Microscopy Techniques Using Fluorescence Imaging

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CHAPTER 1: INTRODUCTION

I have explored three techniques in the field of fluorescence imaging microscopy: adaptive optics microscopy, superresolution imaging microscopy and spatial light modulation with adaptive optics microscopy. All of these techniques aim to produce quality images of biological processes that were previously unable to be imaged due to limitations in resolution, contrast, or refraction. Each technique is specific to various specimen and address critical issues in microscopic imaging of these specimens. In addition to advances in microscopy techniques, each project uniquely aims to provide further understanding and solutions to medical problems affecting many individuals on a daily basis.

Adaptive Optics (AO) microscopy involves the reapplication of AO technology to biological imaging in order to image through thick layers of tissue. The specimen of interest in this project was a *Drosophila melanogaster* (fruit fly) embryo. The particular specimen allows us to image important biological processes such as stem cell division, key events following fertilization, and neurogenesis, all of which occur deep within tissue. With conventional microscopy, there are limitations in resolution when imaging through thick layers of tissue.

A way to resolve this issue is to apply the technology of AO in order to correct for the distortion. Adaptive optics was previously used in astronomical imaging systems to resolve distortions caused by atmospheric turbulence\(^2\). Astronomical AO systems rely on a guide star, which is light from a star that serves as a point source for the system.
Distortion in the point source arises due to the atmosphere changing the index of refraction. The distortion is measured by a wavefront sensor and compensated for by a deformable mirror, which removes distortion.

In order for us to apply AO technology in biological systems, it is necessary to introduce a point source of light within tissue so that we can determine the distortions caused by tissue for a known source. Thus, one-micron fluorescent beads were used as an artificial guide star. These fluorescence beads worked well in reapplying AO to biological imaging as they provided the precise intensity of light needed for the wavefront sensor to measure distortion.

Fluorescence imaging technology also played a key role in the superresolution-imaging project, as it was used to image beyond the maximum resolving power of a conventional microscope. I used the flagella of *Chlamydomonas* cells as my model system. *Chlamydomonas* is a genus of unicellular green algae whose cells have two anterior flagella, which allow them to swim in an aqueous environment. I studied the mechanism and interaction of kinesin and dynein motor proteins in transporting interflagellar cargo (IFT cargo) back and forth along the unipolar array of microtubules. The two motor proteins were labeled with fluorescent dyes, each with a unique dye color, through a process called immunofluorescence. Thereafter, I applied Stochastic Optical Reconstruction Microscopy (STORM), obtaining preliminary images and setting the stage for acquiring superresolution images.

Finally, applying fluorescence when imaging through thick tissue causes a scattering effect, which leads to a loss in coherence of light and degradation of image resolution.
Research in this project aims to study the scattering effect in mouse brain tissue and develop the optical system in conjunction with the AO microscope to correct the effects of scattering and aberrations in thick tissue. The system applies a Spatial Light Modulator (SLM) to modulate the phase of incident light and focus the coherent light by interference. In order to perform calibration of the SLM an interferometric optical system was set-up. A LabView program was designed to allow calibration experiments, which entailed control of stage and camera. Calibrations results have demonstrated 0-2pi phase modulation and 0-255-intensity modulation per SLM pixel. Next, experiments on mouse brain tissue embedded with 1-micron fluorescent beads will allow measurement and correction for scattering effects. Following this step the SLM will be introduced to the AO microscope. A positive outcome from this research will be crucial to the study of neurodegenerative diseases.

MICROSCOPY

Microscopy is the technical use of microscopes, wherein objects or specimens that are otherwise unable to be seen through the naked eye are observed\(^1\). Microscopy’s three branches include optical, electron, and scanning probe microscopy. Optical and electron microscopy use the refraction, reflection and diffraction of photons and electrons respectively, scattering off of a sample to create an image. While optical microscopy uses visible light to attain a reflection, electron microscopy uses an electron beam, both of which result in producing an image. Scanning probe microscopy uses the interface of a scanning probe over the surface of a sample to build an image.
The three branches of microscopy make up the three different methods one can view an object. Optical microscopy is the most developed methodology consisting of various techniques. These techniques were developed in order to further imaging systems limited by refraction, resolution, and contrast. Electron microscopy has been able to produce much higher resolution images than optical microscopy, primarily due to the use of electron beams with smaller wavelength. Yet, electron microscopy still remains limited in practicality as compared to optical microscopy due to high costs and the need for a sample to be in a vacuum. Scanning probe microscopy requires a close to flat surface in order to allow the solid probe tip to scan in the vicinity of the object, and is primarily a technique for imaging beyond the maximum resolving power of a conventional microscope. The numerous microscopy techniques have provided much progress in biological research and continue to be used as important tools in the physical sciences.

**FLUORESCENCE**

In 1852, George Gabriel Stokes called it *fluorescence* when he witnessed the absorption of ultraviolet or invisible light and its emission as visible light. We now understand that fluorescence occurs when light with a particular frequency is absorbed by a particle and emitted at another frequency. From the perspective of photochemistry, fluorescence occurs when an orbital electron of a molecule, atom or nanostructure relaxes to its ground state after being excited to a higher quantum state by some type of energy and then emits a photon of light. Over the years, many fluorescence applications have emerged in biological, chemical and engineering research.
Fluorescence has had an impact in biological sciences in allowing a non-invasive approach to analyzing and tracking biological molecules. This approach is achieved by using fluorescent beads, dyes, and proteins, all of which have exhibited far-reaching improvements in biological research.
CHAPTER 2: ADAPTIVE OPTICS MICROSCOPY: IMAGING THROUGH THICK TISSUE

Many important biological processes occur deep within the tissue of fruit fly embryo. In applying AO microscopy, we will be able to obtain further understanding of key events following fertilization as well as stem cell division and neurogenesis, each of which play a crucial role in the fruit fly developmental process. Adaptive optics can be used to produce clear images of these processes occurring deep within tissue. This work is focused on the reapplication of AO technology in biological imaging. A novel method is used to measure the aberrations introduced by a *Drosophila melanogaster* (fruit fly) embryo with an implanted 1-micron bead that serves as a point source reference beacon.

AN ADAPTIVE OPTICS SYSTEM

In order to gain a better understanding of adaptive optics as it is applied to biological imaging, we must first understand its origins when applied to astronomical imaging systems. A significant limitation in astronomical imaging with ground based telescopic systems is that the atmosphere causes turbulence leading to distorted images. Adaptive optics is able to improve the quality of astronomical imaging from ground based telescopic systems by using real-time measurements of the turbulent medium in the optical path, therefore removing the distortion (Fig 1).
Fig. 1: This image demonstrates correction of an astronomical image when adaptive optics is turned off and on. Image by Nick Law (Caltech) / Craig Mackay (Univ. of Cambridge).

A natural or artificial guide star is used as a point source reference beacon to measure the aberrations introduced by the turbulent medium. As light passes through the turbulent media, the changing refractive index is collected in the resultant wavefront. It is important to mention here that we are correcting for the distorted wavefront as it was affected by the changes in refractive index.

AO systems have generally consisted of three main components: deformable mirror, wavefront sensor, and control computer, as shown in Fig. 2. These components work in a feedback loop system, allowing for continuous real-time image correction to take place.
Fig. 2: This image illustrates a common AO system setup and the optical path along the system. The three main components include: a) Deformable mirror: produces reciprocal wavefront to correct for the aberration. b) Wavefront sensor: measures the light aberration in the optical wavefront. c) Control System: gathers information from sensor and calculates for proper adjustment of the deformable mirror.

In order for us to best understand an AO system, we must be able to trace the optical path. As illustrated in Fig. 2, a distorted wavefront enters into the microscope; this wavefront is reflected onto the deformable mirror, which initially serves as a planar mirror. The deformable mirror reflects the light onto a beam splitter, usually consisting of two filters: a spatial filter and a color filter, which are essential in allowing some light to be imaged by the camera while the other light is reflected onto the wavefront sensor. In the wavefront sensor, the wavefront of the light is collected and the derivative of the
wavefront is calculated and this information is sent into a control system. Due to the non-linearity between the wavefront sensor and deformable mirror, the control system performs necessary computations to provide a reciprocal wavefront for the deformable mirror. Once this process has taken place, the deformable mirror creates the reciprocal wavefront in order to perform wavefront correction. Finally, as correction takes place the light going through the beam splitter enters a lens, which focuses the light onto a high-resolution camera for imaging.

OPTICAL ALIGNMENT AND SETUP

My contribution to the AO microscopy system was primarily to the alignment and setup of the system as shown in Figure 3. Building the optical system consisted of numerous instances of trial and error as misalignment and meeting design goals caused us to restart building the system.

In the following paragraphs, I highlight the three main procedural steps, which were important to this project. Each step served as foundation to the following step, therefore allowing organization and timely completion of the project.

i) Build and align optical system: Through a step-by-step process, the lenses and other optical equipment such as mirrors, filters, and cameras were aligned. One key part while performing this task was the handling of lenses and mirrors. Careful measures were taken in order prevent dust and finger prints on the lenses as well as in the slight
movement of aligned components. These measures were important since any mistake could have resulted in realignment of the whole system.

ii) Testing of system setup: All optical components were centered at their axes, and were tested by passing a laser beam through the system from one end and detecting the beam at the other end of an optical fiber. The intensity of light at the end of the optical fiber illustrated whether all components were centered. Next, the camera and wavefront sensor were centered along the same optical path.

iii) Test images with complete setup: Finally the system was ready for images of microspheres on a glass slide. A laser beam was sent through the system, which caused the fluorescent beads to emit light. This allowed for the camera and wavefront sensor to detect light going through the optical system, therefore concluding the optical alignment.
Fig. 3: The schematic illustrates the setup of the optical system and the path of the laser within the system. The system consists of two cameras, fiber optic laser, multiple lenses, objective lens, specimen stand, multiple mirrors and a central computer. This schematic clearly shows the different stages the light from the laser, would travel in order to perform adaptive optics.

DEEP TISSUE IMAGING OF FRUIT FLY EMBRYO USING AO TECHNOLOGY

Deep tissue imaging of the fruit fly embryo is impractical with conventional microscopy due to the optical aberrations that are caused by cellular structures, such as cytoplasm and the plasma membrane. Fig. 4 demonstrates the distortion of an image from deep within tissue.

Fig. 4: This figure illustrates the extent of distortion when imaging 30μm below the surface of the embryo. Image by Oscar Azucena.
Important biological processes take place deep within the tissue of the fruit fly embryo, including stem cell division, key events following fertilization, and neurogenesis. In order to allow deep tissue imaging using AO technology, we need to obtain the distortion of the wavefront being caused by the thick tissue. The distortion is measured by generating a point source of light deep within tissue to be imaged. A point source in our case was generated from a 1-micron fluorescent bead, which was injected into the embryo. The beads absorb laser light and emit light of a different frequency back into the system AO system. The optical path of the laser light as it passes through the tissue is shown in Fig. 5.

![Diagram showing the path of light through tissue](image)

Fig. 5: The path of the light as it passes through tissue is denoted as $\Delta n$ referring to the change in refractive index. Next, an aberrated wavefront emerges and is imaged onto the objective lens.

The aberrated wavefront of light from the fluorescent bead enters the AO system. The wavefront sensor finds the aberrations along the optical path and communicates this information to the control computer, which calculates the point-spread function (PSF) and produces a Gaussian graph. The PSF measures the intensity of light at each pixel and
the Gaussian curve graphically illustrates the PSF distribution. The Gaussian graph and science camera images in Fig. 6a and 6b demonstrate the ability for the system to detect aberrations along the optical path. Since our system did not contain a deformable mirror we were only able to test for wavefront aberrations. The images obtained have set the stage for further developing this project to obtain actual corrected images of the embryos.

Fig. 6: Images taken with the science camera to the right and a Gaussian graph to the left. 

a) A bead at the surface and b) a bead 100μm deep within the tissue of the fruit fly embryo.

This project allowed me to attain knowledge in the field of optics and microscopy. In particular, it incited my interest towards pursuing a career related to biological imaging, a field open to further advancement. With this technology, we will be able to look evermore closely into the human biological make-up.
In my research with the superresolution-imaging project, I sought to study biological processes that occur beyond the imaging capabilities of a conventional microscope. My particular area of study was the molecular mechanism of cytoplasmic dynein, which is involved in the transport of cargo towards the microtubule minus end of eukaryotic cells. Specifically, we used intraflagellar transport (IFT) in *Chlamydomonas* cells as a model system to study interaction of IFT dynein with kinesin II, which is the opposite polarity motor that moves cargos toward the plus end. To detect the distribution and cargo interaction of single motor proteins along the flagellum, I employed Stochastic Optical Reconstruction Microscopy (STORM) microscopy. STORM will be explained in further detail, but in short, it is able to detect the position of a single-molecule by selectively activating and deactivating fluorescent dyes and determining the position of an activated molecule by finding the center of the distribution of light. This technique has been used to construct superresolution images of precisely positioned fluorophores from single-molecule images. In order to facilitate STORM imaging, an immunofluorescence technique was applied to the Chlamydomonas flagella, which consisted of primary and secondary antibodies. I stained the kinesin II and IFT dynein motors in the *Chlamydomonas* flagella with primary antibodies and photoactive dye-pair labeled secondary antibodies. The cells were imaged under a deconvolution microscope to exhibit successful immunofluorescence. With successful immunofluorescence, I was able to obtain preliminary STORM images demonstrating photoswitching of fluorophores in the flagella. In the future, the quantitative analysis of the STORM images will allow us to determine how many kinesin and dynein motors actively work on a cellular cargo, and how the cargo direction is determined by kinesin-dynein interactions. IFT is a universal process in all eukaryotic cilia and flagella. Defects in this process are the primary causes of polycystic kidney disease and retinal degeneration.
INTERACTION BETWEEN MOTOR PROTEINS AND IFT CARGO

There is still a great deal to be learned about interflagellar systems. Our objectives in this project are to obtain an overall distribution of kinesin and dynein motors along the flagella, to determine the number of dynein and kinesin motors bound to a cellular cargo, and finally to determine what occurs at the turnaround zone where a switch from kinesin to dynein motors takes place. In order to experimentally address these objectives, a superresolution imaging technique known as Stochastic Optical Reconstruction Microscopy (STORM) was applied.

CHLAMYDOMonas MODEL SYSTEM AND IFT SYSTEM

Chlamydomonas is a genus of unicellular green algae (Chlorophyta) as seen in Fig. 7. These algae are found all over the world: in soil, fresh water, oceans, and even in snow on mountaintops. Algae in this genus have a cell wall, a chloroplast, an "eye" that perceives light, and two anterior flagella with which they can swim using a breast-stroke type motion\(^1\).

Fig. 7: An illustration of a common chlamydomonas cell, with its flagella extending out\(^2\). Image from US public health publ. #657 1959.
The interflagellar system assembles, maintains, and disassembles the cilia and flagella of a cell. Eukaryotic flagella are highly specialized for long-range transport where kinesin and dynein motors carry long rafts of intraflagellar cargos (referred to as IFT trains) back and forth along the unipolar array of microtubules shown in Fig. 8. In particular, the IFT particles move towards the plus end by kinesin II and back towards the minus end by IFT dynein. Each IFT particle is 100-400 nm of linear array of discrete subunits.

Fig. 8: The IFT system begins assembly of motors and cargo in the minus end of the microtubule array, next the kinesin motors step in hand-over-hand motion to the plus end of the microtubules. At the tip of the flagella which is the turnaround zone, kinesin motors are released and dynein motors began making their way towards the minus end or the base of the flagella. Along both directions of the microtubule path the motors carry IFT cargo. Image by Inglis P.N. et al.

STOCHASTIC OPTICAL RECONSTRUCTION MICROSCOPY (STORM)

Conventional microscopy is limited in imaging beyond half the wavelength of light, which is known as the diffraction limit. Images become blurry beyond the diffraction limit, primarily due to the point spread function (PSF) of each light particle overlapping one another resulting in a loss of precise positioning of each particle. In order to
overcome the issue of overlapping PSFs, STORM is able to detect the position of a single-molecule by stochastically activating and deactivating fluorescent dyes and determining the position of an activated molecule by finding the center position of the light distribution. The center is found by fitting the PSF of the image into Gaussian function as shown in Fig. 9.

Fig. 9: a) This image illustrates the Gaussian function distribution of eight single molecule fluorescent particles. b) The Gaussian function of a single molecule is shown and the arrow illustrates its center distribution. Image by Yildiz et al.

Photoactivation and photobleaching allow stochastic photoswitching of fluorescent dyes. An image of each instance of stochastic photoswitching is collected. Each image is then sent through an algorithm (Fig. 10), which localizes a single fluorescent particle in its precise location by obtaining the center distribution of the particle (Fig. 9B). A superresolution image is then produced, consisting of precisely positioned single molecules.
Fig. 10: The essential STORM technique is demonstrated here where we have six fluorescent molecules with photoactivation and photobleaching properties. Initially all of the molecules are bleached, then a single flash of light corresponding to the activation property is introduced and then bleached with again, hence photoswitching. This causes stochastic fluorescence of a single molecule, allowing localization. This cycle of photoswitching and localization continues until a final superresolution image is produced\textsuperscript{11}. Image by Michael J Rust et. al.

STORM imaging was applied to our model organism by the use of an immunofluorescent technique. Photoswitchable cyanine reporter and activator molecules were coupled to antibodies against kinesin II and IFT dynein. The cyanine reporter and activator dyes allow photobleaching and photoactivation to occur. Photobleaching is the photochemical deactivation of a fluorophore\textsuperscript{16}, which is caused by high intensity light exposure and results in the fluorescent particle to enter into a dark state. The particle is stochastically recovered back to its fluorescent state through photoactivation, which reactivates the fluorophore by means of light. The role of the reporter dye is to enter the dark state when exposed to a particular wavelength of light and the role of the activator dye is to cause the reporter dye to reactivate when exposed to its own particular wavelength of light\textsuperscript{11}. The phenomenon of the activator dye causing the reporter dye to reactivate is not fully
understood. The coupling of the reporter and activator dyes is further explained in the next section.

**IMMUNOFLUORESCENCE**

Immunofluorescence is a technique primarily used in biological samples. The specificity of antibodies to antigens is used to label fluorescent dyes to specific biological molecules in a cell, therefore allowing visualization of target molecules in the sample. Immunofluorescence makes use of direct and indirect protocols to stain cells with fluorophores. Direct staining binds a primary antibody linked to a fluorophore to the target molecule, whereas indirect staining makes use of both a primary antibody, which binds to the molecule and also a secondary antibody linked to a fluorophore, which binds to the primary. The indirect staining protocol allows more flexibility in the variety of target molecules to be stained, and was therefore employed in my project as illustrated in Fig. 11.

![Immunofluorescence schematic](image)

**Fig. 11:** Shown here is an image of the immunofluorescent setup and the specific location the primary antibody would bind to either kinesin or dynein motors. Bound to the primary antibody is the secondary antibody linked to the activator dye Cy5, and reporter dye Cy2 or Cy3. The dye pairs Cy2-Cy5 or Cy3-Cy5 coupled together allow
photoswitching. Reporter dyes vary in order to allow distinction between kinesin and dynein motors.

Kinesin II and IFT dynein motors were stained in the *Chlamydomonas* flagella with respective primary antibodies and photoactive dye-pair labeled secondary antibodies.

**EXPERIMENTATION AND PHOTOSWITCHING RESULTS**

Secondary antibodies were first labeled with Cy2-Cy5 and Cy3-Cy5 dye-pairs to establish reporter and activator ratios in order to allow a precise number of fluorophore photoswitching at one time. The ratios for Cy2-Cy5 and Cy3-Cy5 were set as 1:3.5 and 1:2.5, respectively. Single-molecule experiments were performed by creating a flow chamber in which secondary antibodies linked with fluorophores were flowed into a glass slide to be imaged and examined for concentration, distribution and photoswitching of fluorophores as shown in Fig. 12.

![Fig. 12: The three images illustrate the three instances photoswitching of fluorophores label onto secondary antibodies took place.](image)

The full staining protocol consisting of both primary and secondary antibodies was applied to the *Chlamydomonas* cells. During this process, I was met with many challenges in attaining successful staining. One of the initial issues was due to autofluorescence, which is light being emitted from other sources besides the fluorophore. This issue was resolved when paraformaldehyde was removed from the protocol. Other
issues with regards to cell preparation and fixing were dealt with by running checks at each stage of the staining protocol (see appendix A). After resolving the errors in each step, the immunofluorescence technique was established so the cells were imaged under a deconvolution microscope shown in Fig. 13 to exhibit successful immunofluorescence.

Fig. 13: The images of the *Chlamydomonas* cells shown here demonstrate successful staining of the kinesin II and IFT dynein motor proteins with primary and secondary antibody.

Next, the immunofluorescence technique was put into the test to demonstrate whether it was applicable to STORM imaging. As a preliminary test I wanted to see photoswitching take place when immunofluorescence is applied and the motor proteins are labeled with specific fluorophores. Fig. 14 demonstrates these successful results.

Fig. 14: Shown here are preliminary photoswitching results of kinesin II motor proteins labeled with Cy3-Cy5 dye-pairs.
In the future I hope to take these experiments to the next step and obtain STORM images with which we can determine the position of motors along flagella, and how many motors are bound per IFT cargo and finally present a model of the interaction occurring between motors and IFT cargo.
CHAPTER 4: AO-SLM MICROSCOPY: MINIMIZING SCATTER EFFECTS THROUGH THICK TISSUE

Fluorescence imaging has become an important tool for biological research and continues to open new avenues in its capabilities. A significant limitation that has arisen in imaging fluorescent particles embedded in tissue is the scattering effect caused by particles smaller than the wavelength of light. A technique which would allow minimizing of the scattering effect is shaping the incident wave spatially or temporally to match the scattering behavior of the tissue. With the scattered wave we can constructively interfere scattering from tissue thus creating a focus point. This method is also known as ‘interferometric focusing’. In order to construct a scattered wave with which to interfere the scattering arising from tissue, a liquid crystal Spatial Light Modulator (SLM) is used. The SLM is able to modulate phase and intensity of light at each pixel. We aim to minimize scattering effects arising from imaging mouse brain tissue and introduce this system to the AO microscope in order to improve resolution in deep tissue imaging.

INTERFEROMETRY

The principle of superposition is used in interferometry in order to bring together separate waves so that the result of the combination would allow some understanding of the initial wave. The constructive and deconstructive resulting attributes of the waves would indicate a phase difference between the two combined waves in the same frequency. It is common for most interferometers to use laser light as the initial source of light.
Most often in interferometric systems coherent light is split into two equal beams by a partial mirror, resulting in the beams traveling in two different paths. Eventually the two beams are recombined as they approach a detector. Phase difference comes about as a result of the difference of distance traveled by each beam. As was indicated above the phase difference is what leads to the interference pattern of the beams. Thus, the phase difference is diagnostic of changes that occur along the paths of the beams, these changes maybe as a result of change in refractive index or path length of beams.

**APPROACH IN MINIMIZING SCATTERING**

Light becomes scattered when entering opaque martial. Scattering arises due to random orientation of nanoparticles (Fig 15). A fluorescent particle is imbedded in tissue and when illuminated scatters light. In order to overcome this issue, constructive interference of light is used to focus light at a point. Scattered light from fluorescent particle and the shaped wave scatters allow the interference to occur (Fig 15). The shaped scattered light under goes phase delay so that the scattering and interference paths are used to focus the light at the point source.
Fig: 15. On the left, the scattering of light when entering an opaque media is illustrated. On the right, fluorescent particle embedded inside opaque media is illuminated via the shaping of the light entering the tissue. (Ivo Vellekoop et al.)

OPTICAL SET-UP FOR INTERFROMETRY

In order to calibrate the SLM an optical system was set up to perform interferometry. Calibration of the SLM meant each pixel in an array of 1024x768 could have a phase modulation of 0 to 2pi and intensity modulation of 0 to 255. In order to achieve this the precise polarizer and analyzer combination need to be attained. The polarizer and analyzer together can construct a wave angle of light that would allow the particular modulation specifics.

The designing of the set-up began and proved timely since all of the optical components did not fit in the original area, and were therefore moved to a more accessible area on the optical table. This allowed re-alignment and designing optics such that all components
were accessible for wiring and alteration. The beam splitter and stage with mirror were added on the design. Finally, after receiving the polarizer and analyzer the optics were all aligned (Fig. 16). In order to test SLM and run SLM software for the first time, functionality tests were done and proved successful. Next I began programing for calibration using LabView.

Fig 16. Shown here is a schematic of current optical set-up at MEMS lab. The laser provides a beam of light, which passes through polarizes P1 and P2 allowing control of the intensity of light. Next, the beam undergoes focusing and collimation through L1 and L2 respectively, in order to resize the diameter of the beam for SLM. At the beam splitter some of the light passes through to the SLM and the rest is reflected onto mirror. The mirror and SLM both reflect light back into beam splitter, wherein the beam is directed towards analyzer for increased variability in intensity and finally focused with lens L3 into the DALSA camera.
LABVIEW PROGRAMMING

In order perform interferometry and calibrate the SLM, I needed to be able to control the camera, and stage. The stage consisted of the mirror which needed to preform a step-wise function. This would allow us to determine phase change. With little experience in labview I began making small programs to capture images from camera and save the images. Then I went on to control the stage, so that it could operate with a step-wise function. I also needed a program to control the intensity modulation on the SLM which required displaying a large image to be reflected on SLM. This image could be varied so that intensity modulation experiments were made possible. Finally, I brought together all the pieces of the program together and created an interface. The interface had a display of the current image, voltage or position input for stage and files in which captured images can be saved (Fig. 17).

![Interface console with calibration experiments](image)

Fig. 17: This is the interface console with which calibration experiments were performed.
PHASE-SHIFT CALIBRATION

The phase-shift calibration of the system was done in partnership with our post doctorate Xiadong Tao, who wrote the Matlab program to perform calibration. This program was based on an algorithm (1), which collected a series of 5 interferograms with equally spaced steps. This algorithm was found in the Optical Shop Testing\textsuperscript{18} book, which demonstrated how to determine the phase-shift.

\[
\alpha(x, y) = \cos^{-1}\left[\frac{1}{2}(I_5 - I_1) / (I_4 - I_2)\right]
\]

Schwider et al 1983, Cheng and Wyant 1985

This meant a series of 5 images, needed to be collected, each image stepping through increasing distance from the initial point (Fig 18).

Fig 18. (a) 0V (b) 50mV (c) 100 mV (d) 150mV (e) 200mV. Each image a-e illustrates the series of images with increasing phase change at each step.
With these images the program is run to find the phase shift. In order to find the 90 degree optical phase shift the following calculation needed to be done:

\[
90^\circ \left( \frac{v_{in}}{\alpha} \right) = v_{out}
\]

Once all of the new images are collected and processed through program, we should end up with a 90 degree phase shift, thus illustrating the calibration of the system. In order to illustrate the calculated results a histogram is produced to show the measured phase shift (Fig. 19).

![Before](image1.png) ![After](image2.png)

Fig. 19: The histogram demonstrates that the phase-shift before and after calculations were performed.

Issues in obtaining consistent stage movement resulted in a few changes were so that all optical parts were stable on the optical table but a drift like movement was still present. We finally came to the conclusion that this was as a result of the particular stage being
used and thus a replacement was made. The phase shift was set at 90\(^\circ\) with the step-size was calibrated to 0.0345 Volts per step.

**SLM CALIBRATION**

With the new found step-size for phase modulation I was ready to begin calibrating the SLM for 0 to 2\(\pi\) phase modulation. Intensity modulation was previously set-up, after the labview program was capable of displaying optical function images on the SLM. Our aim was to obtain maximal intensity for a phase modulation of 0 to 2\(\pi\). In order to obtain these specifics, a particular polarizer and analyzer angle pair need to be formulated. To obtain this we sought to experiment with various polarizer and analyzer combinations to find the optimal combination. These experiments continued to fail in finding the optimal combination. Alternatively, the SLM manufacturer provided us with various methods to obtain the desired modulation.

As a result polarizer and analyzer angles 21\(^\circ\) and 135\(^\circ\) respectively, demonstrated sufficient intensity modulation and phase modulation. Intensity modulation allows a full range of 0-255 gray scale spectrum of modulation with very minimal change in intensity (Fig. 21). Similarly with phase modulation we see a full 0 to 2\(\pi\) or 360 degrees modulation at increasing gray scale intensity (Fig. 22). These results indicate that we are now able relate a particular intensity of light found at the detector to a phase at very high accuracy. This accuracy is as a result of the intensity modulation having very minimal change over a change in grayscale. Therefore, when the intensity is determined from the detector it is very close to the exact intensity and thus can be correlated to the right phase.
Fig. 21: Intensity distribution, on the x-axis is the 0-255 intensity modulation and on the y-axis is the 0 to 1 power spectrum.

Fig. 22: Phase vs. Intensity distribution, on the x-axis is the 0-18 frames of gray scale intensity modulation and on the y-axis is the 0° to 360° phase modulation.
AO-SLM DESIGN

The next steps for this project will be to develop an algorithm with which to perform real-time SLM calibration based on scattering arising from tissue. This algorithm will have to modulate both phase and intensity of each pixel segment on SLM. Experiments will be performed to obtain maximal intensity from an embedded bead within tissue.

The SLM will be implemented into the Adaptive Optics confocal microscope (Fig. 23). The implementation will allow an increase in depth of imaging within tissue as well as an increase in resolution. It is also important to note that the SLM will compensate for AO where it becomes ineffective due to nanostructured media. Finally, a limitation in this system will be due to multiple beads at close range to each other. This limitation may be overcome by focusing on well spaced beads, with minimal proximity to other beads.
Fig. 23: Illustrated here is the schematic of the design for the AO-SLM microscope. A HeNe laser is used to fluoresce the bead embedded in the mouse brain tissue and a solid state green will be used to fluoresce the GFP tagged neurons of the mouse brain tissue.

The AO-SLM combination will be a novel technique in improving deep tissue imaging of mouse brain tissue. These images would be able to provide more detail of neuronal organization and with subwavelength resolution. Images at these depths of detail can have an impact on the study of neurodegenerative diseases.
CHAPTER 5: CONCLUSION

The history of microscopy began along with the development of optical technology since a microscope consists of an orientation of optical components, which allows observation of objects beyond the resolution of the unaided eye. Al-Hazen laid down the foundation of optical technology in his *Book of Optics*, which he wrote in a span of ten years from 1011 to 1021. His ideas in optics flourished and resulted in the development of the microscope¹. As a scholar of high caliber and one of the first scientists who sought answers through experimentation¹⁷, Al-Hazen has been an inspiration for me throughout my studies.

In gaining knowledge of microscopy and fluorescence, I came to an understanding of their impact on physical and, most importantly, in biological sciences. In building the adaptive optics (AO) microscope, I learned the importance of experimental design and setup along with the ingenuity required to introduce a new technique to the field. We were able to demonstrate that deep tissue imaging was possible by applying AO technology to biological imaging through the use of a fluorescent bead as an artificial guide star. Preliminary images without the deformable mirror illustrated that we can measure the aberrations from the biological specimen. The AO microscope has now been developed to its full potential by Oscar Azucena, who was the graduate student whom I worked with in the initial stage of the project. The microscope is now able to measure and correct for aberrations caused by biological specimen. My work in this project and
Al-Hazen’s work on optics inspired me to continue pursuing knowledge in the area of microscopy. Thus, I went on to explore other areas of microscopy, and began working in biophysics lab which focused on single-molecule fluorescence microscopy.

With my new found inspiration from Al-Hazen and I began learning a new technique and performing experiments in order to implement this technique in a new system. I learned that single-molecule fluorescence technology had a significant impact in superresolution imaging as it allowed imaging beyond the diffraction limit. I understood the importance of creating an immunofluorescence technique for superresolution imaging in order to study the IFT system in *Chlamydamonas* flagella. Thus, the immunofluorescent technique with which we can apply STORM to *Chlamydamonas* flagella was developed. The technique was tested and the results showed successful immunostaining. Next, preliminary STORM images were taken to illustrate photoswitching of fluorescent dye pairs stained onto motor proteins. These experiments set the foundation with which to produce superresolution images. In the near future, I plan on completing this project by obtaining the superresolution images and studying the IFT system. With the images we will be able to create a hypothesis of transport processes occurring in the IFT system. Work in this project was presented at the Biophysical Society annual meeting in Baltimore, MD and an abstract was published in the Biophysical Journal.

Finally, in an attempt to further study the particular field of deep tissue imaging using fluorescent technology I delved into utilizing, spatial light modulation (SLM) in order to minimize the effects of scattering. Continuing on with Al-Hazen’s inspiration, the optical
set-up to perform interferometry was completed. I then went on to programming using Labview, which allowed me to control the stage, camera and SLM. The interface was built and the phase-shift step-size was determined. I then went on to calibrating the SLM to obtain 0 to 2π phase modulation and 0 to 255 intensity modulation of each pixel segment of SLM. Results thus far have demonstrated a peak to valley ratio of intensity as 0.46. The project is underway to move into its second phase of developing an algorithm for real-time modulation and its implementation into an adaptive optics confocal microscope. This work has been in partnership Dr. Xiaodong Tao, whoes a post doctorate research in our lab. With his guidance and assistance I have been able to bring this project to this stage. I hope to complete this project and publish the results in the near future.
APPENDIX A: IMMUNOFLOURESCENCE PROTOCOL FOR CHLAMYDOMONAS

Day 1:
1. Block Solution
   - 100% Block = 5% BSA + 1% Cold water fish gelatin or donkey serum in 1X PBS.
   - Set up 2 coupling jars, write labels in front of each jar with sharpie so you know which way the cover slips will be facing (maintain this throughout Protocol).
   - Fill coplin jars with -20C degrees of methanol.
2. Poly-lysine on cover slips
   - Put 200µL of Sigma poly-lysine solution onto each 22x22 coverslip and wait 10 mins.
   - Dip the coverslips into a beaker of pure water.
   - Dry coverslips on a kimwipe and place coverslip on kimwipe with poly-lysine coated side facing up.
   - Use air suction to dry off remaining water.
3. Fixing cells
   - Mix 16% paraformaldehyde 1:3 with healthy-looking cells (check first whether most of them are swimming in the bright field). This step should last as little time as possible; fixation is pretty much instantaneous.
   - Put 100 µL of cells on the coverslips wait 2-5mins or recommended 10mins.
   - Tap coverslips one at a time against kimwipe to get rid of excess cells and media.
   - Make sure to keep the side of coverslips with cells facing the label on jars and place them into the coplin jars with the -20C methanol.
   - Leave jars with coverslips inside in -20 degrees freezer for 5 mins.
   - Transfer coverslips into a second coplin jar of -20c methanol and let it sit for 5mins (optional).
   - Dump the methanol and replace with 50% MeOH/50% PBS and wait for 5 mins in room temp. (Ben style= freezer)
   - Transfer coverslips to another jar and with PBS and wait 5mins.
   - Replace PBS with fresh PBS and wait 10mins.
4. Block
   - Dump PBS and put 100% block and wait 30mins.
   - Dump block and add (2nd block) 10% normal goat serum made in 100% block and wait 30mins.
To be sure about goat serum - you make 100% goat serum by adding 10mL of distilled water to the bottle of serum, then dilute this serum 1:10 in 100% block. You end up with 80% block final concentration.

5. Primary antibody stain
   - Make up antibody dilution to have a final concentration of 20% block (diluted in PBS):
     - When diluting antibodies, calculate 100 µL per coverslip to be stained.
     - You can do 1:20, 1:100 or 1:200 dilutions of antibodies.
   - To set up humid chamber use flat tupperware boxes and place a few kimwipes inside then spray DI water over it to wet the kimwipes.
   - Then place parafilm over wet kimwipes.
   - For each coverslip put 100 µL of antibody into parafilm.
   - Invert the coverslips onto drop so that the side with cells are facing antibody.
   - Close the top of the box and leave over night for staining.
   - If you are in a hurry, an hour is probably good enough.

Day 2:
6. Secondary antibody stain
   - Wash coverslips 6 times with PBS, allowing 5 min per wash as well as dumping and replacing PBS each time.
   - Dilute 1:500 or 1:1000 (or 1:2000) of secondary antibody (anti-mouse for kinesin and anti-rabbit for dynein) in 20% block and complete to 100 µL with PBS.
   - Pipet the 100 µL of secondary antibody onto parafilm and invert coverslips onto the solution.
   - There is no need for humid chamber this time but you should block the room light by placing a box over the samples.
   - Allow staining for 1 hour.
   - Maintain samples in dark throughout the rest of the experiment to prevent bleaching.
   - Once again wash coverslips 6 times with PBS, allowing 5 min per wash as well as dumping and replacing PBS each time.

7. Mount
   - Clean slides by washing them in H2O and drying them with a kimwipe first and then brushing over bunsen burner to get rid of dust particles.
   - Deposit 15-20 µL of imaging buffer onto slide.
   - Tap coverslips on a kimwipe to let excess PBS run off.
   - Invert coverslips onto a slide.
   - Seal slide with nail polish and let it dry for 5 mins be sure to cover from room light.
   - The sample is now ready for viewing under microscope.
Suggestions:
1. Good cells checkpoints:
   - When picking the culture
   - After PFA fixation
   - After poly Lysine step
   - After 2nd block
   - After first wash before secondary staining
   - Right before going down to the basement!

2. Cleanliness might be very important (cf. Stefan’s rotation)

3. If you have problems with PFA, you can use iodine (1:3 cells). It’s labeled “Lugol’s solution”, there’s a small tube of filtered solution in aluminum foil in the cupboard under my bench.

4. To get straight, nicely extended flagella, you may try adhering the cells onto the poly lysine coated coverslip before fixing them:
   - Put 200µl poly Lysine onto a coverslip. Let dry for 10 min. Dip in ultrapure water etc…
   - Spread 100µl live cells onto the coverslip. 2 min (up to 10).
   - Dip the coverslip onto a 4% paraformaldehyde solution (under the hood!!!).
   - Then go on with methanol washes etc.
APPENDIX B: MANUAL CONTROL OF MIRROR STAGE

1. Began by Clicking:
   a. Start on desktop-> All Programs->national instrument->measurement and automation.

2. Once there:
   a. In blue, under my system click device and interfaces

3. Then click on NI PCIe-6323"Dev1"

4. On the right you will see the items boxed in red:
   a. click on "Test Panels" and on Test Panel window click analog input

5. Select Analog Output and under Channel name select Dev1/ao2
   a. Now set Min output limit to 0.
   b. You can now step throw the y-axis of stage
APPENDIX C: MANUAL CONTROL OF DELSA CAMERA

- **Running Dalsa Camera Software**
  1. Began by Click:
     
     Start->All Programs-> DALSA -> Sapera LT-> CamExpert
  2. Once on CamExpert window simply click Grab (boxed in red) to start cam and freeze to stop
  3. In order to save an image:
     Click freeze then right click over the image and select save as…

Fig 1. This is an illustration of the CamExpert software, highlighted in red is the button, which alternates between Grab which began imaging with camera and Freeze which stops collecting images.
APPENDIX D: RUNNING SOFTWARE TO GENERATE OPTICAL FUNCTION ON SLM

1. Began by clicking:
   Start on Desktop-> All Programs -> HoloEye SLM Application Software Version 2.2 for the LC-R 2500 -> HOLOEYE SLM Application Software 2.2 for the LC-R 2500

2. Once on HoloEye window-> Click Elementary Optical Functions and choose function of interest

3. After choosing an option, the window with selected pattern will appear. If you scroll to your right you will see more options to manipulate pattern and once ready simply click signal light show in red box (fig 5a) to apply function to SLM.

4. An image of camera shows function on SLM in fig 5b

Fig 5a. Screen-shot of HoloEye software with function pattern.

Fig 5b. Image of function on SLM
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