from the distance of the center of the Airy disc to the first minimum (first white ring) and can be found from the relation:

$$r_{xy} = \frac{1.22\lambda}{NA}$$

And the axial resolution is determined from the center of the Airy disc to the first minimum in vertical space. Given by the relation:

$$r_z = \frac{1.4\lambda\eta}{(NA)^2}$$

Where λ is the wavelength of the beam and η is the refractive index of the media (η=1 for air). Of course, this is not the case in reality, but it is a good approximation to the actual resolution of the system.

Layout of the confocal optics

Figure 8 below, shows an above view of the layout of the optics. The output end of the fiber is mounted using a 3D printed mount to a raised optical breadboard that is now the platform for the rest of the optical components. The beam then comes into contact with a Brightline 532nm single edge dichroic mirror (Di02-R532-25x36) that reflects wavelengths under 532nm and transmits anything of a higher wavelength. Once reflected from the dichroic mirror, two aluminum mirrors are used for precision control to guide the beam to the scanning mirror (SN3834). The scanning mirror directs the beam through two positive thin lenses (L_1= 50 mm and L_2= 200 mm), in a Keplerian design, to give the beam a 4x magnification to ensure we completely fill the back aperture of the objective lens. L_1 is mounted to the optical bread board while L_2 is mounted a distance f_1+f_2 away on a custom designed 3D printed microscope head on top of the microscope. Along with L_2 is an angled mirror mounted on the head to direct the beam downward through the objective lens (RMS40X-PF) (NA=0.75) and onto the sample.

The microscope we used is an out of production accu-scope 3000 LED microscope (080096). We dismantled the eyepieces from microscope head and used our own custom designed 3D printed head to mount L_2 and a mirror. As well, we 3D printed a custom stage to mount a (LPS710E) piezo actuating stage for precise control over the axial dimension.

After leaving the sample, the light is of a longer wavelength and is de-scanned back through the same path. This time, however, it is transmitted through the dichroic mirror and using two more mirrors (for precision) is directed towards the spatial filter. Placed in between the mirrors is an EdgeBasic 532nm long pass edge filter (BLP 01-532R-25) to cancel any source light that could interfere with the imaging. The spatial filter consists of another objective lens (NA=) and a 20μm pinhole (P20D). The objective lens focuses the light through the pinhole where it is incident on a Hamamatsu current-to-voltage converter avalanche photodiode (APD) (C10508-01). The scanning mirror scans the sample point by point and the APD then converts the light into a photocurrent and then a voltage signal. Using a myRIO from National Instruments the voltage signal is converted into our final image.

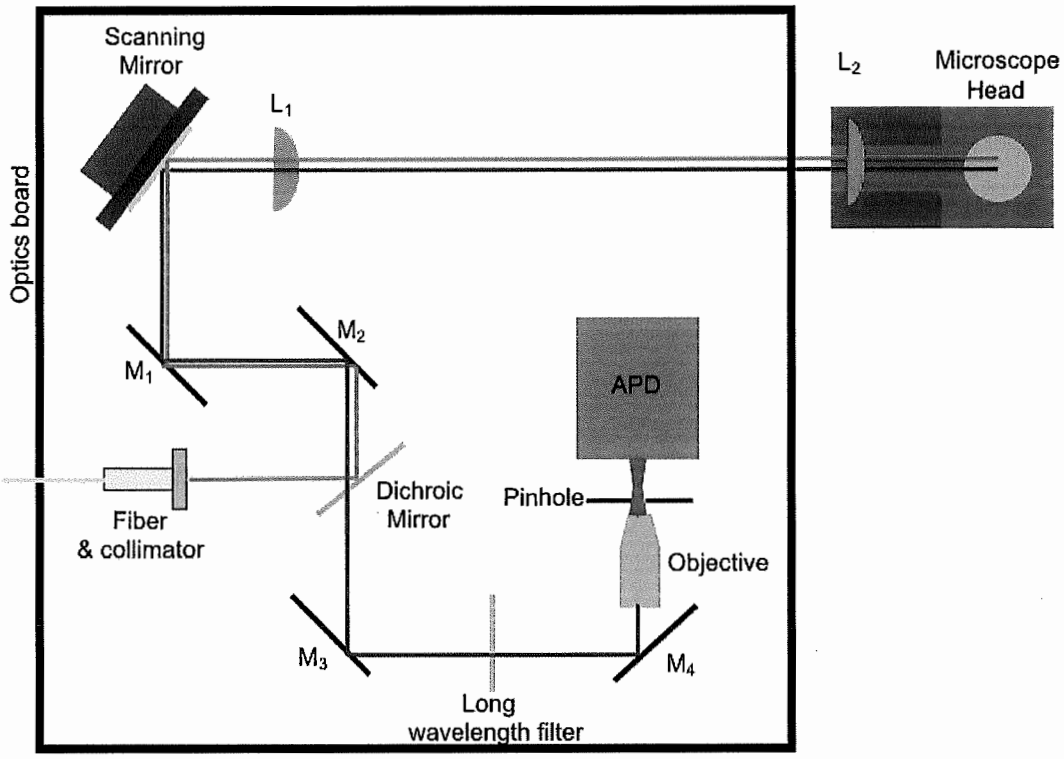

Figure 8. A schematic overhead diagram of the arrangement of the confocal optics. The circularized collimated beam (bright green ray) leaves the fiber and is directed towards the microscope head where it is directed downward and onto the sample. The dark green ray represents the emitted fluorescent light. It passes through the dichroic mirror and through the long wave length filter to ultimately be focused through the spatial filter and onto the APD.

Development of Transient Absorption System

The methods described above and the diagram remain roughly the same except for the removal of the spatial filter and the insertion of an optical chopper before the fiber launching stage (shown schematically in figures 9 and 10 below).

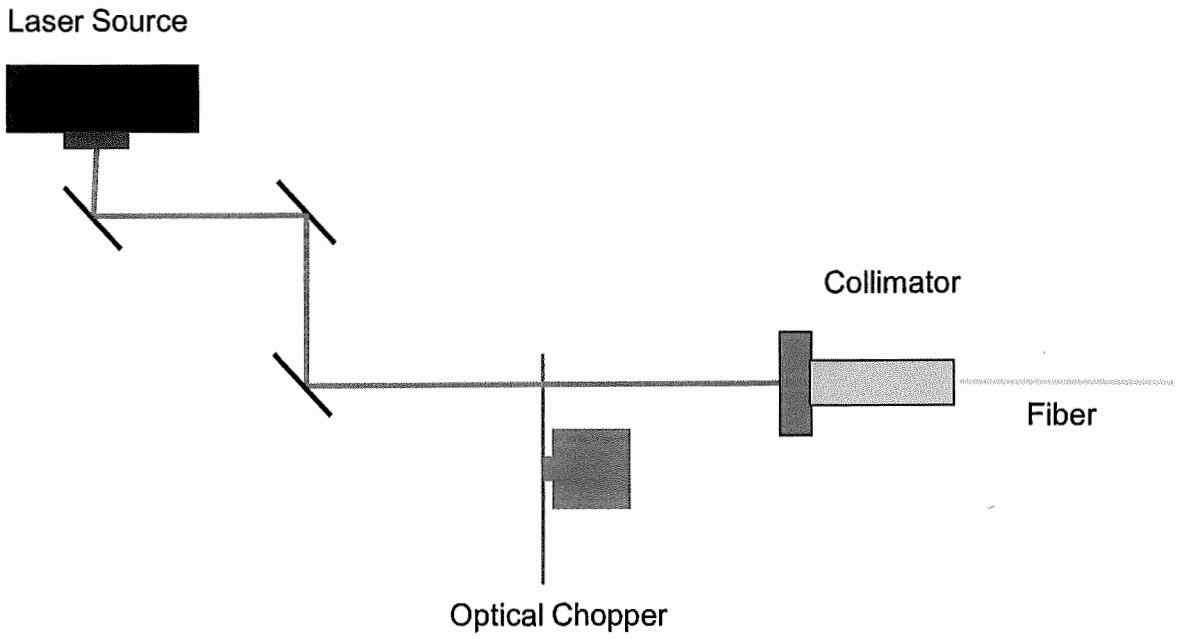

Figure 9. A schematic overhead diagram of the source laser and placement of the optical chopper before the fiber launching stage.

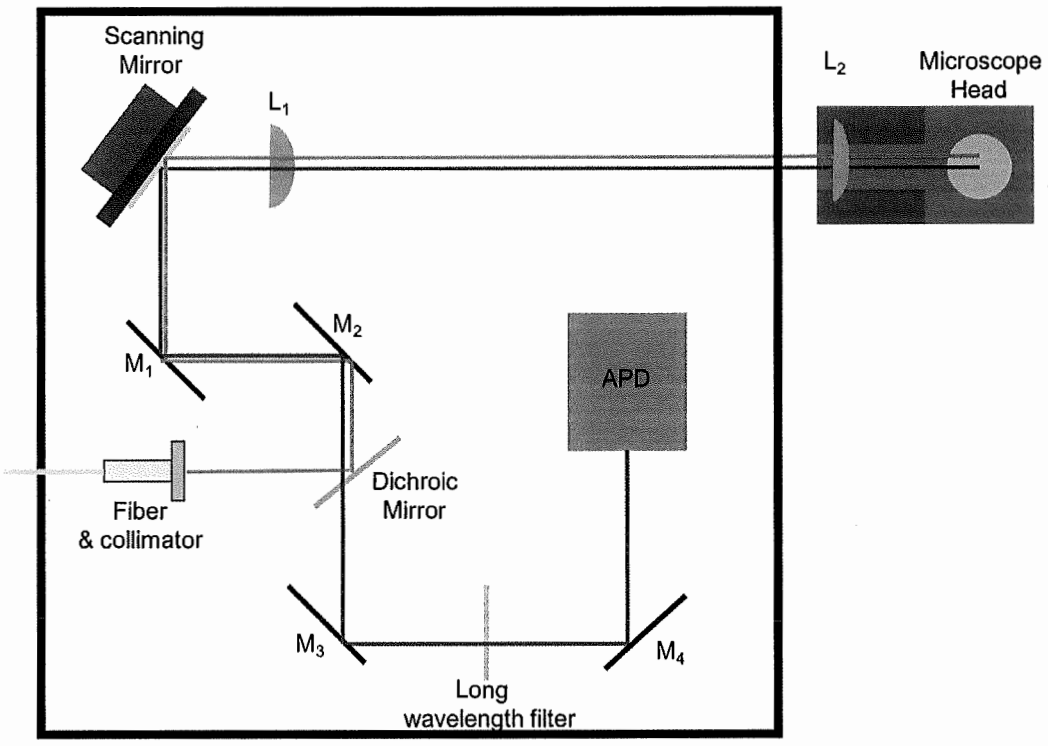

Figure 10. A schematic overhead diagram similar to figure 8, but without the spatial filter.

A typical system utilizing TAM, employs pulsed laser sources. This allows for the measurement of the rapid processes that occur in biological systems. Common laser sources used produce pulses ranging from picoseconds to femtoseconds [15]. Then some amplitude modulator is used to modulate the beam. With our system, we continue to use the same continuous laser source as before and an optical chopper to modulate the beam.

When exciting some of the electrons in the ground state with the pump we are depleting the number of electrons that can fluoresce. The probe then comes in and monitors the depletion. The chopper acts to modulate the excitation beam which, in turn, enables us to isolate the signals generated by the pump and the probe and the pump-probe interactions. The interaction between pump and probe alter the absorption

properties of the molecules. This variation in the absorption properties may be extracted from the beat of the pump and probe beams. When the pump and probe beams are modulated to the same frequency, the transient absorption signal appears at the second harmonic of the chopping frequency.

By placing an optical chopper in the beam path, we are modulating our Gaussian beam with a square wave. The Fourier series of a square wave only has odd harmonics. Modulating the beam produces another beam with a Gaussian intensity distribution and the frequency of a sine wave. The difference in beam intensities from our modulated Gaussian beam and the emitted fluorescence results in a signal that's gives rise to the same PSF as that of a confocal system. The signal appears as a second harmonic in an otherwise purely odd harmonic series.

The optical chopper is of our own custom design and printed from our in-house 3D printer. It is powered by a variable voltage source and driven by a simple DC motor. It consists of fifty rectangular slits, each measuring 2.6 mm by 5 mm, and rotates at a speed of 400 RPM which produces a chopping frequency of ω_p= 2 KHz.

The beam is directed in the same manner as before to the sample. The sample we used was a capillary tube filled with rhodamine dye, a dye that is commonly used in fluorescence microscopy. The beam is encoded with a frequency ω_p by the optical chopper. Using the same ADP, the signal is amplified by a signal processor amplifier with a gain of 200. The signal is then Fourier transformed via the fast Fourier transform to convert the signal to the frequency domain where we look for the second harmonic.

CHAPTER III

RESULTS AND DISCUSSION

Resolution of Confocal Microscope

Upon completing the final alignment of the spatial filter the we were ready to take measurements of the axial and lateral resolutions. To measure the axial resolution, a mirror was placed on the piezo stage to reflect the laser light back through the system. The long wavelength filter is removed and the dichroic mirror will allow the transmittance of a weak signal so long as the incoming light is of a high enough intensity. The stage is raised to place the mirror in the focal plane of the objective. Then, using the APD and a signal amplifier, the intensity of the reflected light is measured as the mirror is lowered away in 0.2 μm steps from the objective.

The signal received from the reflected light is a convolution of the PSF and the mirror. The signal is then de-convolved with a step function and the data is then collected. The graphs below show the normalized magnitude of the intensity versus the axial position of the mirror. The curve is then approximated by a Gaussian curve to show that the beam is indeed, Gaussian. To avoid the possibility of hitting the objective with the mirror, the stage was only lowered and not raised. Had we chosen to raise the mirror we would see a symmetric Gaussian curve. The axial resolution (or axial spot size) can be determined from the Full Width at Half Maximum (FWHM). By observing the graph, the intensity drops to half its maximum approximately at 0.75 μm. Thus, the axial resolution determined from the FWHM is approximately 1.5 μm which is also in accordance with the calculated value of 1.3 μm.

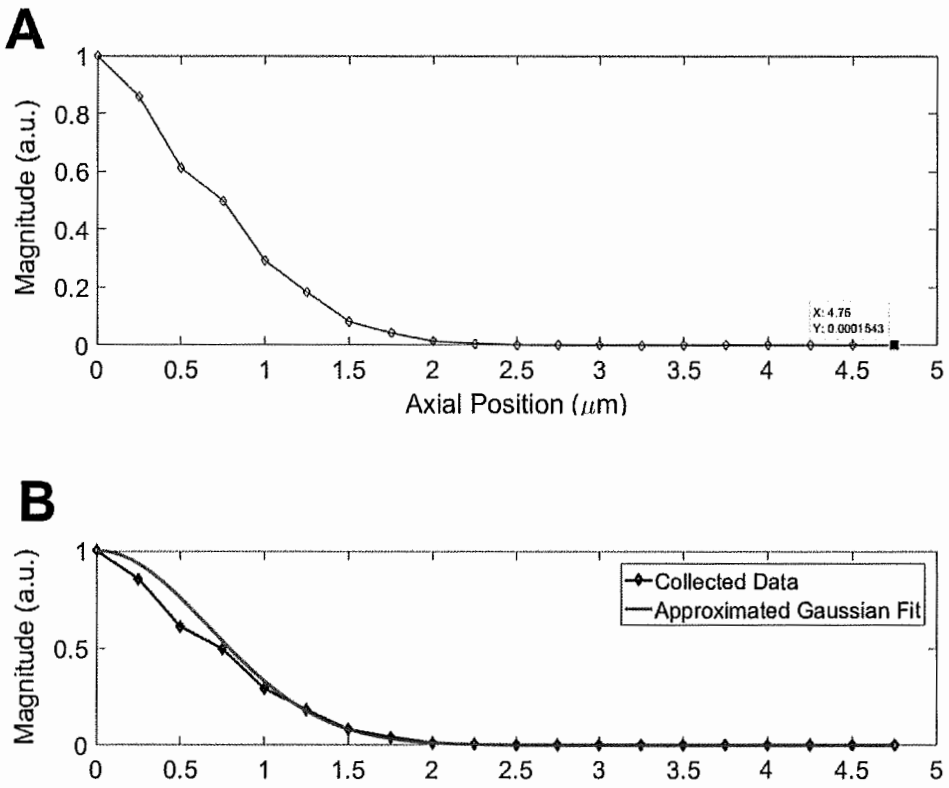

Figure 11. (A) Graph of the normalized intensity vs axial position of the mirror. (B) The normalized intensity compared to a Gaussian curve.

Next to find the lateral resolution we used a USAF resolution test chart, a commonly used procedure to determine lateral resolution of a system. The chart consists of a series of horizontal and vertical lines of a known thickness and spacing. By identifying the largest set of non-distinguishable lines, we can then find the lateral resolution of the microscope.

The chart was printed at 1200 dots per inch (dpi) which is roughly 21 μm. We then imaged group 1, element 2 that has a line spacing of 222.72 μm. We sampled a 100-

pixel x 100-pixel image of this region and took care to ensure that we scanned the entire region of space between the lines. Using just ±2V of power for the scan mirror we able to sample this region and obtain an image of the positive space between the lines. Using a de-speckle algorithm built into imageJ, a widely used imaging suite, we were able to remove some of the noise caused by using a continuous laser source. Then, by measuring the spacing of the positive region we can estimate the scan range of the microscope.

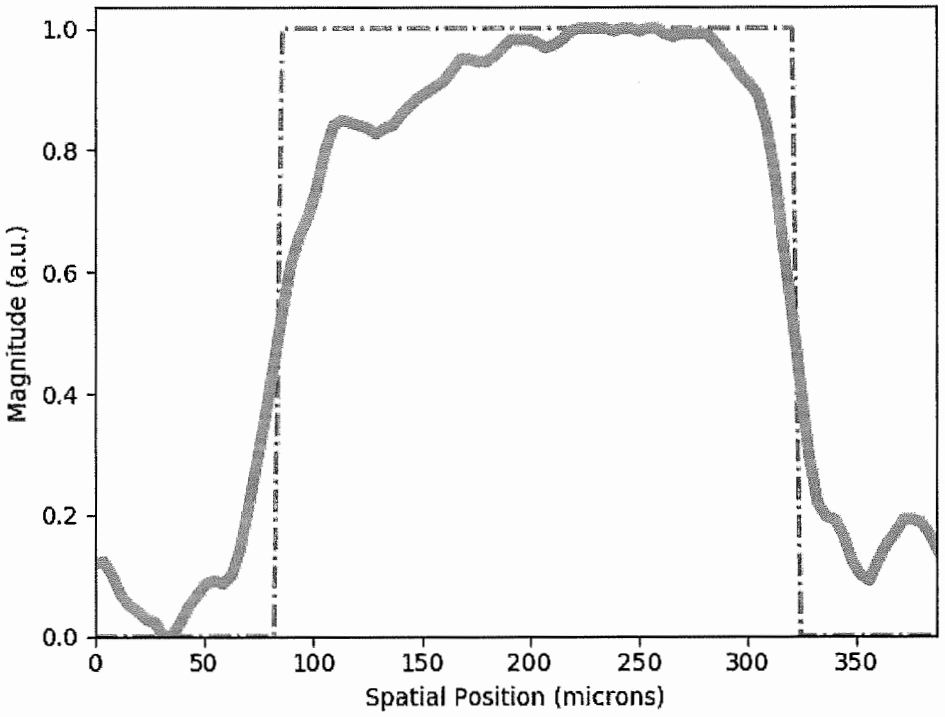

Figure 12. Normalized magnitude of intensity scanned across positive region of USAF resolution test chart. Group 1, element 2.

The image above shows a single line through our image. We us +/- 2V for a scan range of roughly 386 μm. The boxed region is used to calculate the percent of the image it took up. 222.72 μm is divided by that fractional value to obtain the full image spacing. The range of voltage used means that we were scanning 96.74 μm per volt for a total scan range of approximately 2 mm. Below is the image we acquired of the spacing between lines.

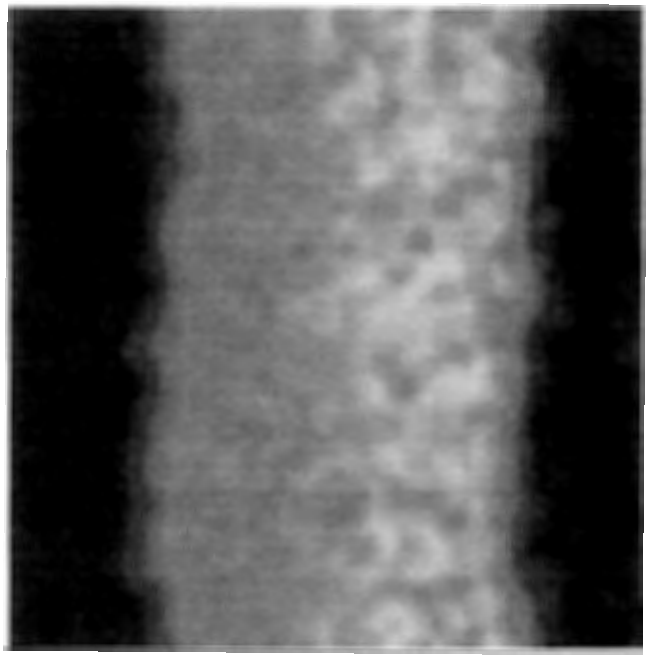

Figure 13. The image acquired from scanning positive region of USAF resolution test chart. Group 1, element 2.

The goal with our initial design was to produce a working confocal laser scanning microscope, which we did. However, we also wanted to leave room for

possible future projects to improve upon the microscope. One such opportunity for improvement is the size, complexity, and openness of the system. The system is rather cumbersome and difficult to use. It requires regular maintenance by an experienced user and the optics are completely exposed. A reduction in the size of the layout and an improvement on the stability of the optics will make the microscope more user friendly. A housing unit to enclose the exposed optics, too, will reduce the system's susceptibility to vibrations and becoming misaligned.

Another opportunity for improvement is the immobility of the microscope. Currently the microscope itself and the rest of the optics are bolted down to an optical table. A future goal would be to produce a design where we could construct a stable housing unit for the transportation of the microscope thus making it more accessible to other departments of interest.

TAM System

The spatial filter was removed after determining the resolution of the microscope and the optical chopper was placed right before the fiber launching stage to modulate the continuous beam. A capillary tube filled with rhodamine dye was placed onto the piezo stage at the focal plane of the objective. However, with the chopper in place a clear peak was present in the second harmonic of the chopping frequency even without the presence of the sample. Thus, the signal to noise ratio was too high and we were not able to isolate the second harmonic signal. With this noise peak present, we were unable to isolate the transient absorption parameter and thus would not be able to reproduce the same images as before with the spatial filter in place.

We suspect this peak likely arose from using an open loop design to the optical chopper as well as mechanical instabilities that arise from using plastic rather than metal for the chopper itself. In addition, the chopper wheel may not have laid flat enough to produce a clean unfluctuating chopping frequency. Alternatively the slits themselves could have inconsistences and may have all been of slightly different dimensions. Using sturdier materials and precision instruments a more refined chopper could be made and produce the desired chopping frequencies to acquire a confocal parameter.

Another option would be to use an acousto-optic modulator (AOM). AOMs can be used to control the frequency of light by making use of diffraction that occurs between sound waves and light. The acousto-optic interaction can be used to modulate the beam spatially and temporally. By spatially modulating the beam, one can create sources for the pump and probe and induce transient absorption without the need for an optical chopper.

Conclusion

I have demonstrated that one can successfully build a confocal laser-scanning microscope using common optical components one can find in an optics lab, by making use of recycled microscopes, and simple 3D printed parts. We have characterized the efficacy of this system to provide a measured axial resolution of 1.5 μm which matches well with the theoretical value of this device. Furthermore, we have characterized the scanning properties of the system to acquire accurate measurements of the system. In addition, I have laid out the ground works for possible future projects to modify and improve upon the microscope. As well, I laid the initial groundwork for experiments

involving transient absorption. The microscope design and layout can serve as a base for further exploration into the possibilities of fluorescence microscopy.

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