The Design, Construction, And Operation Of A Two-Photon Laser Scanning Confocal Microscope

Benjamin Alexander

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THE DESIGN, CONSTRUCTION, AND OPERATION OF A TWO-PHOTON LASER SCANNING CONFOCAL MICROSCOPE

by

Benjamin Alexander, B.S.

A thesis presented to the School of Engineering of Washington University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011
Saint Louis, Missouri
ABSTRACT OF THE THESIS

The Design, Construction, and Operation of a Two-Photon Laser Scanning Confocal Microscope

by

Benjamin Alexander

Master of Science in Mechanical Engineering

Washington University in St. Louis, 2011

Research Advisor: Professor Guy Genin

Abstract: Confocal microscopes have become valuable tools in many fields of study. This work is intended to serve as a primer for basic confocal microscopy theory as well as a description of the design and construction of a homebuilt system in the Elson Lab at the Washington University School of Medicine. A description of the different designs of confocal microscopes is provided, and the capabilities, benefits, and detriments of each is described. The thought process behind each design decision is explained leading to the final system design.

A more in depth description of the final design is provided in Chapters Two and Three, but, in short, the final design is a non-descanned, laser scanning, two-photon confocal microscope. The system is designed to integrate as seamlessly as possible with the existing system and to allow for all existing functionality to remain. When completed, the system will be capable of one- and two-photon fluorescence correlation
spectroscopy (FCS); low-speed, one-photon confocal imaging using a piezo-stage; and high-speed, two-photon confocal imaging using the laser scanning system.

Finally, future directions as well as the limitations of the system are also described. All resources necessary to continue work on the system as well as those necessary to use it are provided. This includes:

- System diagrams
- Optical layout plots
- Component data sheets
- LabVIEW program

These resources are intended to make using, modifying, and improving the system much simpler.
Acknowledgments

I wish to express my sincere gratitude to a number of people who helped me during this project. Without their help, the success I achieved would not have been possible.

I would like to thank Drs. Eric Galburt and Mark Miller for their help in explaining the design and construction of the optical system. Their insights and knowledge were invaluable in getting me going on the right track.

I would also like to thank Drs. Tony Pryse, Tom Stump, and Artem Melnykov for their constant support. Their extensive, practical knowledge and their availability as a resource to me made virtually every aspect of this project easier. Without their support, this project would not have been possible.

And finally, I would like to thank Dr. Elliot Elson for the opportunity to play such and integral role in his lab, and to Dr. Guy Genin for all of his support and encouragement throughout my academic career.

Benjamin Alexander

Washington University in Saint Louis
August 2011
Dedicated to my wife, for her love and patience during this time.
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Chapter 1

Introduction

Confocal microscopy is an advanced imaging process which allows three dimensional images of biological structures to be obtained. In the field of biochemistry and molecular biophysics, it is used to view the movements of living cells through three dimensional environments and the response of living cells to external stimuli like stress and strain, among other things. In Dr. Elliot Elson’s lab, confocal microscopy and the associated experiment, fluorescence correlation spectroscopy (FCS), is used extensively to study the effect of stress and strain on cells. In particular, it is desirable to study the regions, referred to as focal adhesions, where the cells attach to the extracellular matrix (ECM).

For reasons which will be discussed further in the Theory section, the commercial confocal microscope the lab has access to is not capable of performing the specific type of imaging desired to study focal adhesions. However, there was a homebuilt system which had most of the components needed for a microscope which would be capable of the type of imaging needed. My task was to design and build the additional components and systems which would allow the homebuilt system to perform this function. By using a custom solution, rather than a commercial product, the project is much more cost effective, integrates more smoothly with the existing system, and allows for future customization [1].
1.1 Overview of Thesis

In the following thesis, the design, construction, and testing of a two-photon, scanning, confocal microscope is described. The basic theory behind the optical design and function is described, and the design process I followed is detailed. Finally, the operational procedure is described and a basic verification experiment is described to show the system performs as designed.

1.2 Challenges

A great number of challenges presented themselves throughout the life of this project. Some were very technical in nature, while others were more logistical. Most of the technical challenges will be described in more detail later, once the underlying theory has been explained. At this point, they are mentioned briefly to provide insight into the thought process behind the design.

1.2.1 Finding Information

Initially, I possessed very little specialized knowledge regarding the operation and design of confocal microscopes. To gain the necessary knowledge, I turned to journal articles and books, references provided on the web courtesy of other research labs, and the advice of Drs. Eric Galburt and Mark Miller.

Gaining access to these resources was not really that difficult. What was challenging was finding intermediate information which would allow me to interpret the information they provided. Drs. Galburt and Miller were invaluable in this respect. Dr. Miller has a very similar system in his lab, and he graciously showed it to me and explained the basic principles behind its operation. Dr. Galburt utilizes an optical tweezer in his lab. The optical characteristics of that system are very similar to those of the system I sought to build. He was able to further explain the principles, as well as provide advice on how to go about performing the arduous task of aligning all the optical components.
In addition to difficulties obtaining information about the system I was trying to design, it was also difficult to obtain information about the Zeiss microscope the system would be built around. Particularly, it was difficult to obtain details about the internal optical components of the microscope. In the end, I was able to move forward without obtaining all the details as I was able to infer all the necessary information from external components in the existing system.

Over the course of about two weeks, I was able to go from knowing very little about confocal microscope design to having a thorough understanding of all the basic principles. This foundation of knowledge served as an excellent tool throughout the project.

1.2.2 Maintaining Current Functionality

One of the requirements from the very beginning was to adapt the microscope to expand its functionality without compromising any of its current abilities. The system in use in the Elson Lab was already quite extensive. The laser I planned to use was being shared by the confocal microscope and a fluorescence lifetime measurement (FLM) apparatus. The confocal microscope was already equipped to perform FCS studies as well as basic, confocal scanning. Since the system I was building would be a vast improvement on the current scanning abilities, interfering with that system was not an issue; however, the ability to perform FCS and FLM experiments absolutely could not be compromised.

These concerns left little room on the table with which to work. The optical paths for the FLM and FCS systems contained numerous lenses, mirrors, and other components which could not be moved. In the end, it was possible to make some room by removing certain components whose function was no longer necessary.

Another challenge involved the placement of the scanning mirrors. Because of the layout of the microscope, it was necessary for the excitation pathway to run directly above the emission/detection pathway. The only way to accomplish this was to design a bracket which could be mounted above the detectors and serve as a platform for the scanning mirrors.
1.2.3 Equipment Failure

The final major challenge encountered in this project was the failure of the laser during the final stages of the project. The laser for which the system is designed malfunctioned and became inoperable. In order to move forward, a different, less optimal laser was used to perform the rough alignment so that only minor adjustments would need to be made when the primary laser was repaired. A major obstacle to this was the fact that the primary laser has a wavelength of around 800 nm whereas the only other lasers available were in the 480-514 nm range. Because mirrors that have a high reflectivity for the longer wavelengths have a very poor reflectivity in the shorter wavelengths, several of the mirrors had to be temporarily replaced when using the short wavelength source in order to provide sufficient intensity throughout the optical pathway.
Chapter 2

Background and Design Principles

To begin, it is necessary to set forth the basic principles behind how a confocal microscope operates. While all confocal microscopes operate on the same basic principles, there are several different methods and configurations by which the technique can be employed. Understanding the methods and configurations and their corresponding capabilities, benefits, and detriments is necessary in order to follow the explanation of the design decisions made throughout the project.

2.1 Confocal Microscopy

Confocal microscopy is an imaging process based on the use of fluorescent dyes which are excited with light of one wavelength and emit light at a different wavelength, allowing the excitation light to be filtered out leaving a pure emission signal. Since the fluorescent dyes are excited by a very specific wavelength of high intensity light, lasers are an ideal source of excitation light. The use of lasers provides further utility through the use of an auxiliary optical system by allowing the imaged region to be restricted to a well-defined focal plane. This ability to obtain two-dimensional images from well-defined, parallel planes allows stacks of images to be obtained which can then be reconstructed to produce accurate, three-dimensional models.

Since, with this method, the excitation light is focused down to a very small region, images are acquired by scanning that focused point across the field of view, monitoring the emission from each point of focus, and processing that data to form an image. Physically, there are two ways of accomplishing this. The first is to move the specimen
with respect to a stationary focal point. The second is to move the focal point with respect to a stationary specimen. The latter requires the motion of only two small mirrors which comprise relatively little mass when compared to the mass of the stage and specimen which are what move in the former method. This makes the beam scanning method inherently faster and more well suited for the application at hand [2].

In general, a confocal microscopy system consists of an excitation method and an emission or detection pathway. However, there are two different ways of approaching both components. Concerning the excitation method, the two approaches are known as one-photon and two-photon excitation. Concerning the emission pathway component, the two approaches are known as descanned and non-descanned pathways.

2.1.1 One-Photon and Two-Photon Excitation

As mentioned previously, confocal microscopy operates on the principle of exciting fluorescent molecules and monitoring the emission to produce an image. In one-photon excitation, a high frequency excitation beam is used to excite the fluorescent molecules which then emit light at a lower frequency. At the molecular level, each fluorescent molecule absorbs a single photon which allows it to transition to a higher energy, unstable state. When the molecule returns to its lower energy, stable state, it emits a single photon [2]. The fact that each molecule absorbs a single, high energy photon is what gives this method its name.

A consequence of this process is that it requires a relatively lower intensity of light to induce fluorescence. Therefore, even though the beam is focused down to a very small focal plane, there is still some out-of-plane excitation which occurs on both sides of the focal plane [2]. This effect is illustrated in Figure 2.1. In order to accommodate this phenomenon, an optical pinhole placed at a conjugate image plane is used to selectively pass the emission from the focal plane and attenuate all the out of focus emission. Since this method necessitates the use of a pinhole in order to attenuate the out of focus emission, the emission beam must remain stationary at the point in the optical path where it passes through the pinhole. However, since the emission is coming from different locations in the specimen, something must be done to take the moving beam and render it stationary so the pinhole can be used. This is usually
accomplished by using the same mirrors used to scan the excitation beam and is known as “descanning.”

Alternatively, two-photon excitation can be used. In two-photon excitation, each fluorescent molecule must absorb two, rather than one, photon in order to fluoresce. Since the molecule is absorbing two photons, each photon has half the energy of a single photon capable of exciting the molecule. However, because two photons are required, the intensity level necessary to cause fluorescence is much higher than with one-photon excitation. As a result, the region of sufficient intensity is highly localized to the focal point. The fluorescence is so localized, that virtually no out of focus fluorescence occurs. This is illustrated in Figure 2.1. It should be noted that a consequence of two-photon excitation is the emission is a higher frequency than the excitation. Often, high powered, infrared lasers are used for the excitation with emission ending up somewhere in the visible spectrum. This is exactly opposite to what occurs in one-photon excitation, and special attention should be paid to ensuring the proper dichroics are used.

Figure 2.1: Comparison of (left) one-photon excitation to (right) two-photon excitation demonstrating the absence of out of plane fluorescence with two-photon excitation (http://microscopy.berkeley.edu)
You will recall, the presence of out of focus fluorescence with one-photon excitation necessitated the use of an optical pinhole to attenuate out of focus signal. Since two-photon excitation induces no out of focus signal, the optical pinhole is not required in order to obtain a clean, in focus measurement. Without the optical pinhole, there is not necessarily a reason to descan the emission beam. With a large enough detector area and a just a couple of lenses, the emission can be allowed to remain in motion and still acquire accurate measurements. Sometimes, however, it may still be necessary or desirable to descan the emission beam, and this can still be done just as it is for one-photon excitation.

2.1.2 Descanned and Non-Descanned Emission Pathways

As mentioned above, in order to utilize a pinhole with one-photon excitation, the emission beam must be rendered stationary through descanning. This method simply directs the emission signal back along the same path as the excitation beam toward the scanning mirrors. Since the mirrors remain essentially motionless relative to the speed at which the fluorescence occurs and the light travels, the excitation and emission beams follow the same path through the mirrors. The result is a stationary emission beam after passing backward through the scanners.

Once the beam has been descanned, it is possible to place a dichroic mirror in the beam to separate the two signals. In the case of one-photon excitation, a pinhole can now be used to selectively attenuate the out of focus signal. This configuration is known as a descanned configuration.

When performing one-photon laser scanning confocal microscopy, the descanned configuration is the only option since the pinhole is necessary. However, since the pinhole is not required for two-photon excitation, there is another possible two-photon configuration. This configuration, known as the non-descanned configuration, uses optics to place an image of the mirrors and back focal plane of the objective lens on the detector. Notice that at the mirrors, the collimated beam is “pivoting” about an approximately stationary point. Through the use of a scan lens and tube lens, an image of this point is created at the back focal plane of the objective, again creating an optical pivot point about which the collimated beam rotates as it is scanned. The
planes in which these pivot points are located are known as telecentric planes. By creating a third, telecentric plane on the detector, the collimated beam again pivots about a stationary point on the detector. This allows for a dramatically simpler, more efficient emission pathway.

In all there are three different possible configurations. One using one-photon excitation, and two using two-photon excitation. These different configurations are summarized in Figure 2.2. Each configuration has its benefits in different applications. Since the application at hand requires the use of two-photon excitation, the non-descanned configuration was chosen for its simplicity, efficiency, and its ability to integrate easily with the existing system.

![Figure 2.2: Hierarchical Arrangement of Confocal Microscope Configurations](image)

2.2 Optics Simulations

When beginning to work with these ideas of conjugate image and telecentric planes, my intuition concerning them was very weak. To provide a better understanding of what exactly the terms meant and to better visualize how the optical system would behave, I used a student copy of Lambda Research’s OSLO® optical design software to simulate the basic optical path. With this software package, I was able to simulate the path the light would take as it was scanned by the mirrors and generate diagrams like those shown in Figure 2.3. As you can see, Figure 2.3 illustrates what the image
Figure 2.3: Basic optical diagram illustrating what the image and telecentric planes are. Note that $F_1$ is the focal length of the scan lens and $F_2$ is the focal length of the tube lens.

and telecentric planes are. On the image plane, the beam is focused down to a point which moves in that plane depending on the angle of the incident light. On the telecentric plane, the beam is collimated and passes through the plane at different angles, but always at the same stationary location.

From the diagram in Figure 2.3, it is easy to see that the scanning mirrors themselves are located on a telecentric plane. This is the principle referred to at the end of section 2.1.2 when discussing the non-descanned emission pathway. Even though the beam is not motionless, it would always remain directed on a sufficiently large detector area so long as that detector was located on a telecentric plane.

### 2.3 Final Optical Layout

In the end, the primary goal of this design was to build a system which could perform two-photon, confocal imaging. Since one-photon imaging was not required, the non-descanned layout was an option. Since the non-descanned system is simpler and places fewer elements in the emission pathway, that layout was chosen. It was also the case that the non-descanned layout could be made to integrate more closely with the existing system, allowing for virtually all of the previous functionality to be retained.
Due to the critical nature of the placement of the scanners and intermediate optics with respect to the back aperture of the microscope objective and to each other, their locations were all predetermined since the microscope was already on the table. The excitation pathway enters the microscope through a custom port built into the turret. Along this pathway, the tube lens was placed one focal distance (200 millimeters) from the back aperture of the objective. The scan lens was mounted on a plate with the scanning mirrors such that the midpoint between the two mirrors was a focal distance away (along the optical path) from the scan lens. This plate was cantilevered from a two inch post above the detectors used on the current system. The entire post and cantilever bracket assembly was mounted to the table via a translation stage. This allowed the mirrors and scan lens to be moved together into position. The assembly was positioned such that the scan lens was a distance to the sum of the scan and tube lenses’ focal lengths (40 and 200 millimeters, respectively.) These two lenses perform a 5x beam expansion so that the back aperture of the objective is fully illuminated.

In summary, the excitation components are arranged according to the schematic diagram in Figure 2.3. The excitation path enters the microscope on the left side at the level of turret. The path is directly above the emission path which exits the microscope from the standard, left-side port. Figure 2.4 is a view of the components from the left side. The two inch post supporting the bracket on which the scanning mirrors and scan lens are mounted is in the bottom-left corner of the image. The custom port on the turret as well as the standard, left-side port are shown as well.
Figure 2.4: Image of the excitation path components and their arrangement with respect to the microscope, other elements previously on the table, and each other.
Chapter 3

System Description, Usage, and Theory of Operation

The entire laser scanning system consists of several separate components: the servo board and scanning galvonometers, a hamamatsu photon-counting head, and a National Instruments DAC board. Additionally, there are a few different Virtual Instruments which use the National Instruments board to communicate with the other hardware components.

3.1 Derivative Feedback Controlled Galvanometers

The scanning mirrors and the servo board which controls them came as a tuned pair from Cambridge Technology. As a tuned pair, the system is tuned to produce an optimal response time along with other various parameters such as overshoot and steady-state error. The result is a highly accurate and repeatable system which is capable of precisely positioning the two mirrors.

The system uses proportional and derivative feedback control with feedback signals coming from optical encoders built into both galvanometers. The servo board uses the signal from the encoders to precisely control the motion of the scanners. The servo board is a single board which supports both channels and is powered by a single power supply. The power supply provides $\pm 24$ volt power to the servo board. The power supply and the servo board are both mounted in an enclosure which is located above
the PC used to generate the control signal. The servo board is mounted internally to a heat sink per the specifications.

The servo board has four cables attached to it. The first is the power cable which provides +24VDC, -24VDC, and a system ground. The second is the input signal cable. This cable consists of two separate cables which are joined by a single connector at the board and have separate BNC connectors at the other ends for connecting to the analog output terminals on the National Instruments board. The last two cables are the cables which run to the galvanometers. These cables are responsible for both powering the motors and communicating the feedback signal from the optical sensors to the servo board.

### 3.2 National Instruments DAC Board

As mentioned previously, a National Instruments 6025E board is used to generate an analog signal which controls the position of the galvanometers. By generating various waveforms, the mirrors can reflect the laser in a variety of patterns. For instance, sawtooth waveforms can be used to generate raster scans, and 90-degree out of phase sine waves can be used to produce orbital scans. It should be noted that the signal generated consists of a series of discrete, analog voltage values ranging from -10 to +10 VDC. Except in the case when the points in a waveform are so close they overlap partially, the galvanometers react so quickly, each individual point appears separate from the others, rather than a continuous path. Therefore, each point is essentially a pixel which works well for this application since each point represents a single defined location at which a photon-count is measured. Figure 3.1 shows the difference between a signal with few points per cycle and one with many. In the former, each individual point is resolvable. In the latter, there are sufficient points per cycle to produce what approximates a continuous path. In reality, the beam does traverse the space between the points, but it does so quickly enough that any fluorescence generated is insignificant.

The analog output on the National Instruments board is triggered to move to the next point in the waveform by one of the two counters built into the board. This counter serves both as the update clock for the analog output responsible for scanning
each row and as the bin timer by triggering the second clock to start counting pulses from the photon counting head. This arrangement assures synchronicity between the position of the laser and the recorded values for each pixel. After each row is scanned, a one time update of the second analog output is performed to advance to the next row. In this case, since both analog outputs are being triggered (resetting the row and advancing to the next row), the second analog output can be triggered independently since the resetting of the first output is still triggered along with the bin counter by the bin timer. Figure 3.2 illustrates this process and the relationships between the counters, analog outputs, and the respective hardware.

\section{LabVIEW Program}

The LabVIEW program used to test the system is provided in Appendix A. This program takes as inputs basic parameters which define the number of pixels in the raster scan, the spacing between each pixel, and the bin time. Using these inputs, a waveform is constructed and cycled through for each row. At each point, the bin counter records a photon count at the end of a vector. At the end of each row, this vector is appended beneath the previous row’s vector to form a two-dimensional array and the second analog output is updated to assume a value referring to the next row.
Figure 3.2: A schematic representation of the different hardware and software components used in image acquisition system.

When all the rows have been scanned and the photon count array is fully populated, the file is exported as a text file. To generate an image, the text file was imported into MATLAB where the imagesc function generate a scaled value image.

It should be noted that by default, the analog outputs utilize the dedicated analog output clock. To operate in the fashion described here, the Analog Output VIs must be reconfigured to be triggered by the desired clock. Either of the two onboard clocks may be used. Also, the Analog Output can be configured to be triggered by an external clock connected to a PFI pin.

### 3.4 Usage Instructions

To perform an image acquisition with the system, the first thing to do is to power on the laser. This is done following the standard procedures which have been previously documented. Next, power on all the scanning components. Start up the computer and get LabVIEW running, turn on the power to the galvanometers by flipping the labeled switch on the power supply above the computer, and plug the power supply
for the PMT into a 120VAC wall outlet. Finally, select the appropriate filter for your application. The system is now ready to run.

The current procedure for obtaining an image is to open the LabVIEW program described previously and in Appendix A. Using the eyepiece on the microscope, locate the region you wish to image in the center of the field of view. Turn the selector on the microscope so the side-port is active. Once this is done, run the LabVIEW program. You can select the number of points per row, the number of rows, the resolution (i.e., the distance between the points), and the time the laser dwells at each point. When all the parameters have been entered, run the program to obtain the image data which you will be prompted to save as a text file. To convert the image data into an image, import the data file to MATLAB and use imagesc to generate a scaled value image based on the relative values of each datum in the text file; each datum corresponds to the photon count at each respective pixel. The raw data file can also be otherwise post-processed using the data analysis tool of your choice.

The process which was just described is the process which must be followed at this point in time. In the future, the galvanometer control and image acquisition system will be built into the image acquisition LabVIEW VI which was used with the original system. All inputs and outputs will be the same. The only difference will be the internal workings of the VI. The system was designed to be integrated into this existing VI to make the system easier to adopt since the user interface remains unchanged. The steps required to fully integrate the new image acquisition system with the existing VI is detailed in Section 4.2.2.
Chapter 4

Limitations and Future Directions

Every attempt was made to provide the maximum amount of functionality from this system. Ultimately, the system will add several new capabilities to the overall system while maintaining all previous functionality. While a great deal of work was accomplished towards the completion of the project, there are still several areas which remain to be completed in order to provide full usefulness. These limitations and further work are described here.

4.1 Limitations

The main limitation, if it can be considered as such, is the inability to perform one-photon scanning and FCS using the galvanometers and PMT. This is because the use of a non-descanned optical pathway does not allow the use of an optical pinhole as described in Section 2.1.2. However, if the pinhole is inserted into the pathway in the same location as it was with the original system, the piezostage and APDs may be used to acquire images and perform FCS measurements in exactly the same way as with the original system.

Therefore, while it is considered a limitation of the laser scanning system designed and built for this project, the ability to perform one-photon image acquisition and FCS is not a limitation of the overall system. As mentioned previously, it was a capability of the original system and is therefore maintained in this system. All that is required is to insert and align the optical pinhole.
4.2 Further Work

While the limitations of the system are very few in the sense that all the capabilities which were desired are, at least in some way, achievable, the possibilities for performance above and beyond those initially specified are more numerous. The following areas of further work are opportunities to provide capabilities which will further enhance the usefulness of the overall system.

4.2.1 Integrate the Piezostage’s Z-Scan

The first area for future work is to integrate the piezostage’s z-scan capabilities. One of the biggest advantages of confocal microscopy is the ability to image on well-defined planes. This allows images from multiple, parallel planes to be reconstructed to produce a three-dimensional image. By using the z-scan capability of the piezostage, this process can be fully automated. Furthermore, this functionality is already built into the LabVIEW VI used with the original image acquisition system. If the work described in Section is completed, the z-scan capability will also be gained.

4.2.2 Improve the LabVIEW UI

The biggest opportunity for further work involves improvement of the LabVIEW user interface. The original system had a very well developed user interface which is very familiar to many of the systems most common operators. The new scanning system was designed to operate off of the same inputs as are provided through this well developed interface. Additionally, the system can be configured with minimal effort to output the same data as the scanning sub-VI does in the program used with the original system. By performing the necessary modifications to the VI currently used to control the scanning system, it can be dropped into the original program in the place of the scanning sub-VI which uses the piezostage for scanning. This will allow the new system to provide all the same capabilities as the original system, including the ability to do z-scans, with the added benefit of drastically reduced acquisition time.
4.2.3 Fully Automated Cell Tracking

The final area of future work which I believe would provide valuable additional functionality is the capability to obtain long-term, time-lapse z-scans of a specific cell using a fully automated cell tracking algorithm. Being able to image a living cell as it migrates through a three-dimensional extracellular matrix over periods of time too long to be conveniently performed manually would provide valuable information to researchers. Because this system has two separate scanning system (i.e., the galvanometers and piezostage), a three-dimensional image can be quickly obtained using the galvanometers, that image data can be analyzed to determine where in space a particular cell is, and the piezostage can be used to bring that cell back into the center of the field of view. By tracking the movement of the piezostage, the path taken by the cell over many hours can be reconstructed and analyzed.

While many challenges exist in designing an algorithm which achieves the required cell identification, it is not something which is outside the realm of possibility. Additionally, this capability would be limited by the range of motion of the piezostage. This range could be increased slightly by allowing the the galvanometers to scan regions not centered in the field of view. The extent to which this can be done remains to be seen as the affects of operating outside of the center of the optical path may be too severe to allow accurate image acquisition.
Appendix A

LabVIEW Program

The LabVIEW program shown in Figure A.1 on the next page was used to perform preliminary tests of the image acquisition system. It takes as inputs the x- and y-dimensions in pixels, the resolution in nanometers per pixel, the bin time, and the hardware configuration. The volts per nanometer node is a calibration constant and can be adjusted as needed.

The x-dimension and resolution inputs are used by the For Loop to generate an array of analog voltage values corresponding to each point in a row. This array is passed on to the analog output sub-VIs in the Sequence structure as well as output to the front end of the VI for troubleshooting purposes.

The y-dimension and resolution inputs are used by the larger For Loop to update the value of the second analog output each time the loop iterates. The y-dimension also serves to define the number of times the loop iterates. Within the larger For Loop is a Sequence Structure. This structure ensures that the row is scanned and the photon counts recorded before the scanner advances to the next row. See Section 3.3 for further information.

Once all the rows have been scanned, a dialog window appears prompting you to select the directory and file name where the data should be stored. Once saved, the data can be accessed to produce an image or otherwise post-processed.
Figure A.1: The LabVIEW program used to test the image acquisition system.
Appendix B

Component Diagrams

A custom mounting bracket was designed and built for this system. This bracket allows the scanner, scan lens, and other optics to be mounted in the correct location directly above the APDs. Due to the presence of the APDs, the components could not be directly supported, requiring the mounting bracket. The bracket attaches to a two-inch post which is in turn mounted on two translation stages to allow for accurate positioning of the scanner in two dimensions. The horizontal part of the bracket has a series of holes so that the scanner and scan lens can be placed over a wider range without having to change the location of the mounting post (effectively increasing the range of the translation stages). The vertical portion has a standard four hole pattern intended to work with standard two-inch mounting clamps.

In order to allow for easier access to the components below the mounting bracket, provisions were made to facilitate easy and repeatable removal and installation of the bracket. As mentioned before, the bracket is designed to attach to a standard two-inch mounting clamp. The vertical portion of the bracket extends beyond the lower edge of the mounting clamp. By placing a second clamp on the post below the one attached to the bracket, the position of the bracket can be retained by leaving the lower clamp in place and removing the upper clamp which is attached to the bracket. When replacing the clamp and bracket, simply ensure that the two clamps are aligned angularly and that the top one is firmly seated on the lower. There is a small amount of play between the lower part of the vertical portion of the bracket and the lower mounting clamp. By pushing the bracket firmly to a side and then tightening the upper clamp, the precise location can be easily recovered.
The following drawings detail the bracket design. They can be used to fabricate a new bracket if another is needed or the current one is damaged. Note that the clearance holes used to attach the horizontal part to the vertical part are not shown in the drawings of the horizontal part.

Additionally, the diagram which was provided with the scanner is shown in Figure B.5. This assembly contains the galvanometers and scanning mirrors in a mount for which the bracket described above is designed to interface with.

Finally, a wiring diagram is provided in Figure B.6. This is similar to Figure 3.2, but with an emphasis on hardware and wiring. Note that the pinouts for each connector are shown. The pinouts for the cables between the driver board and the galvanometers are not provided because these cables were provided with the system pre-assembled.
Figure B.1: Vertical portion of the bracket
Figure B.3: Horizontal portion of the bracket (2 of 3)
Figure B.5: Scanner assembly; diagram provided by Cambridge Technology
Figure B.6: This diagram details the wiring of the system. The pinouts are provided so replacement cables may be easily fabricated.
References


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Publications

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