RICE UNIVERSITY

A Multi-Photon Microscope
for Three Dimensional Functional Recording
of Fast Neuronal Activity

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A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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MAY 2007
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ABSTRACT

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Gaddum Duemani Reddy

A thorough understanding of how neurons work is one of the greatest scientific
goals in the field of experimental neuroscience. However, four fundamental technical
limitations complicate any attempt to study neuronal function with sub-cellular
resolution: First, neurons and neuronal processes are small, second, in realistic
experimental situations they can be located deep within optically scattering tissue, third,
the chemical and electrical signaling that characterizes neuronal behavior happens
quickly, and fourth, neurons and neuronal processes have very three dimensional (3D)
shapes. Here we develop a tool that overcomes all four listed limitations by combining
the technique of multi-photon microscopy with a unique method for 3D laser beam
steering. The result is an instrument capable of monitoring physiological signals at
multiple locations in the volume of space occupied by a neuron, a task that is
unachievable with any other available instrument.
Acknowledgements

These last four years have been a great learning experience and for that, I am grateful to many people. First and foremost is my advisor Dr. Peter Saggau. There is no telling where this project would have ended up or how long it might have taken had Peter not kept pushing me to continue in spite of the road bumps we ran into along the way. I am truly grateful for the friendship, support, and advice he has given me throughout this journey. I also owe a debt of gratitude to several members of the Saggau lab, both current and past, including Vivek Bansal, Rudy Fink, Brad Losavio, James Manusco, Tycho Hoogland, Vijay Iyer, Rob Gaddi, Olga Gliko, and XanWen Chen. They have collectively been a source of camaraderie and advice that has made the lab a pleasurable place to work. I would especially like to thank Keith Kelleher for his help with the biology in the later stages of the project.

I am also very grateful to the Bioengineering department in general and my committee members in particular for allowing me to pursue a project that intrigued me and giving me the flexibility to define research goals that matched the project. I also owe a special thanks to the administrative staff of the Rice Bioengineering department, the Baylor Neuroscience Department, and the Baylor MD/PhD office, especially Kathy Crawford, for the helpful resources they have been to me throughout the years. I would also like to extend this gratitude to my MD/PhD advisor, Dr. Matzuk, who has also been a source of great advice in pursuing the at times completely frustrating goals of a double doctorate.

Finally, I express my deepest thanks to the friends and family that have been with me through the highs and lows of the past years. To my parents and brothers in particular
I am forever indebted. I have no doubt that without your support very little I have ever done in my life would have been possible.

Duemani Reddy
April 9, 2007
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Chapter 1: Introduction

1.1 Overview

The human brain is arguably the most fascinatingly complex system known to man. With conservative estimates of it containing a hundred billion neurons, the fundamental building blocks of the nervous system, each with approximately ten thousand connections, any attempt to study brain function necessitates a bottom-up approach. One of the most fundamental of such approaches is to study the physiology of a single neuron. Indeed, single neuron studies have been the focus of much of the neurobiology community since the advancement of the neuron doctrine by Cajal in 1906 [1].

The primary goal of this subfield of neurobiology is the development of an accurate “input/output” relationship for a neuron. This endeavor is complicated by several factors, which, in addition to the average neuron having thousands of synapses (inputs) that can be located almost anywhere on its surface, include the fact that the effect of the synapses themselves can be graded in intensity, from strongly inhibitory to strongly excitatory, as well as dynamic, i.e., changing in intensity from one experiment to another. While these processes make the neuron inherently more difficult to experimentally characterize, they also dramatically increase its computational abilities since, for the majority of cases, it is not only the synaptic influences the neuron receives, but also the process by which those influences interact with each other as well as with the neuron itself that determines whether or not the neuron will fire an action potential (output).
The role thin neuronal structures called dendrites play in this process has changed drastically over the past few decades. Indeed, initial work by Rall and others modeled them as resistive and capacitive networks, which made them passive conduits for electrophysiological influences propagating from the synapse to the soma [2,3]. However, recent experimental results have shown that far from being simple passive cables, dendrites are active and dynamic entities that can not only shape synaptic influences, but also modulate the interactions between them [4-7]. In addition, it has further been shown that the active properties that give dendrites this power are governed by the density of the voltage gated ion channels present on the dendrites and the biophysical characteristics of these channels [8], both of which can be modulated by the activity of the neuron [9,10] itself. This gives individual neurons the ability to dynamically change the way they process information, which further complicates attempts to understand their response characteristics.

Given this complex integrative nature of neurons, any attempt to develop a quantifying input/output relationship for even a single neuron would benefit from as much signal information as could be recorded. In the ideal case, it would help to know the complete electrochemical characteristics everywhere on a neuron. This would allow one to see how synaptic responses are modulated as they propagate along dendrites. It would also allow one to analyze where and how subthreshold responses integrate to affect the generation of an action potential. In addition, with pharmacological modification, one could also characterize the effects of various ion channels on this process.
However, a recording mechanism that would allow one to know either the electrical or the chemical characteristics of a neuron at more than just a handful of sites does not yet exist. Indeed, while there are imaging techniques such as confocal microscopy and multi-photon microscopy that provide the spatial resolution necessary to study detailed neuronal processes, they do not have the temporal resolution needed to follow multiple fast neurophysiological phenomena, such as calcium transients, that occur on the order of milliseconds. In addition, while significant steps have been taken to improve the temporal resolution by using high speed galvanometer based [11] or acousto-optic based scanning [12,13], all methods developed to date restrict fast scanning to the two lateral dimensions (x,y). This severely limits attempts to monitor more than just a few sites on a neuron, since most neurons have complex three dimensional (3D) shapes.

To truly be able to map enough locations on a single neuron to effectively study the properties of neuronal computation, a recording instrument must be capable of rapidly scanning in all three dimensions. In this dissertation, we design and build the first known tool for accomplishing this task by developing a fast 3D laser beam steering scheme and combining it with a multi-photon microscope. Using this tool, we are able to monitor physiological parameters at multiple different axial and lateral locations on a neuron, which represents a major technological step towards the development of the ideal instrument for studying single neuron computation.
1.2 Neuroscience Background

1.2.1 The Brain (Gross Anatomy)

The study of modern neuroscience has its beginnings in the work of Camillo Golgi and Ramon y Cajal at the end of the nineteenth century. Using a silver based staining technique developed by Golgi, Cajal was able to label individual neurons and show that, contrary to popular belief at the time, nervous tissue was actually composed of discrete cells [1,14]. Cajal’s work would later serve as the basis for the principle known as the neuron doctrine, in which neurons are identified as basic signaling elements with distinct interaction points between them known as synapses. Today, the human brain has been conservatively estimated to contain approximately 100 billion neurons with the average neuron (excluding Purkinje neurons) containing between 1 and 30 thousand synapses.

The complexity of the system is at least somewhat reduced by the fact that brain can be compartmentalized into developmentally distinguishable regions and even further compartmentalized into functionally specialized regions (see Fig. 1-1). The classic method of doing this involves first separating the brain into three regions that are first identifiable as distinct protuberances during development, i.e., hindbrain, the midbrain, and forebrain. The hindbrain can then be further subdivided based on function into the medulla, the pons, and the cerebellum. The forebrain can similarly be further subdivided into the diencephalon, which is composed of the thalamus and the hypothalamus, and two cerebral hemispheres, each of which contains a basal ganglia, a hippocampus, an amygdala, and overlying cerebral cortex. Anatomically, the cerebral hemispheres can
Figure 1-1: Gross anatomy of the brain (adapted from Kandel 1991 [15])
Primary divisions of the brain (forebrain, midbrain, and hindbrain) are labeled on the right side of the figure. Secondary divisions of the hindbrain and forebrain are labeled on the left side of the figure. See text for more details.
also be divided into the frontal, temporal, parietal, and occipital lobes. It is beyond the scope of this introduction to discuss the roles of each of these regions [15]. Instead, particular attention will be paid to the hippocampus, since cells in this region have become great models for studying neuronal computation.

**The Hippocampus**

The hippocampus, which is located in the temporal lobe, has been linked to memory since the work of Brenda Milner. In 1966, she showed that epileptic patients who had their hippocampi bilaterally removed for therapeutic reasons were subsequently unable to form certain long term memories [16]. Studies done to investigate this unique link have resulted in the hippocampus being a very well characterized structure.

Anatomically, there are several ways to divide the hippocampus, the most common of which is to separate it into the dentate region, which is primarily composed of granule cells, the CA3 region, which predominantly contains pyramidal cells (known as CA3 pyramidal cells) and interneurons, and the CA1 region which, like the CA3 region, is mainly pyramidal cells and interneurons (see Fig. 1-2, [17]). There are three major excitatory pathways (axonal bundles) in the hippocampus that connect these three regions; the perforant pathway, which runs from other parts of the brain into the dentate region, the mossy fiber pathway, which runs from the dentate region to the CA3 region, and the Schaffer collaterals, which run from the CA3 region to the CA1 region.

The physiological connection between these pathways and memory lies in processes known as long term potentiation (LTP), in which specific synapses are strengthened for a significant duration (hours), and long term depression (LTD), in which
**Figure 1-2: Gross anatomy of the hippocampus** (adapted from Fleming 2000 [17]). Illustration of the rat hippocampus showing the Dentate gyrus, CA1 and CA3 regions, as well as the primary axonal bundles.
specific synapses are weakened for approximately the same amount of time. The ability to change synaptic strength, via either LTP or LTD, has been labeled synaptic plasticity. It has been shown that high frequency stimuli to any one of the excitatory pathways listed above can produce LTP in the receiving region (for example, stimulation of the Schaffer collaterals produces LTP in CA1 pyramidal cells). The specific processes required for LTP and LTD as well as the exact mechanisms behind their induction are well studied and will not be covered here (for review see [18]). However, largely as a result of the work done to understand plasticity, a lot of information has been gained on the characteristics of pyramidal cells in the hippocampus which, in addition to their relatively simple structure, makes them ideal for studying neuronal processes in general.

1.2.2 The Neuron (Microanatomy)

While there are actually many more glial cells in the brain than neurons, neurons have been far better studied and are more directly implicated in information processing mechanisms. Thus, this review will focus solely on them (for a review of glial cells see [19]). The basic anatomy of a neuron is divided into three distinct regions, the soma, the dendrites, and the axon (see Fig. 1-3).

Soma

The soma (i.e., the cell body) is the metabolic center of the neuron and contains the nucleus as well as other organelles necessary for producing proteins and RNA. It is typically roughly modeled as a sphere. For CA1 pyramidal neurons, data from various reconstruction studies done on rats have estimated the average soma diameter to be
between 20\(\mu m\) to 54\(\mu m\), with a surface area that ranges from 932\(\mu m^2\) to 1300\(\mu m^2\) and a volume that falls between 1800\(\mu m^3\) and 2900\(\mu m^3\) (for review see [20]). This is approximately 27\% of the total neuronal volume but only 4\% of the total surface area. It is important to note that this value is very different for different neuronal subtypes (for review [21]), however, in most neurons the soma contributes very little to the overall surface area.

**Dendrites**

Most of the surface area of a neuron comes from the dendrites, which in CA1 pyramidal neurons are further classified as apical dendrites and basal dendrites. Apical dendrites, which emerge from the apex of the soma, synapse proximally with Schaffer collaterals from the CA3 region in a location known as the strata radiatum. Distal apical dendrites synapse with inputs from the entorhinal cortex as well as subcortical structures such as amygdala in the strata lacunosum-moleculare. Basal dendrites, which emerge from the base of the soma, synapse proximally with the Schaffer collaterals in a location known as the oriens, however, they also synapse with recurrent collaterals from the CA1 region as well. Studies have shown that the apical dendrites in rats can have surface areas of up to 42000 \(\mu m^2\) and 72\% of the total surface area of the neuron while basal dendrites can have surface areas of up to 24000 \(\mu m^2\) and 40\% of the total surface area [20]. For both the apical and basal dendritic trees, further divisions can be made by separating the main dendritic trunks, which have diameters on the order of one to two microns, from the smaller oblique dendrites whose diameters are significantly less than a micron. In this regard, it has further been shown that the majority of the surface area of a CA1 pyramidal neuron is actually from the small oblique dendrites and not the larger
trunks [22]. Thus, it seems logical that the majority of synapses would occur on these dendrites. Indeed, despite their small size, it is estimated that in the apical dendrites approximately 85% of the excitatory synapses occur on oblique dendrites [22]. Thus, their role in neuronal computation is crucial, however, while a lot of work has gone into characterizing the properties of large dendrites (for an extensive review see [23]), oblique dendrites have been less significantly studied, predominantly because their small size makes them difficult to record.

*Dendritic Spines*

Dendritic spines are specialized micro-extensions of a dendrite and are believed to compartmentalize individual synaptic responses. They are approximately 0.5µm in diameter, 0.5-2µm in length and have a wide range of morphologies (see *Fig 1-3*), one of the most common being a round spherical head attached to a smaller cylindrical neck. Optical studies have shown that they are continuously created and destroyed (or pruned) [24]. They are also continuously distorting in shape and moving in both immature and mature cells (for reviews see [25,26]). Particularly interesting is that the time scale for any of these processes can be within minutes to an hour. Thus, they are believed to be especially important in the formation of memories, and several studies have investigated their link to synaptic plasticity (for review see [27,28]).

*The Axon*

The axon arises from a region of the soma known as the axon hillock. The diameter of an axon can range from 200nm to 20µm and, in certain animals, it can be up to meters in length. For rat CA3 axons which make up the Schaffer collaterals, the
typical axonal diameter is between 20\textmu m to 40\textmu m and the length is approximately 700\textmu m [29]. In addition to being the beginning of the axon, the hillock is also the generation site of an action potential, a neuronal signal which once generated quickly descends down the axon due to presence of myelin (a product of neighboring glial cells). The end of an axon divides into many thin processes, which contain protuberances known as boutons (also known as presynaptic terminals) where neurotransmitter is released. These are the presynaptic counterparts to dendritic spines, however unlike spines, studies have shown that they are largely immobile [26]. Neurotransmitter release has also been shown to be localized to other areas of the axon, known as axonal varicosities. These segments of axon are approximately 50% larger in diameter than neighboring segments and are not distinctly associated with a dendritic spine. They are also the subject of much study since, as of yet, their role in a variety of neuronal functions is not well understood [29].
Figure 1-3: Neuron microanatomy  (a) Maximum projection image of a CA1 pyramidal neuron filled with a fluorescent structural indicator showing locations of basal and apical dendritic trees relative to the soma (image taken by Tycho Hoogland) (b) Fluorescent image of a dendritic branch with numerous visible spines; “thin” type and “stubby” type specifically identified (adapted from Zuo 2005 [24]).
1.2.3 Neuronal Preparations

Most neuroscience studies use neural tissue from developing invertebrates, chickens, and rodents primarily because of the similarity of their nervous systems to ours. In addition, these animals are also relatively easy to maintain in groups, as opposed to less often used larger animals. For hippocampal studies, the standard model is the rodent. However, since different types of preparations are typically used, it is important to gain an understanding of the advantages and disadvantages of each of them. Here I briefly introduce three different preparation types which are commonly used (for review see [30]).

Cell Culture

This category is itself composed of several subcategories, such as disassociated cultures and organotypic cultures (for review see [31]). However, most all studies which use neuronal cultures, regardless of their type, benefit from their reduced and adjustable complexity. Indeed, since the external environment is user defined, several parameters of a cell culture, such as the density of cells or number of synapses, are at least somewhat controlled by the supplied conditions. However, this is also the primary drawback of cell cultures, since supplied conditions can be far from the conditions in vivo. Indeed most cell cultures lose the inherent architecture of the tissue. One definitive example of this is the fact that cell cultures are relatively 2D, since they are basically individual cells plated on a substrate. While this is advantageous because it allows several recording techniques to be used that would otherwise be unavailable, it also simultaneously sheds doubt on the accurateness and relevance of the data obtained, since in vivo cells develop in extensive
3D networks. The importance of this particular point is exemplified by recent advances in cell culture techniques which are aimed at incorporating the 3D nature of biological systems (for review see [32]).

**Brain Slice**

Brain slices offer a method of maintaining cellular architecture while still preserving some of the flexibility of cultures. In the hippocampus, transverse slices (i.e. slices which are cut so as to maintain the three excitatory pathways) are arguably the most common preparations used (for review see [33]). The thickness of these slices can vary from 150μm to 600μm. Thinner slices tend to be used for structural imaging of single cells, whereas thicker slices are used for electrophysiological recordings since they ideally should have higher levels of intact circuitry. The cells in the middle portion of slices that are too thick tend to die from lack of oxygen, since there is no circulatory system to effectively deliver perfusing solution to them. This is a prime example of the limitations of slices. In addition, since brain tissue is a highly scattering optical media, optical imaging techniques that are used to image brain slices must be capable of isolating specific sections of the slice.

**Live Animal**

While brain slices tell us a lot about single neurons and small neuronal networks, *in vivo*, neurons operate in large network. Thus, the most realistic neurophysiologic data is achieved from studies done on completely intact brains. However, as can be guessed, this preparation is the most difficult to manage and the least flexible to experimental
manipulations. Indeed, since access to the brain is achieved via a craniotomy opening while the animal is under anesthesia, preparations are far less stable than either a slice or culture because the pulse and respiratory cycle tend to interfere with the data being recorded. Thus, the information that can be recorded is typically restricted to large signals, such as action potentials. Despite this limitation live animal studies have been used for a long time to study the visual system [34], and their use in studying other neuronal systems has been steadily increasing (for review see [35]).
1.2.4 Neuronal Electrophysiological Techniques

Electrophysiological techniques are designed to measure membrane potential changes, which are the mechanisms neurons use to communicate with each other. Indeed, since the resting potential of a neuron is usually around 70mV, any change in this value is indicative of a process done by or on the neuron. Given the relatively small sizes of these changes, particularly for subthreshold events which can be only a few tenths of a millivolt, any technique designed to record them must be capable of high signal/noise (S/N) ratios. In addition, electrophysiological signals occur relatively fast (i.e., on the order of milliseconds), so techniques to measure them must also have high temporal resolution. In this regard, there are primarily two distinct choices, the patch clamp technique and the optical recording approach.

**Patch Clamp Technique**

While the idea of using a microelectrode to study potential changes in a neuron had been well developed before Neher and Sakmann created the patch clamp technique [36], this method quickly became the standard for monitoring electrophysiological experiments. This technique places a large diameter (1-3μm) low resistance (1-10Mohm) glass electrode on a single visualized cell (for review see [37]). The low resistance of these electrodes results in high S/N ratios. Once the pipette is placed on the cell, brief suction is used to form a high resistance seal (approximately 10GΩ) with the membrane. This tight seal minimizes the leak current and further increases the S/N ratio, which allows the currents through single channels to be recorded. If the seal is ruptured, then the electrode and the cytoplasm are in electrical contact, and intracellular or whole-cell
recordings are permitted. When combined to a feedback amplifier, this allows the user to clamp either the voltage (voltage clamp) of the cell and monitor the current or deliver a fixed amount of current to the cell (current clamp) and monitor the voltage.

While the patch clamp technique is great for not only monitoring intracellular conditions, but also selectively controlling them, the extent to which the patch clamp can be used is severely spatially limited. Indeed, despite the fact that several studies have shown the ability to patch dendrites [38-40], even with experienced electrophysiologists, only a few sites on the entire neuron can be studied simultaneously. Thus, for studies of neuronal computation, the patch clamp technique alone is inadequate.

**Fluorescence Imaging**

Biological fluorescence imaging, which represents a subcategory of a large collection of optical techniques designed to generate multi-site minimally invasive recordings, is an extensively used method in neuroscience for collecting electrophysiological data either in addition or as a substitute for the patch clamp. The principle of fluorescence which governs it relies on molecules which absorb light at a given frequency and emit it at a shorter frequency. The degree to which different molecules can do this is determined by their molecular structure and is characterized by their extinction coefficient ($\varepsilon$), which describes how many photons they can absorb, and their quantum efficiency ($Q$), which quantifies the emitted photons to absorbed photons ratio. Biological studies which use fluorescence imaging benefit from the inherent contrast it provides. Indeed, in neuroscience, since individual neurons can be selectively
labeled with fluorescent indicator, fluorescent imaging has been very effective in structural imaging.

While fluorescence imaging represents a wide variety of techniques, this review will focus primarily on fluorescence microscopy, and even more specifically on fluorescence microscopy techniques that are capable of 3D imaging. The microscopy principles will be discussed in the next section, but here I give a brief description of both the fluorescent indicators and detectors used in optical electrophysiological experiments.

*Fluorescent Indicators*

There are primarily two types of fluorescent indicators used in electrophysiological imaging studies, voltage-sensitive dyes and calcium-sensitive dyes.

*Voltage Sensitive Dyes (VSDs)*

Fluorescent VSDs are a class of indicators whose levels of fluorescence change with changes in the surrounding electrical field. As such, they have been shown to be effective in monitoring changes in membrane potential as well as neural activity (for review [41,42]). They can be further subdivided into slow-response and fast-response based on how fast they are able to change in response to membrane potential changes.

Slow response VSDs (e.g., DiSC(3)-5) are positively charged and membrane permeable. Therefore they separate, like other cations such as sodium and potassium, into an intracellular store and an extracellular store. The exact distribution is determined by their Nernst potential, which is dependent upon the membrane potential. Thus, changes in membrane potential cause redistribution of the dye between the two stores,
which changes the fluorescence. Although the fluorescent change is substantial, because this redistribution requires the dye to physically cross the membrane, their response time is on the order of seconds, which severely limits their use in neurophysiological studies.

Fast VSDs (e.g., RH-414, di-4-ANEPPS, di-8-ANEPPS) are membrane impermeable and thus attach to the outside of membranes if bath applied or the inside of membranes if loaded via a patch pipette. Their molecular structure is very sensitive to the surrounding electric field and thus changes with changing membrane potential. This results in a change in either their extinction coefficient or their quantum efficiency, which changes their fluorescence. Since the molecular changes can occur very quickly, fast VSDs have response times on the order of microseconds, however their change in fluorescence is much smaller than slow VSDs, making them very difficult to use as well.

*Calcium-Sensitive Dyes*

Calcium-sensitive dyes represent the most commonly used ion-sensitive indicator involved in optically monitoring neural activity (for review see [43]). They can also be subdivided into two separate classes, metallochromic dyes (e.g., Arsenazo III, Antipyrylazo III, Azol) and tetracarboxylic dyes (e.g., Calcium-green 1, Fura-2). Both types of dyes work by changing their optical properties when they bind calcium, however tetracarboxylic dyes are more often used since they are more selective. Indeed, since metallochromic dyes bind to various different types of cations, including single protons, they are less reliable than tetracarboxylic dyes for accurate measurement of calcium. Tetracarboxylic dyes also benefit from the availability of membrane-permeable derivatives, such as the acetoxyethyl (AM) ester form (i.e. Fura-2 AM). In these
variants, the calcium binding capability is only available once intracellular esterases have cleaved the inactivating terminals. However, this cleavage also makes the dye membrane-impermeable, thereby trapping it in the cell. Thus, these derivatives offer a noninvasive method of loading calcium dyes that obviates using a micropipette.

**Detectors**

There are three primary types of detectors used in optical neuroscience experiments, cameras, photodiodes, and photomultiplier tubes (for review see [44]). While there are several parameters that characterize them, they can be distinguished based on their spatial resolution, their speed, and their noise (which includes the noise generated by photons released without the presence of light, e.g. dark noise).

**Cameras**

Cameras, which include the charge coupled device (CCD) camera, are the most often used imaging detectors. Indeed, since they are, in essence, an array of several individual pixel sized photodetectors coupled through a shared readout mechanism, cameras are the only detectors listed here that allow detection at more than just a single site of interest. However, they are rarely fast enough to be used for monitoring electrophysiological signals. In fact, most cameras are limited to 60 frames per second, which is more than a factor of 10 too slow for monitoring physiological signals, such as calcium dynamics. However, modern CCD cameras allow the user to increase this rate by either decreasing their resolution using a process called binning, in which multiple pixels are grouped together, or by decreasing their field of view, by selectively covering a
portion of the active photodetectors. In addition, the dark noise of a CCD camera can also be reduced by cooling it, which reduces thermal fluctuations. With these techniques, different groups have been able to use CCD cameras to study some neurophysiological processes [45,46], but have usually been restricted to monitoring relatively large structures (i.e. main dendritic branches).

Photodiodes

Photodiodes are some of the most commonly used non-imaging detectors. Unlike cameras, they have no spatial resolution. Therefore, when used to acquire images, they must be coupled to a mechanism that serializes the light detection process. However, they also have no readout requirement, which makes them much faster than cameras. In addition, they have a relatively high quantum efficiency, which is a measure of the input photon/output electron ratio, and are therefore very sensitive. Their noise level is lower than that of a camera primarily because they have fewer components which would generate thermal fluctuations. As a result, they have also been extensively used by different groups in various neuroscience experiments.

Photomultiplier Tube (PMT)

PMTs are photodetectors that offer high internal gain for low light applications, which also make them some of the most common detectors in optical neuroscience. They consist of a photocathode and a series of electrodes. Unlike a photodiode where the output electron is converted to an output signal, the output electron in the PMT photocathode strikes a secondary electrode, which generates another electron. Each
successive electrode is held at a higher potential, which serves to draw the emitted electrons towards them. This cascading effect results in an amplification of approximately $10^5$ to $10^7$ electrons per incident photon. This is also true for photons generated by thermal noise therefore, the dark noise of the system is also amplified. Since this process is very fast PMTs, like photodiodes, are capable of operating at physiological speeds. However, also like photodiodes, they are not imaging detectors and therefore have no spatial resolution. But when combined with scanning mechanisms, such as those described below, PMTs have also been shown effective in several optical neuroscience studies.
1.3 Microscopy Background

As mentioned above, the most effective studies of neuronal physiology are done on live animals or neuronal preparations that mimic the in vivo environment as much as possible. However, most of these preparations have many layers of cells and therefore require imaging mechanisms that are capable of isolating an optical section inside an extended specimen. Over the past few decades, techniques that can perform this function, such as confocal microscopy and multi-photon microscopy, have become important in almost all fields of biology. Here we briefly describe the principles behind both these techniques as well as their application to experimental neuroscience (for confocal review see [47,48] for multi-photon review see [49-51]).

1.3.1 Confocal Microscopy

The confocal principle, in which point illumination is combined with point detection to isolate a plane of observation, was initially described by Minsky in 1961 [52]. As seen in Fig. 1-4, this technique spatially filters out of focus light from planes above and below the plane of interest. In addition, as can be seen from the figure, it also spatially filters light from points laterally displaced from the point of interest. Thus, in addition to providing optical sectioning and improving axial resolution, it also improves lateral resolution. It is important to note that since images are acquired point by point, they are typically reconstructed via a computer.

In most confocal microscopes, point illumination is obtained via a focused light source, which is usually, but not necessarily, a laser, and point detection is obtained via a
pinhole. Multiple points on an image are acquired by scanning the light source, which if done correctly, allows the sample and the pinhole to remain fixed. This is true for all forms of confocal microscopy, including fluorescence-based confocal microscopy. This particular form of confocal microscopy has had great impact in the structural imaging of optically thick biological tissue. However, given the fact that the excitation volume is the same as in widefield microscopy, out-of-focus fluorescence light is still generated with this technique. Therefore, confocal fluorescence microscopy suffers from high levels of phototoxicity, where the direct and indirect effects of laser light lead to tissue damage, and photobleaching, where the fluorescence of molecules drops over time.

Given the presence of fluorophores capable of binding specific physiologically important ions, such as calcium-sensitive dyes, or measuring voltages, such as VSDs, the field of functional imaging (in which biological function instead of structure is recorded) has benefited greatly from the resolution enhancement and optical sectioning capabilities of confocal microscopy.

1.3.2 Multi-photon Microscopy

The principle of multi-photon microscopy allows the wide-field and confocal problems of phototoxicity and photobleaching to be overcome. Two-photon absorption, which was initially proposed by Maria Goppert-Mayer in 1931, is based on the idea that within a sufficiently small temporal window, a molecule can absorb two photons in the same quantum event. However, the probability of having two photons interact with a molecule within this time window is rare, unless the flux of photons is extraordinarily
**Figure 1-4: Spatial filtering in confocal microscopy**  (a) Points at the lateral and axial focal position of the objective lens form an image at the pinhole, but (b) the image of a point that is laterally offset at the focal plane is laterally offset from the pinhole, and the (c) image of a point that is axially offset from the focal plane is axially displaced relative to the pinhole.
high. Thus, the practice of two photon excitation/absorption did not take hold until the advent of high power laser sources.

Even with such powerful laser sources, the photon flux needed for effective two photon absorption is only achieved if the laser beam is focused. This is the inherent sectioning ability that is utilized in multi-photon microscopy. An advantage of this sectioning mechanism, when compared to the spatial filtering mechanism available in confocal microscopy, is the fact that fluorophores that are not within the multi-photon cross section are not excited (see Fig. 1-5). Therefore, both phototoxicity and photobleaching are reduced.

Another advantage is that since at least two photons are absorbed, these photons must have lower energy and longer wavelength. Therefore, since Rayleigh scattering is inversely proportional to the fourth power of the wavelength, they are less scattered by optically dense biological tissues such as brain slices. Because of this, multi-photon microscopy has a larger penetration depth than confocal microscopy. Thus, multi-photon imaging has extensive applicability in structural imaging and has potentially even more impact in functional imaging than confocal microscopy.

1.3.3 Scanning Mechanisms

To an extent, both confocal and multiphoton imaging have been used to functionally study neurons. Indeed, physiological parameters such as membrane potential and calcium dynamics have effectively been investigated using both methods ([53,54]). However the extent of their use in neurophysiological studies has been severely
Figure 1-5: Characteristics of multi-photon excitation (a) Jablonsky diagram of the two photon absorption process. Note that the fluorophore can be excited to either the first or second excited state, with a rapid decay in the latter case. Also note the frequency of the emitted photon is less than twice that of the exciting photon. (b) Illustration of the multi-photon (two photon) focal volume in comparison with a single photon focal volume.
limited by their speed, which in turn is limited by the laser scanning mechanisms they use.

**Lateral Scanning Mechanisms**

*Galvanometers*

Most commercial based systems use mirrors attached to galvanometers to laterally scan the laser beam. A galvanometer, which uses a current carrying wire in a magnetic field to create a torque, can be a very precise mechanism for positioning a laser beam if operated in a feedback controlled system. Also, compared to other conventional methods of positioning a laser beam, including flying optics systems, in which most of the optical setup is moved, or fixed-beam systems, in which the preparation is moved, galvanometers are relatively fast since they are much less massive and, thus, have less inertia. However, for monitoring neurophysiological process, which occur on the millisecond time scale, galvanometers are far to slow for spatially widespread studies. Indeed, since the frame rates of galvanometer based systems are less than 1 kHz, in order to functionally study multiple sites, most users typically restrict their scan region to a single line. While this does allow them to view sites that are on the line with speeds sufficient enough to record physiological data, it severely restricts the number of sites that a user can visit on complex highly-nonlinear structures such as neurons. In fact, usually no more than a handful of sites are recorded during a single scan.

*Acousto-Optic Deflectors*

Acousto-optic deflectors (AODs) are devices which use the interaction of sound
and light waves within an medium to create tunable diffraction gratings (see Fig. 1-6). They consist of a transducer attached to an acousto-optic medium, typically a crystal. When an acoustic wave is propagated through the crystal, it creates a series of compressions and rarefactions with separation distances proportional to the wavelength of the sound wave.

Light propagating through a crystal undergoing this effect selectively interferes in a way that mimics a diffraction grating (for review see [55,56]). Therefore, an AOD can be represented as a diffraction grating with a grating constant proportional to the wavelength of the acoustic wave. Implicit in this definition is the fact that for an AOD, the deflection angle depends only on the wavelength of the acoustic wave and the angle of incidence. In fact, the angle of deflection for the first order beam (when operated in the Bragg regime where the angle of incidence equals the angle of deflection) can be calculated using the following equation:

\[ \theta = \frac{\lambda}{\Lambda} = \frac{\lambda f}{v} \]  

(1-1)

Since the transition time for an AOD is effectively governed by the time it takes the acoustic wave to cross the aperture i.e., the aperture time (~15\(\mu\)s), AODs offer a method of selectively deflecting an incoming laser beam which is faster than galvanometer-based scanning. In addition, AODs allow one to scan non-contiguous points at the same speed, thereby removing the line scan constraint. It is important to realize that since they are diffraction gratings, the output power from an AOD is always
appreciably less than the input power. However, if this reduction in power can be tolerated, AODs offer an attractive method for lateral scanning and have recently been combined with confocal and multi-photon microscopy to create fast two dimensional (2D) neuronal imaging setups ([12,13,57,58]).
Figure 1-6: **Principles of acousto-optic deflection** (a) Illustration of isotropic Bragg diffraction in an acousto-optic deflector. Note that although not shown, the angle of incidence in this situation must be equal to the first order angle of deflection $\theta$, which is given by the equation in (b), where $\lambda$ is the wavelength of the light, and $f, \Lambda, v$ are the frequency, wavelength, and velocity of the acoustic wave respectively.
**Axial Scanning Mechanisms**

*Micropositioners/Nanopositioners*

While galvanometers are the typical lateral scanning mechanism used in most commercial based confocal and multi-photon microscopes, the most common axial scanning mechanisms are positioners. These are piezoelectric stepper motors which, like galvanometers, can have very high degrees of precision (nanometers to micrometers) if operated in a feedback controlled method. However, while these positioners offer high precision, they are relatively massive and thus slow (typically ~10ms). Therefore, they limit any attempt to monitor fast neurophysiological changes in three dimensions. This is a severe restraint in neuroscience studies since the dendrites of neurons are complex 3D structures which dive in an out of many planes.

*Strategic scanning via stepper motor and galvanometers*

A recent advancement has combined sinusoidal positioning with an objective stepper motor with strategic positioning via galvanometers to generate novel scan patterns that extend the ability of fast scanning capabilities to three dimensions [59]. While, this system is still limited to relatively moderate axial scanning speeds (10Hz) due to the inertia limited temporal bandwidth of the objective stepper motor, its has been used to demonstrate viable calcium recordings from neuron cell bodies *in vivo*.

*Liquid-Filled Lens*

There have been several methods that have been introduced that could increase the speed of axial scanning. One of the most developed is the variable focal-length liquid-filled lens. The principal mechanism for changing the focal length within this type
of lens relies on changing the pressure within a lens chamber, which in turn deforms an elastic membrane and changes the curvature of the lens [60]. Recent developments have increased the maximum numerical aperture [61] and the speed [62] at which the focal position can be changed, but despite these improvements, the fastest variable focal length liquid-filled lenses still require at least one millisecond to change the focal position, which, while 10 times faster than motorized positioners, is not fast enough to track neurophysiological signals at more than just a few sites. Variable focal length lenses which use nematic liquid crystals or electro-optic materials to change the refractive index of the lens rather than the shape have also been developed [63-65]. In general, for these types of lenses, generating fast response times requires restricting the thickness of the lens cell, which in turn severely limits the maximum change in focal length.

*Deformable Mirror*

Another documented approach to achieving high speed axial scans involves using a deformable mirror (DM) [66]. These devices have launched the field of adaptive optics, particularly in astronomy [67,68] and ocular imaging [69-72], where they have been used to adapt for atmospheric and ocular aberrations. They have also been implemented in confocal microscopy based schemes to correct for spherical aberration in different samples [73,74]. In this regard, they are one of the few fast focusing schemes that allow for diffraction limited resolution at all axial planes, since they can simultaneously change the axial focus as well as correct for aberrations. However, even with the most modern technology, these devices are always inertia limited by the amount of time it takes to physically move the mirror components. Indeed, the fastest
commercially available DM known to this author has a frame rate of 1kHz (Boston Micromachining, Mini-DM, high frame rate option).

Temporal Focusing

Temporal focusing, also known as simultaneous spatial and temporal focusing (SSTF), is a multi-photon microscopy based technique in which the different optical frequencies in an ultrafast pulse are separated using a diffraction grating, collimated with a cylindrical lens and refocused using an objective [75,76]. Since the separated frequencies only spatially recombine at the focal distance of the objective lens, the temporal pulse width is only transformed limited (i.e., as short as the frequency bandwidth will allow) at this location. It has been shown that introducing group velocity dispersion (GVD), an optical frequency based phase delay that broadens ultrafast pulses when they travel through materials, into an SSTF system increases the optical path length of the system, which displaces the temporal focus away from the objective focus [77]. However, to date, the change in dispersion has been done via manipulation of the prisms in a pre-chirper, making the process relatively slow.

Axicon based Scanning

Possibly the most direct way to increase the speed of axial scanning is to increase the depth of field of the focused beam, in essence acquiring multiple axial sections simultaneously. This is the basis behind techniques which use an annular mask to reduce the effective numerical aperture of the objective lens. However a more power efficient way of broadening the axial dimension of the focus is to use an axicon [78]. An axicon is
a conical prism that transforms an incident Gaussian beam waveform into one represented by a zeroth-order Bessel function, where the central peak of the Bessel function represents the focus along the axial direction. This technique has been implemented in multi-photon microscopy to quickly generate 2D maximum projection images of 3D structures [79]. However, the drawback of this technique is two fold. First, in structures that have layers, the individual sections cannot be isolated. Second, the energy of the beam is distributed across the entire axial length of the focus, requiring increased amounts of power to excite multi-photon fluorescence.

Of the available techniques for generating quick axial scans, none seem to have the speed or random access ability that acousto-optic deflectors offer for lateral positioning. While it might be possible to combine AOD based lateral imaging with one of the above mentioned techniques for axial positioning, the ideal solution would allow for AOD based axial positioning as well as lateral positioning. This idea is further described in the next chapter.
Chapter 2: Three Dimensional (3D) Acousto-Optic Scanning

This chapter proposes a technique in which AODs are used to generate a variable focal length cylindrical lens. In turn, this fast focusing cylindrical lens is used, in conjunction with a second fixed lens, such as an objective lens, to vary the focal position around the inherent focus point of the second lens, thus allowing for quick axial positioning. The theory behind how a focus could be deterministically positioned in the axial dimension as well as in both the axial and lateral dimensions is developed. In addition, experimental evidence is shown that demonstrates the ability of a simplified scanner to accomplish both these tasks in close agreement with the theoretical predictions. Also, this technique is shown to compensate for spatial dispersion, a resolution degrading process that occurs when AODs are utilized in multi-photon laser scanning microscopy (MPLSM).

2.1 AOD Based Axial Scanning

2.1.1 Theoretical Considerations

As mentioned in section Chapter 1, AODs can be viewed as tunable diffraction gratings, where the wavelength of the sound wave propagating through the AO medium, e.g., a tellurium dioxide (TeO₂) crystal, corresponds to the grating constant. Therefore, changing the frequency of the sound wave changes the angle by which the incoming beam is deflected (Fig. 1-7). When operated in the Bragg regime, where the Klein and Cook parameter, Q, [56] is much greater than one, the first order angle of deviation $\theta$
can be described by Eqn. 1-1 in Chapter 1, which is repeated here as Eqn. 2-1 for convenience.

\[
\theta = \frac{\lambda}{\Lambda} = \frac{\lambda}{\nu} f,
\]  

(2-1)

Once again, \( \lambda \) is the wavelength of the laser light, \( \Lambda \) is the acoustic wavelength, \( f \) is the acoustic frequency (Note: throughout this document, frequencies and changes in frequencies will be denoted by \( f \), whereas focal lengths and changes in focal length will be denoted by \( F \)), and \( \nu \) is the acoustic velocity in the AOD.

If we change the acoustic frequency in time, we can represent both the frequency and the angle of deviation as time-dependent functions \( f(t) \) and \( \theta(t) \), where Eqn. 2-1 still holds. If we then consider chirped acoustic frequencies, where \( f(t) \) constantly changes, we can define the deflection angle \( \theta(x,t) \) at any point \( x \) in the aperture of the AOD, as follows:

\[
\theta(x,t) = \frac{\lambda}{\nu} f \left( t - \frac{x}{\nu} \right),
\]  

(2-2)

where \( x = 0 \) has, without loss of generality, been chosen to be at the center of the AOD aperture and the positive \( x \) direction has been chosen to be in the direction of the deflection. We can see from this equation that if we linearly change the acoustic frequency in time (chirp) with a given frequency/time value of \( \alpha \), i.e.,
\[ f(t) = f_{\min} + \alpha t, \] where \( f_{\min} \) is the lowest acoustic frequency in the bandwidth, \\
\[ \alpha = \frac{(f - f_{\min})}{T_{\text{scan}}} = \Delta f/T_{\text{scan}}, \] and \( T_{\text{scan}} \) is the duration of the chirp, then the angle of 
\[ \theta(x,t) = \frac{\lambda}{v} (f_{\min} + \alpha t) - \frac{\lambda \alpha}{v^2} x \] 
\[ (2-3) \]

This equation describes a converging cylindrical lens with a focal length equal 
to \( F = \frac{v^2}{\lambda \alpha} = \frac{v^2 T_{\text{scan}}}{\lambda \Delta f} \), whose central axis of propagation changes from a minimum 
angle of \( \theta = \frac{\lambda f_{\min}}{v} \) at the beginning of the scan to a maximum of \( \theta = \frac{\lambda f}{v} \) at the end 
of the scan. Note that we can also make a diverging cylindrical lens (as shown in Fig. 2- 
1a) by using a negative chirp (i.e., let \( f(t) = f - \alpha t \)).Thus, a single AOD with a 
linearly chirped acoustic signal can be modeled as a cylindrical lens with a time-varying 
central optical axis.

If we then optically relay the aperture of this AOD to another AOD, we can 
describe the total deflection angle by:

\[ \theta(x,t) = \frac{\lambda}{v} \left[ -f_1 \left( t + \frac{x}{v} \right) + f_2 \left( t - \frac{x}{v} \right) \right], \] 
\[ (2-4) \]

where \( f_1(t) \) is the time-dependent acoustic frequency in the first AOD and \( f_2(t) \) is the time-
dependent acoustic frequency in the second AOD. It is important to note that by
**Figure 2-1: AOD methods of operation** (a) Spread of the deflection angles when an AOD is operated with a chirped frequency input. (b) Elimination of the lateral scan by using two AODs and relay optics (note that the optical relay makes the second acoustic wave effectively counter-propagating relative to the first).
reversing the light beam and deviation angles, the optical relay effectively makes the second acoustic wave counter-propagating relative to the first.

If we let the acoustic frequencies \( f_1(t) = f_2(t) = f_{\text{min}} + \alpha t \), the total deviation angle can be represented as follows:

\[
\theta(x,t) = -\left(2\alpha \frac{A}{v^2}\right)x
\]  

(2-5)

This equation now describes a converging cylindrical lens with a central axis of propagation that is time-independent. The focal length of this acousto-optic lens (AOL) is

\[
F_{\text{AOL,conv}} = \frac{v^2}{2\lambda \alpha} = \frac{v^2 T_{\text{scan}}}{2\lambda \Delta f}.
\]  

(2-6)

in agreement with published results [80] but arranged so that no off-axis deviation is introduced. Thus, for each chirp value \( \alpha \), we get a specific axial focal position. Note that if we use \( f_1(t) = f_2(t) = f - \alpha t \) as our chirp frequencies, this results in a diverging cylindrical lens (as shown in Fig. 2-1b) with a focal length of \( F_{\text{AOL,div}} = -\frac{v^2}{2\lambda \alpha} = -\frac{v^2 T_{\text{scan}}}{2\lambda \Delta f} \).
2.1.2 Experimental Evaluation

To demonstrate the effectiveness of the axial scanning scheme, we optically relayed the exit aperture of the AOL to the back focal aperture of an infinity-corrected air objective lens (10X, 0.30NA Zeiss Plan Neofluar). Therefore we expect a total focal length equal to

$$F_{Total} = \frac{F_{Obj} F_{AOL}}{F_{obj} + F_{AOL}}, \quad (2-7)$$

where $F_{Obj}$ is the focal length of the objective (~16.5mm).

Using a continuous wave (CW) argon laser ($\lambda = 488\text{nm}$) and two TeO$_2$ cylindrical aperture AODs (LS55V, Isomet) with acoustic velocities of $v = 619\text{m/s}$, we generated axial scan patterns using a scan time ($T_{scan}$) of $30\mu\text{s}$ and maximal frequency bandwidths of $\Delta f = 30\text{Mhz}$ (giving us a range of $\alpha$ values from -1MHz/µs to 1MHz/µs). Specifically, in Fig 2-2., we created a five-point axial scan pattern using the CW laser and AO characteristics described above. We imaged and reconstructed a 3D representation of the scan pattern using the imaging setup described below. Figure 2-2a shows the axio-lateral projection of the five-point pattern. Figure 2-2b compares predicted (based on Eqn. 2-7) versus experimentally obtained values for $F_{Total}$. 
Imaging setup:

To acquire our data, we focused the image obtained from an infinity-corrected (32X, 0.6NA Leitz) air objective lens onto a commercial video camera (WV-1550, Panasonic). Both camera and objective lens were mounted on a translation stage with a resolution of 10\(\mu\)m in the axial direction (see Fig. 2-2c). Two-dimensional images were taken at every 10\(\mu\)m axial step to determine the axial distances of the focal points. Lateral distances were derived from the pixel count. The pixel size was determined by imaging a stage micrometer to assess and normalize for the magnification of the optics. Reconstructed maximum projections were generated using 3D reconstructing software (Amira 3.0, Template Graphics Software). Intensity plots were generated either by selectively identifying camera pixels onto which focal spots were formed and monitoring the intensity at those pixels as the image stack was acquired or by post-acquisition image analysis (Matlab 7.0, Mathworks).
Figure 2-2: Axial Scan (a) Reconstructed axio-lateral projection image of a five-point axial scan pattern (Focus 1-5) using the 10X Zeiss objective lens mentioned in the text (b) Experimental (marker) and theoretical (line) plot of the total focal length change ($F_{\text{Total}} - F_{\text{Obj}}$) versus chirp value ($\alpha$) for the AOL scanning scheme (c) Setup used to acquire images in a (for description, see text).
2.2 AOD Based Axial and Lateral Scanning

2.2.1 Theoretical Considerations

If we use the AOD scheme shown in Fig. 2.1b and introduce offsets in the acoustic frequency (i.e., let \( f_1(t) = f_{\text{min}} + \alpha t + f_{1\text{offset}} \) and \( f_2(t) = f_{\text{min}} + \alpha t + f_{2\text{offset}} \)), we find that the total angle of deflection according to Eqn. 2-4 can be described by

\[
\theta(x, t) = -(2\alpha \frac{\lambda}{v^2}) x + (f_{2\text{offset}} - f_{1\text{offset}}) \frac{\lambda}{v}
\]  

(2-8)

The first part of the right hand side of this equation is identical to the purely axial case (Eqn. 2-5) and once again describes a cylindrical lens with a focal length defined by Eqn. 2-6. The second part of the equation defines a time-independent lateral deflection angle

\[
\theta_{\text{lateral}} = (f_{2\text{offset}} - f_{1\text{offset}}) \frac{\lambda}{v} = \Delta f_{\text{offset}} \frac{\lambda}{v}
\]  

(2-9)

that is mathematically independent of the focal length and, therefore, the axial focal position. We can see that when \( f_{2\text{offset}} = f_{\text{max}} \{ f_{\text{min}} \} \), where \( f_{\text{max}} \) is the maximum frequency in the acoustic bandwidth, and \( f_{1\text{offset}} = f_{\text{min}} \{ f_{\text{max}} \} \), we get a maximal {minimal} scan angle of \( \theta_{\text{lateral}} = (\Delta f_{\text{bandwidth}}) \lambda/v \{- (\Delta f_{\text{bandwidth}}) \lambda/v \} \), where
$\Delta f_{\text{bandwidth}}$ is a change in acoustic frequency which is equal to the entire acoustic bandwidth. This gives a total scan range of $\Delta \theta_{\text{lateral}} = 2(\Delta f_{\text{bandwidth}}) \frac{\lambda}{v}$. It is important to note that to utilize the maximal lateral scan range, there can be no frequency chirp and thus no axial scanning.

2.2.2 Experimental Evaluation

To demonstrate both axial and lateral scanning, we generated a 3-position axio-lateral scan pattern (Fig. 2-3) using the AOL and a 10X objective lens ($F_{\text{obj}} = 16.5\text{mm}$). We dwelled $\sim 30\mu$s at each focal position, thus enabling us to visit all three spots within the frame rate of the camera. The offset and chirp values used, as well as the predicted (based on Eqs. 2-6, 2-7 and 2-9) and measured lateral and axial focal positions relative to the inherent focal position of the objective lens (i.e., the focal position if a non-deflected collimated beam is incident on the back focal aperture), are shown in table below.

<table>
<thead>
<tr>
<th>Focus</th>
<th>Chirp parameter ($\alpha$)</th>
<th>Offset parameter ($\Delta f_{\text{offset}}$)</th>
<th>Predicted axial focal position*</th>
<th>Measured axial focal position*</th>
<th>Predicted lateral focal position*</th>
<th>Measured lateral focal position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.75 Mhz/µs</td>
<td>7.5 Mhz</td>
<td>-504µm</td>
<td>-510µm</td>
<td>97.4µm</td>
<td>100µm</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>-0.75 Mhz/µs</td>
<td>-7.5 Mhz</td>
<td>537µm</td>
<td>550µm</td>
<td>-97.4µm</td>
<td>-96µm</td>
</tr>
</tbody>
</table>

* Both lateral and axial positions are calculated relative to the inherent focal position of the objective lens, which is equivalent to the position of the second focus since it has neither a chirp nor an offset parameter.
To visualize our scan, we used the imaging setup described in Sect. 2.1.2 and shown in Fig. 2-2c. As is seen from the maximum projection image and intensity plots shown in Fig. 2-3, three foci are laterally and axially distinguishable. Since the image acquisition was not started at a particular location, only relative distances are important. In this regard, the calculated axial (537\(\mu\)m and -504\(\mu\)m) and lateral (\(\pm\)97.4\(\mu\)m) inter-focal distances are close to their measured axial (550\(\mu\)m and -510\(\mu\)m) and lateral (-96\(\mu\)m and 100\(\mu\)m) values. Also, since the differing levels of the AOD diffraction efficiency at each position were not compensated during the acquisition of this image, the inherent objective lens focus has a higher intensity than either off-axis focus. In general, the differing level of diffraction efficiency can be compensated by utilizing acoustic wave generating methods that are capable of modulating the acoustic power. However, here, to attain intensity values that were similar, and thus able to be visualized in the same image, we allowed the objective focus to saturate the camera. As a result, accurate resolution quantification (particularly in regards to comparisons between the on-axis lens focus and the off-axis foci) cannot be attained from this image.
**Figure 2-3: Axio-lateral scan** (a) X-Z maximum projection of a three-point axio-lateral scan pattern (Focus 1-3). The reference position (0,0) is set at the non-deflected and unchirped focus, which is equivalent to the inherent focal position of the objective lens. Distance were calculated using the intensity plots in Fig. 3c (b) X-Y images at particular focus planes showing both the spherical focus at the reference position and the cylindrical focus in the X dimension at the off-center foci, which is a result of the AOL (c) Intensity plot generated by image analysis of a maximum projection similar to the one in Fig. 3a. Side projections of the intensity plot show that the lateral and axial spacing between the foci approximately agrees with predictions. Note that, as described in the text, due to differences in diffraction efficiency, the center focus (focus 2) is of higher intensity than either side foci and saturates the camera at its peak.
Figure 2-3: continued
2.3 Spatial Dispersion Compensation

2.3.1 Theoretical Considerations

As has been shown previously [81], spatial dispersion is a serious limitation when utilizing AODs with MPLSM. It is a direct consequence of the frequency bandwidth found in the ultra-fast laser pulses used in MPLSM. The resolution of an AOD is typically defined by the number of resolvable points (NRP) that can be obtained in the far field, which equals the NRP that can be obtained in the focal plane of an objective lens (if the back focal aperture is filled). This value is defined by the total scan range of the AOD ($\Delta \theta_{\text{scan}}$), which for single AOD operation is defined as

$$\Delta \theta_{\text{scan}} = \frac{\lambda \Delta f_{\text{bandwidth}}}{v},$$  \hfill (2-10)

(where $\Delta f_{\text{bandwidth}}$ is the acoustic bandwidth) divided by the minimum spot size. When monochromatic light sources such as CW lasers are used, the minimum spot size is the diffraction limit which, using the Rayleigh criterion, can be approximated by

$$\Delta \theta_{\text{diffraction}} \sim \frac{\lambda}{d},$$  \hfill (2-11)

where $d$ is the effective aperture size of the AOD. However, when light with a significant frequency bandwidth propagates through an AOD, there is an angular spread (spatial dispersion) at any given acoustic frequency which can be described as
\[ \Delta \theta_{\text{dispersion}} = \frac{\Delta \lambda f}{v} \]  

(2-12)

When this dispersion is significantly larger than the diffraction limit, it almost exclusively determines the effective spot size. For a tellurium dioxide (TeO₂) AOD with an acoustic velocity \( v \approx 620 \text{ m/s} \) and an acoustic bandwidth from 60Mhz to 100Mhz, even at the lowest acoustic frequency, the dispersion angular spread is already \( \sim 10 \) times the size of the diffraction limited angular spread (assuming a beam diameter of \( \sim 10 \text{mm} \) and a 100fs Gaussian pulse with a time bandwidth product of 0.441 and \( \lambda \sim 850\text{nm} \), which makes \( \Delta \lambda \sim 10.6\text{nm} \)). This dispersion decreases the resolution (the number of resolvable spots) by at least a factor of 10.

However with our 3D scanning scheme, the lateral scan position is determined by Eqn. 2-9. Thus, the effective bandwidth of the system is from \(-\Delta f_{\text{bandwidth}}\) to \(+\Delta f_{\text{bandwidth}}\), which for the TeO₂ AOD described above is from -40Mhz to +40Mhz. Using Eqn. 2-12, we see that this implies that at the center frequency, the angular spread due to dispersion is completely eliminated while at extreme scan angles (worst case), the angular spread is only 2/3 of the minimum spread of the single AOD case (see Fig 2-4a). However, by utilizing two AODs, we have also effectively doubled our scan range from \( \Delta \theta_{\text{scan}} \) to \( 2\Delta \theta_{\text{scan}} \). Therefore, this scheme results in a minimum improvement in NRP of three-fold over the uncompensated case at the extreme scan angles and a progressive increase in NRP towards the center.
2.3.2 Experimental Evaluation

To demonstrate the effect of spatial dispersion, we coupled a multi-line laser (main lines of 488nm and 514nm) to a single TeO₂ AOD to simulate the bandwidth of an ultra-fast laser that would be used with MPLSM. Using three separate acoustic frequencies \( f = 60 \text{Mhz}, 75\text{Mhz}, \text{and } 90\text{Mhz} \) and a 10X objective lens \( (F_{\text{obj}} = 16.5\text{mm}) \), we generated a three point lateral scan pattern which we imaged and reconstructed using the scheme described in Sect. 2.1.2 and shown in Fig. 2-2c. An axio-lateral projection image is shown in Fig. 2-4b. At each individual location, the laser lines are spectrally separated in the lateral dimension, which is expected since each wavelength has a specific deviation angle determined from Eqn. 2-1. We then coupled our two AOD scanner to the multi-line laser and re-scanned the three positions (using offset acoustic frequency values of \( \Delta f_{\text{offset}} = 15\text{Mhz}, 0\text{Mhz}, \text{and } -15\text{ Mhz respectively} \) ). The result is shown in Fig. 2-4b. The separate laser lines perfectly recombine at the center, indicative of complete dispersion compensation. In addition, the spacing between the laser lines at the edges has also significantly decreased. To further quantify this effect, we measured the inter-focal spacing between the two major lines of the laser at multiple points in the bandwidth of both the uncompensated single AOD case and the compensated two AOD case. Using the deviation angles predicted by Eqns. 2-1 and 2-9, we can also calculate the separation distance between the foci formed by these two frequencies. Figure 2-4c shows the result of both the calculation (lines) and the measurement (markers). Both show a minimum decrease of \( \sim 2/3 \) in interfocal-distance (and hence dispersion) when using the two AOD scheme. As mentioned above, since we have also doubled our scan range, this indicates a resolution enhancement of at least three-fold over the single AOD case.
Figure 2-4: Spatial Dispersion (a) Calculated spatial dispersion (as characterized by $\Delta \Theta_{\text{disp}}$) versus acoustic frequency for the multi-photon scheme described in the text ($\lambda = 850\text{nm}$, $\Delta \lambda = 10.6\text{nm}$) (b) Axio-lateral projections of a three point lateral scan pattern with a multi-line laser (top) uncompensated scan obtained using a single AOD (bottom) compensated scan obtained using the two AOD scheme (c) Predicted (lines) and measured values (markers) of spatial dispersion (as characterized by inter-focal distances) versus acoustic frequency for both a single AOD (uncompensated) and the two AOD (compensated) scheme when using a multi-line laser (main lines of 488nm, 514nm) to mimic the spectral width of an ultra-fast laser.
Chapter 3: Development Of A Multi-photon Microscope Using 3D Acousto-Optic Scanning

This chapter describes the development of a multi-photon microscope that uses the principle of 3D acousto-optic scanning developed in the last chapter as its axial and lateral beam steering method. In this regard, it serves two purposes. The first is to describe the design considerations that went into the construction of the microscope and the second is to document the final design of the microscope, including its software and hardware. Because of this last purpose, several sections of this chapter (Sects. 3.3, 3.4, and 3.5) are written expressly for a user of the multi-photon microscope, with the goal of serving partially as a user manual.

3.1 Design Criteria: Component Selection

In the previous chapter, it was experimentally shown using two counter-propagating AODs that it is possible to quickly and deterministically position a cylindrical focus in a given axio-lateral plane. However, to be able to functionally image from neurons, we must be able to deterministically position a spherical focus within a full 3D volume. To do this requires two AODs per dimension, for a total of four AODs. Since each AOD must be operated independently, driving the AODs requires four separate high power radio frequencies (RF) sources. In addition, to be able to use the scanner for multi-photon fluorescence excitation, without excessive amounts of power, requires an ultra-fast laser source.
3.1.1 Acousto-Optic Deflectors

The AOD units selected were two shear-acoustic wave based TeO$_2$ XY AOD deflector pairs (OAD-1121XY, Isomet; Springfield, VA). Each of these AOD pairs consists of two identical deflectors, separated by a distance of approximately 4cm from each other. These deflectors were chosen for two reasons. First TeO$_2$ was chosen again because of its high efficiency-bandwidth based acousto-optic figure of merit M1 [56] which characterizes both the diffraction efficiency and the available frequency range. Indeed, each of these deflectors is capable of approximately 70% diffraction efficiency across the RF range of 60-100Mhz. The second reason these deflectors were chosen was because of their large active apertures of 9mm by 9mm, since as discussed in Sect. 2.3.1 on spatial dispersion compensation, the size of the aperture is inversely proportional to the attainable resolution. The square aperture allows us to use spherical lenses, rather than cylindrical lenses as would be the case with cylindrical aperture AODs, to relay between AOD sets without clipping the laser beam.

One consequence of using TeO$_2$ as the acoustic medium is its birefringence. Indeed at 800nm, the refractive index of the ordinary axis and the extraordinary axis of TeO$_2$ can be calculated using the Sellmeier equations [82]

\[
 n_o^2 = 1 + \frac{3.7088\lambda^2}{\lambda^2 - 0.038575} \tag{3-1}
\]

and

\[
 n_e^2 = 1 + \frac{4.3252\lambda^2}{\lambda^2 - 0.040959} \tag{3-2}
\]
By implementing these equations for 800nm, the refractive index along the ordinary axis of the TeO$_2$ crystal is calculated to be 2.22, whereas the refractive index along the extraordinary axis of the TeO$_2$ crystal is 2.37. AOD manufactures have utilized this property to manufacture shear acoustic wave based TeO$_2$ deflectors, like the OAD-1121s, that operate via anisotropic diffraction, which allows for a large acoustic interaction bandwidth using a single transducer. As a consequence though, the polarization of the beam is rotated by 90 degrees after passing through the deflector and each AOD is polarization sensitive to the incident laser beam. Thus, a specified order of deflectors has to be maintained.

It should be noted here that in different experiments these deflectors were utilized in two fundamentally different ways, either as a pair, i.e. the X and Y deflector were kept together as a unit, or as separated individual units. In circumstances where they were used as a pair, there are four primary diffracted orders, the 0,0 order, the 1,0 order, the 0,1 order and the 1,1 order. Of these, only the last is used as it corresponds to 1$^{\text{st}}$ order of each AOD. However, when the deflectors are separated, there are only two visible orders, the 0 order and the 1$^{\text{st}}$ order. Thus, depending upon which situation was implemented, the AOD diffraction orders will be described differently.

### 3.1.2 Radio Frequency Generation

Radio frequency waves for driving acousto-optic deflectors have been generated using primarily two distinct methods, voltage controlled oscillators (VCOs) [83] and direct digital synthesizers (DDSs) [84].
Voltage Controlled Oscillator

VCOs are oscillators that use varactor diodes, or tuner diodes, as a primary tuning element in an oscillating circuit. A varactor diode when reversed biased generates a capacitance. Altering the amount of reverse bias alters the capacitance and changes the oscillation frequency of the circuit. Thus by changing the level of a stable and clean DC voltage source, the frequency of the output sine wave can be changed.

Direct Digital Synthesis

DDS solutions produce RF waves by first generating the signal in a digital form and then using very high speed D/A converters to convert it to an analog signal. The digital signal in turn is generated using a phase accumulating system in which every clock cycle incrementally increases the accumulated phase (by an amount determined by a specified tuning word), which in turn is mapped via a look up table to a specific digitally defined amplitude. This digital amplitude is then converted using the D/A channel to an analog output. As the phase accumulates, it repeats with a frequency determined by the system clock and specified tuning word, which determines the frequency of the output sine wave. DDS systems have become very popular in recent years since they offer cheap solutions for generating highly stable RF waves. However, there are drawbacks in choosing a DDS system. One is the sparseness of available prepackaged solutions. Indeed, since the system design for any DDS solution is very dependent upon the specifications, (i.e., frequency ranges, number of samples needed to be generated), fully developed DDS solutions are rare, particularly for the 3D AOD
application where a large number of points in the form of frequencies ramps is required, which necessitates a large amount of on-board memory.

Primarily for purposes of simplicity and compatibility with the selected AODs, the solution chosen here is VCO based (VCO-200A, Isomet; Springfield VA). The specifics of these VCOs will be presented in the Sect. 3.4 on hardware.

3.1.3 Laser Source

While multi-photon excitation can theoretically be accomplished with a high power CW laser, modern multi-photon microscopes utilize pulsed laser sources to generate high peak power while maintaining moderate levels of average power. Because of this, since its introduction in 1986 [85], titanium doped sapphire (Ti:Saph), which has a very high gain bandwidth that allows for very short pulses and a large range of wavelength tunablility, has become the preeminent gain medium for multi-photon laser sources. Indeed, using this medium, lasers capable of delivering pulses on the order of 5 femtoseconds(fs) have been developed [86,87], although most commercial devices generate pulses on the order of 100fs.

Primarily for reasons of keeping with previous lab experience, one of two versions of the same Ti:Saph laser oscillator were used as the laser source for this setup. Initial tests were done with the Mira 900 (Coherent; Santa Clara, CA). Later test were performed with the Mira HP (Coherent). The pump source for both situations was an 18Watt(W) 532nm laser (Verdi 18W, Coherent). The combined Mira 900:Verdi oscillator:pump combination is capable of producing 2 Watts of modelocked power at
800nm. The 900 oscillator also allows for user adjustable wavelengths from 680nm to 900nm as well as pulsewidths from approximately 100fs to 200fs. It also has an optional cavity mode that allows for pulses to be generated in the picosecond(ps) range. In addition, the 900 has the off-label ability to increase its output pulsewidth all the way to 1ps in the femtosecond cavity at the expense of output power. This can be done by intentionally misaligning an end mirror. The HP:Verdi oscillator:pump combination on the other hand is capable of generating up to 4 Watts of modelocked power and will be discussed in a later section. It will suffice to say here that except for the optional picosecond cavity layout and off-label pulsewidth tuning ability, it has the same wavelength and pulsewidth adjustability as the 900.
3.2 Three dimensional scanning verification

To demonstrate the ability of the system to in fact scan a spherical focus in three dimensions, as opposed to the cylindrical focus in a single axial-lateral plane described in the previous chapter, we designed a simple test experiment using a similar protocol to the one mentioned in Chapter 2.

As this optical layout was just temporally used, only brief mention will be given to its description. In essence, the 2W output of the MIRA 900 laser was collimated and deflected by the first set of XY AODs (OAD1121-XY, Isomet). The 1,1 output of this deflector set was reflected by a single mirror and optically relayed to the second set of XY AODs. The 1,1 output of this last deflector set was then optically relayed to the back focal aperture of a 10X objective lens (Plan Neofluar, Zeiss) and images were taken using a second 32X objective lens (Quarzgl, Leitz) mounted with a coupled camera on a positioning stage.

As can be seen in the animation for the anticipated scan pattern in Fig. 3-1, the nine point scan pattern we generated had the center point at the closest axial position, the four points around it at a further axial position, and the four points around those at the furthest axial position. By using a movable imaging setup similar to the one described in Sect. 2.1.2, we were able to image the focus spots at different axial positions. From the images of Fig. 3-1, it can be seen that for the first axial position, only the center position is in focus, for the second axial position, the four spots around the center spot come into focus and for the final axial position, the last four spots come into focus. Since the dwell time for each point is 20μs, all nine points are visited within 180μs, which is much faster than the refresh rate of the camera. Thus, all nine points are visualized in each section.
Figure 3-1: Nine Point Scan Pattern (Left) Animation of a scan pattern scheme showing nine separate focal positions color coded by axial depth. Silver rectangle indicates the section at which an image is taken (Right) Image corresponding to the silver rectangle in the animation on the left.
This gives clear evidence of the 3D scanning ability of the system. Also, as can be seen in the images, the spots are round at the focal positions, indicative of the inherent spatial dispersion compensation of the scanning system.

3.2.1 Special Considerations

Scan Pattern Rotations

As mentioned in the section above, there is a single mirror after each deflector pair. By adjusting the angle and position of this mirror relative to the AOD pair that precedes it and the lens that comes after it, it is possible to orient the center of the scan pattern through the center of the lens. However, if the deflecting beam has an angle of deflection in both the X and Y directions, then, the resulting pattern will be rotated. This is a serious problem for AOD based focusing, since the second deflector set will no longer see the expected angular scan range, but rather a smaller one due to the rotation. The result is a mismatch in the first and second counter-propagating deflectors, which distorts the focus and decreases the resolution. To avoid this problem, it is necessary to use a full beam aligner, with two mirrors each to give four independent degrees of freedom, two to control the position of the beam, and two to control the rotation.

Astigmatism

While the two AOD deflector pairs offer increased convenience for the optical layout, they also create the general problem of astigmatism for foci which are not the inherent focus of the objective lens. This is due to the fact with the AOD based focusing scheme, the total spherical focus is actually composed of two separate cylindrical foci,
one in the X dimension and another in the Y dimension. Each of these is in turn created by the counter-propagating acoustic waves in the deflector pairs oriented in that respective direction. Since, by the principal of relay optics, the counter-propagating deflectors are imaged onto each other despite not being located at the exact focal points of the intervening lenses, the generated cylindrical lenses will have the same focal lengths. However, since there is a difference between where they are located, there will be a difference in where they generate a focus. In essence, the overall effect is of two cylindrical lenses with the same focal length placed at different points along the optical path, which will lead to the formation of the X and Y foci at different places, an optical aberration commonly known as astigmatism. It is possible to compensate for this effect by making the focal lengths in the X and Y dimension different so that they take into account this difference in position, however, it is more practical to separate the deflector pairs, which allows them to be optically relayed not only between counter-propagating deflectors but also between X and Y deflectors.

**Bragg Angle Mismatch**

The explanation of the acousto-optic deflector principle given in Chapter 1 is useful for a variety of situations. However, there are several simplifications implicit in it. The most important for AOD based focusing is the fact that in a useful deflector, either one of two processes must happen for deflection to occur across the entire RF frequency range, there must be a divergence of the acoustic wave or the acoustic wave must be steered. This is because for effective first order diffraction efficiencies, the AOD must be operating in the Bragg regime, where the input and output angles are equal. With a single direction of acoustic wave propagation and a single input angle, this would give rise to
only a single output angle. AODs such as the OAD-1121 are able to circumvent this issue by creating a diverging acoustic field, thus giving rise to several acoustic beams at the same time. The user then adjusts the gross incident angle to ensure the full range of deflection angles for the deflector. However this only works for a single incident angle. If the incident angle is now changed (as is necessary for the AOD focusing scheme), the Bragg angle condition is no longer matched, and the efficiency of the deflected beams drops. The degree to which the incident angle can be varied while still maintaining relatively constant levels of diffraction is known as the incident aperture of the deflector. In AODs utilizing anisotropic diffraction, like the OAD-1121s, the incident aperture is particularly small. The effect for the 3D focusing scheme is that the further one is from the inherent focus of the objective lens, the less the available power. This could possibly be overcome by using deflectors with arrays of transducers that steer the acoustic beam to match the differing input angles, which will be discussed in Chapter 5. However, this situation would require more sophisticated RF driving electronics.

**Temporal Dispersion**

A common problem when using any material in the field of ultrafast optics is temporal dispersion. This is a consequence of the dependence of the refractive index of a material on the frequency of light. Most material exhibits what is known as normal dispersion in the visible and near infrared frequency ranges, wherein the refractive index increases with increasing optical frequency. As a consequence, higher optical frequency light, “i.e. bluer light” has a higher refractive index than lower frequency light, “i.e. redder light”. As a result, in a multi-chromatic ultrafast optical pulse traveling through a
normal dispersive material, the red portion of the pulse will travel faster than the blue portion of the pulse, which in turn will lead to an increased pulse length as the pulse becomes chirped.

A method of characterizing the degree to which a pulse will be broadened by a given material is through the amount of accumulated second-order phase, which is commonly known as the group delay dispersion (GDD) of the material. This value can be normalized for the length of the material, in which case, it is renamed the group velocity dispersion (GVD). Mathematically, this value can be expressed as

\[ GVD = \frac{\varphi'(\omega)}{L} \]

(3-3)

Where \( \varphi \) is the phase of the light wave with respect to the angular frequency \( (\omega) \) and \( L \) is the total distance. By expressing the phase in terms of refractive index,

\[ \varphi(\omega) = \frac{n(\omega) L \omega}{c}, \]

(3-4)

we see that the GVD can also be expressed as

\[ GVD = \frac{d^2}{d\omega^2} \left( n(\omega) \frac{\omega}{c} \right) = \frac{\lambda^3}{2\pi c^2} n'(\lambda) \]

(3-5)
Note that this since the wave vector $k$ is just the accumulated phase per unit length (i.e., $k = \frac{\varphi(\omega)}{L}$), the GVD can also be expressed as the second order derivative of the wavevector with respect to the angular frequency or $\frac{d^2k}{d\omega^2}$, or, since the first derivative of the wavevector with respect to the angular frequency is the inverse of the group velocity (i.e., $\frac{dk}{d\omega} = \frac{1}{v_{\text{group}}}$), as $\frac{d}{d\omega} \left( \frac{1}{v_{\text{group}}} \right)$. Sometimes in the literature [88], the second derivative is taken with respect to the wavelength ($\lambda$) instead of the angular frequency. This necessitates a conversion factor of $\frac{d\lambda}{d\omega} = \frac{-\lambda^2}{2\pi c}$ when switching between the two conventions.

The wavelength dependent value of the refractive index can be determined from the empirically derived Sellmeier equations which for TeO$_2$, the material used in the AODs, were described above in Eqns. 3.1 and 3.2 for the ordinary and the extraordinary axis respectively. Using these equations, the total GVD introduced by the 14cm of TeO$_2$ crystal (3.5cm per AOD * 4 AODs) in the scanner can be calculated to be a maximum of 14cm*6065.6 fs$^2$/cm = 84918fs$^2$ (this is using the worst case scenario of ordinary axis only deflection) at 800nm. The output pulsewidth ($\tau_{\text{out}}$) after the dispersion by the AODs can be calculated using the following equation [89]

$$\tau_{\text{out}} = \tau_{\text{in}} \left[ 1 + 7.68 \frac{(GVD* L)^2}{\tau_{\text{in}}^4} \right]^{-0.5} \quad (3-6)$$
where $\tau_{\text{in}}$ is the input pulsewidth. If we assume an input pulsewidth of $\sim 130$ fs, then the output pulsewidth after all four AODs will be $1.81$ ps, which is over 10X longer than the input pulsewidth. (Note that while this equation assumes a Gaussian profile when the real profile of the laser output is hyperbolic secant, it is only being used for a rough estimate of the output pulse).

Since the multi-photon excitation rate is inversely related to the pulsewidth and directly related to the square of the incident power, this increase in pulsewidth would require a 3.5X increase in incident power. However, there are several ways to compensate for the broadening in pulsewidth, such as using prism based prechirpers [88,90-93], grating based prechirpers [94-96,96], coated mirrors [97], photonic crystal fibers [98,99], and even particular arrangements of AODs [100-104] and prism-AOD combinations [57,105,106].

It is important to realize that while pulse broadening results in increased power requirements for the same rate of multi-photon activity, the level of photo-damage and the quality of the image obtained has been shown to be the roughly the same for pulse durations that range from 75 fs to 3.2 ps [107], assuming low excitation rates. This is irrespective of whether the pulses themselves were transform-limited (i.e., unchirped) or not. As a result, several studies have been done on biological specimens using picosecond imaging either with an increased repetition rate [108,109] or without [110]. Indeed, some studies have even demonstrated efficient multi-photon excitation with continuous wave lasers [111,112], although this requires highly concentrated staining with dyes that have large two-photon cross sections. Thus, if sufficient excitation power
is available, the easiest solution to compensate for pulse broadening is simply to increase the excitation power accordingly.

3.3 Optical Layout for Fluorescence Imaging

Taking into account the considerations noted above for the preliminary test, a new optical layout was implemented (see Fig. 3-2). As this is the eventual setup that was used for the experimentation, more detail will be used to document it.

Mira HP

As shown in Fig. 3-2, the light source for the microscope is provided by the Mira HP mentioned above. For the majority of experiments conducted, the wavelength was based around 800nm and the average output power was approximately 4 Watts. A portion of the beam (<1%) is sent to a spectrum analyzer (Laser Spectrum Analyzer, IST instruments) for bandwidth analysis. The typical bandwidth of the laser output was approximately 5nm, corresponding to a minimum wavelength of 797.5nm (or frequency of 3.76 x 10^{14} Hz) and a maximum wavelength of 802.5nm (or frequency of 3.74 x 10^{14} Hz). Assuming a transform-limited pulse, we can use the time-bandwidth product of a hyperbolic secant pulse (which is what the output pulse of the Mira HP most closely resembles[113]),

\[ \Delta \nu \tau_p = 0.315 \]  (3-7)
where $\Delta \nu$ is the frequency bandwidth of the pulse and $\tau_p$ is the temporal bandwidth of the pulse to estimate the pulse duration to be approximately 134fs. For independent pulse duration analysis, an autocorrelator (APE, Mini; Berlin Germany) is periodically used to measure the pulsewidth (~200fs) before experiments and subsequently removed from the beam path during experimentation. Since the autocorrelation does not measure the exact shape, and hence the exact temporal profile, adjustments to the autocorrelation values are used based upon models for particular shapes. For example, in the case of the hyperbolic secant pulse, the relationship between the autocorrelated pulse and the actual time pulse can be described by Eqn. 3.8 below,

$$\frac{\tau_p}{\tau_{ac}} = 0.648$$

(3-8)

Where once again, $\tau_p$ is the temporal bandwidth of the pulse and $\tau_{ac}$ is the autocorrelated temporal bandwidth. Using this equation and the 200fs value for the measured autocorrelated bandwidth, we can define the actual temporal bandwidth to be 130fs, which is very close to the value predicted by the time-bandwidth product described by Eqn. 3.1. The output beam size of the laser is 0.8mm. An reflective ND filter (M-925B, Newport; Irvine, CA) was initially used in the design right after the laser to control for power modulation, but was subsequently removed since the intensity of the laser beam 795.7W/cm² exceeded its threshold (75W/cm²) and led to burning.
Figure 3-2: Optical Table Layout  See text for details *(Inset)* Expansion of the 3D AOD scanner box, see text for details.
Figure 3-2: Optical Table Layout, continued
**Beam Magnification**

To fill the first aperture of AOD with the laser beam, which provides for the highest resolution, two sets of telescopes were used for a beam magnification factor of 3:1 (see Beam Magnification Optics section of Fig. 3-3). The first telescope was composed of a first lens with a focal length of 50mm (f50) and a second lens with a focal length of 75mm (f75) (All telescopes will hereby be abbreviated as f1:f2 where f1 is the focal length of the first lens in mm, and f2 is the focal length of the second lens in mm. Using this notation, this telescope can be described as a f50:f75). The general characterizations of all the lenses and mirror used in the scanner will be given later, but it will suffice to mention here that all lenses used in the design were achromats and antireflective coated for the near infrared wavelength ranges. This first telescope was located approximately 30cm away from the laser. While the laser is specified to have an output beam diameter of 0.8mm, it also has a divergence. In this regard, the first telescope served two purposes, 1) to initially collimate and align the laser beam and 2) to provide for an initial 1.5X magnification (For explanation of how collimation and alignment was tested, see Section 3.3.1). The second telescope in the initial beam expansion was a f75:f150, which provided the remaining 2X magnification. This telescope was located approximately 1.5 meters away from the first telescope and 20cm away from the first AOD. The appropriate distance between the two lenses in the second telescope was determined by retesting the collimation after the telescope.
1st X AOD

The expanded beam filled the 9mm aperture of the first Tellurium oxide (TeO$_2$) AOD (OAD-1121, Isomet), which is arbitrarily oriented in the X direction (see Fig. 3-3). Alignment through this AOD was done using the procedure for AOD aligning and positioning described in Section 3.3.1. In addition to providing for deflection in the X direction, this first AOD also served as a gross modulator for the power output. This is accomplished by adjusting the amplitude of the RF frequency by changing the voltage to a preamplifier connected between the VCO and the AOD (this will be further discussed in the Section 3.3). For this AOD only, the voltage to the preamplifier is changed through an adjustable voltage divider (to generate this, a 5V supply is voltage divided using a 3Ω resistor and a 50Ω potentiometer) that is conveniently located by the controlling computer. This allows for easy modulation of the power during experimentation. To optimize for the AOD incidence polarization a half wave plate was used (AHWP05M, Thorlabs; Newton, New Jersey) between the first and second telescopes of the initial beam expansion. In initial design stages, the optimal setting for this half wave plate was determined by rotating it to optimize for deflected power after the first AOD.

1st Optical Relay

The entire optical layout of the scanner is based around a series of relay telescopes. These telescopes serve the purpose of successively imaging each AOD onto each other, and then onto the back focal aperture of the objective. For most cases, since there is enough distance given by the focal length of the lenses, the beam path is folded
using mirrors. This allows the physical path length to be shortened and also allows for
the deflected beam to be directed down the center of the lenses (see Sect. 3.3.1). In this
regard, the 1st order output from the first deflector is reflected and directed using a mirror,
optically relayed with a f150:f150 telescope, and reflected again with a second mirror
before hitting the second deflector (see Fig. 3-3). Two important points must be
considered here. First, note that scan pattern rotation is not an issue in this situation since
the deflection is only in the X dimension. Second, the polarization after the first X AOD
is vertical due to polarization rotation by the TeO₂ crystal. This is the optimal
polarization direction for a second AOD oriented in the Y direction and, as discussed
below, in essence requires the deflector to be oriented in this direction.

1st Y AOD

While it would be convenient to have the non-orthogonal counter-propagating
AODs placed one after in the optical path to ensure focus formation in each dimension
separately, the polarization rotation for each AOD requires that for this to happen, the
polarization must be rotated again by 90 degrees between the deflectors themselves to
ensure optimal polarization for the second deflector. While this can be done within the
optical layout using mirrors (for example, by first deflecting the beam up, and then
deflecting sideways), doing so while preserving the angular deflection direction of the
AODs is not possible. Thus, the only feasible way to adjust the polarization between the
deflectors would be with another half wave plate. There are two problems with this
approach. First is that it would require a total of at least two more half wave plates, one
between the two X AODs and another between the two Y AODs, and second, since the
beam size has now been expanded to 9mm, any half wave plate must be at least this large to avoid clipping on the plate itself (note that attempts to place the half wave plate between the lenses of the intervening telescope would require the plate to be large enough to encompass the entire scan image, which, for the f150 lenses is larger than 9mm).

In order to avoid these issues, the polarization rotation provided by the AODs is maintained and the successive placement of the defectors (1st X, 1st Y, 2nd X, 2nd Y), ensures optimal polarization orientation (as long as no polarization rotations are induced by the optics). Thus the second deflector in the path is oriented in the Y direction (see Fig. 3-3). Alignment through this AOD, similar to the 1st X AOD, is once again done using the AOD alignment procedure discussed in Section 3.3.1. However, unlike the 1st X AOD, this AOD is mounted on an axial positioning stage, which allows it to be moved relative to the “pivot point” (i.e., the image of 1st AOD, where all beams “pivot” around) formed by the preceding telescope. The reason this stage was used was to allow for exact placement of the deflector at the pivot point thereby mapping the X deflector exactly onto the Y deflector. By maintaining this positioning ability with all the deflectors in the path, the amount of astigmatism introduced by the differing locations of the X and Y deflectors can be drastically reduced.

2nd Optical Relay

The second relay telescope between the 1st Y AOD and the 2nd X AOD is composed of a f200:f200 system (see Fig. 3-4). In this case, rather than using a single mirror to orient the deflected beam after the 1st Y deflector, it is necessary to use a complete beam aligner to avoid scan pattern rotation as described above. The beam
Figure 3-3: Optical Table Components, First Set  See text for details.
aligner technique used in this case consists of a first mirror that reflects the beam upwards and a second mirror that reflects it laterally. The mirrors are placed in a custom designed mounting apparatus that allows for independent positioning of the vertical and horizontal location of the mirrors. This combined with the ability to adjust the rotation and tilt of the mirrors gives the full four degrees of freedom that allows for alignment through the telescope as well as avoidance of scan pattern rotations, aside from the 90 degree scan pattern rotation that results from the upward then lateral deflection. In order to remove this 90 degree rotation, another set of mirrors is used after the telescope to reorient the polarization and scan pattern directions. It is crucial to ensure that the scan pattern is not re-rotated by this second set of mirrors, which is avoidable since, like the first set, the four degrees of freedom allow for positioning and rotation control.

2nd X AOD

The beam is aligned using this second mirror set through the third AOD in the optical path, which for the reasons of polarization mention above is oriented in the X direction (see Fig. 3-4). The other aspect of direction that needs to be considered is the direction the acoustic beam enters the crystal. Indeed, as was described in Chapter 2, in order to eliminate the continuous lateral movement and generate random access positioning in both the lateral and axial dimensions, the acoustic beam in the 2nd X AOD must be propagating in a direction that is opposite to the first. This can be accomplished either by physically rotating this AOD 180 degrees relative to the first such that the sound enters on the opposite side, or by having the sound enter both AODs on the same side and optically relaying the AODs with a single telescope which rotates the beam by 180
degrees and thereby creates “effectively” counter-propagating acoustic waves between the two crystals.

Here, since we have already used a relay telescope between the 1st X AOD and the 1st Y AOD, which rotates the beam by 180 degrees, a second relay telescope between the 1st Y AOD and the 2nd X AOD, which rotates the beam by another 180 degrees, and a step-up step-down beam aligner system, which again rotates the beam by 180 degrees, the total rotation is 540 degrees, which is equivalent to the “effectively” counter-propagating situation. Thus the 2nd X AOD (third AOD overall) must be oriented such that the acoustic wave enters the crystal from the same side as the 1st X AOD.

Like the 1st Y AOD, this deflector is also placed on a positioning stage. However, here, both the lateral and axial positions are controllable. The axial control allows the AOD to be aligned exactly on the pivot point created by the previous deflectors, as was the case for the 1st Y deflector. The lateral control on the other hand allows the acoustic wavefront in the 2nd X AOD to be mapped identically to the wavefront propagating in the 1st X AOD. Note that if the acoustic waves were not mapped exactly between these two AODs (but the acoustic waves still maintained the same frequency slope between them) then there would be a lateral offset in the beam position since the acoustic frequency seen by a portion of the beam in the second deflector would be shifted by a certain value from the expected frequency. While it is possible to compensate for this by adjusting the frequency between the two AODs, the more direct approach is to position the second deflector such that this offset is eliminated (this will be discussed more on in Sect. 3.3.1 and in Fig. 3-5).
3\textsuperscript{rd} Optical Relay

The 3\textsuperscript{rd} relay telescope in the path is composed of another f150:f150 system (see Fig. 3-4). In appearances, it is identical to the 1\textsuperscript{st} telescope system, with the beam alignment through and after the telescope being accomplished through single mirrors. Note that scan pattern rotation is not a problem in this situation. Although this might seem contrary to what has been said previously since the beam is still being deflected in both the X and Y directions, the difference lies in the fact that the scan pattern is already aligned along the center of the Y axis. In this regard it identically resembles the 1\textsuperscript{st} relay telescope where only the X deflection needs to be realigned along the center of the telescope.

2\textsuperscript{nd} Y AOD

The fourth AOD in the system is oriented in the Y direction, once again for reasons of polarization (see Fig. 3-4). Just like the 2\textsuperscript{nd} X AOD, there is a question as to which orientation the deflector should be mounted in regards to the direction of the acoustic wave. In a similar argument to that presented for the 2\textsuperscript{nd} X deflector, since between the 1\textsuperscript{st} Y deflector and the 2\textsuperscript{nd} Y deflector there are two telescopes and a step-up step-down beam aligner, the beam is rotated by 540 degrees, which corresponds to an “effectively” counter-propagating situation. Thus, the acoustic wave of the 2\textsuperscript{nd} Y deflector must enter from the same side of the crystal as the 1\textsuperscript{st} Y deflector. Also like the 2\textsuperscript{nd} X AOD, this deflector is mounted on a lateral and axial positioning stage, which allows the acoustic wave in the 1\textsuperscript{st} Y deflector to be mapped directly onto the 2\textsuperscript{nd} Y deflector and eliminates any lateral offset.
Scan Angle Magnification

After the 2\textsuperscript{nd} Y AOD, scan pattern rotation is no longer a critical issue since it will not affect the focus quality. For this reason, a single mirror is used to reflect and align the beam after the 2\textsuperscript{nd} Y deflector. The beam is directed along a f120:f100 telescope (see Fig. 3-4), which generates a scan angle magnification of 1.2X. Since the output beam from the last AOD is 9.0mm in size and the back focal aperture of the objective lens is 7.0mm, this value is very close to the largest angle magnification value that can be used while still maintaining a beam size large enough to fill the back focal aperture of the objective lens to ensure maximum resolution at the focal plane. This specific telescope also serves another function. Using the principle of relay optics it is easily shown that by moving this telescope forward or backward relative to the last AOD, it is possible to axially adjust the pivot point such that it is placed exactly at the back focal aperture of the objective lens. This is useful since a misplaced pivot point will lead to compression and expansions of the scan pattern at different axial locations.

Pre-Microscope Relay Chain

The final relays to the microscope are accomplished using two more relay telescope systems. The first is a f100:f100 oriented in the vertical direction. The second telescope is a f200:f200 system which utilizes a f200 lens inside the "double port" of the microscope (see Fig. 3-4).
Microscope Coupling

The scanner system was coupled to an upright microscope (E600FN, Nikon; Tokyo, Japan) expressly designed for electrophysiological studies. The coupling between the optics in the scanner and the microscope itself occurred at the “double port”, an optional part that is usually used to combine a galvanometer scanner and a camera system. The excitation light was reflected by a short-pass dichroic (750DCSPXR, Chroma; Rockingham, VT) within this unit and subsequently transmitted through a long pass dichroic (700DCXR, Chroma; Rockingham, VT) that is located in the filter slider of the optional “Intermediate Port” to the back focal aperture of a 60X, 1.0 NA water immersion objective lens (Fluor, Nikon). With the long pass dichroic filter in place, epifluorescent emission light was reflected by the dichroic back through an emission filter (either a 600/200 (Chroma) for structural imaging or a 500/100 (Chroma) plus 535/50 (Chroma) combination for functional imaging) and a f140 lens in the “intermediate port” to a modified exit port where it was focused onto a PMT (R6357, Hamamatsu; Hamamatsu City, Japan) using a series of two f50 lens placed back to back to produce an effective f25 lens. The combination of the f140 lens with the f25 lens in essence demagnifies the back focal aperture of the objective by a factor of 5.6X, making the 7.5mm aperture approximately 1.33mm, which is smaller than the size of the photocathode on the PMT (3.7mm X 13mm).
Figure 3-4: Optical Table Components, Second Set
See text for details.
3.3.1 Alignment and Collimation

As can be seen by the number of references to alignment and collimation adjustment in the previous section, adjusting for these parameters is very important in getting the highest quality out of any optical system, particularly the 3D AOD scanner. The alignment protocol can be broken down into three subsections depending upon whether the user is aligning a beam through a telescope bench, through an AOD, or through the microscope.

Alignment

Telescope

The basic tools for alignment through a telescope bench are drop-in pinholes. To use these tools for alignment, the user drops them in the bench as far from each other as possible and adjusts the first degree of freedom (for example the location of a reflecting mirror) for alignment through the first pinhole and the second degree of freedom (for example, the angle of reflection for the mirror) for alignment through the second pinhole. For the 3D AOD scanner, there are two sets of drop-in pinholes used. The first are designed for the Microbench (Linos; Milford, MA) optical mounting components. These components are utilized in mounting the lenses for the beam magnification, the second telescope, and the scan angle magnifier. The second set of drop in pinholes are custom built for use with the Minioptic optical mounting components (AF Optical; Fremont, CA), which are used in the larger telescopes between the 1st X and 1st Y AODs as well as the 2nd X and 2nd Y AODs.
Deflector

Alignment through a deflector is also done using the pinholes, however in this case, the Microbench mount is made around the deflector to create a place for the pinholes to be “dropped” in. Before the deflector is oriented, the beam is aligned through both pinholes using the two degrees of freedom described above for the telescope situation. To align through the deflector, the pinholes should be moved such that the first pinhole is before the deflector and the second after it, while the deflector is rotated such that the zero order passes through both pinholes. Note that this procedure ensures that the deflector is normal to the incident beam, thus ensuring that lateral displacement of the deflector will not change the output beam and that adjustment of the Bragg angle will not change the axis of the deflection.

Microscope

To align through the microscope, a two step approach is used. First, gross alignment is done using a microscope to Microbench adaptor, which has an objective thread on one side and connects to a Microbench frame on another. This frame is then connected via rods, to two other frames in which two Microbench pinholes are placed. This, in essence, allows these pinholes to be suspended below the microscope. Using two degrees of freedom, which in this case are the first two mirrors behind the microscope, the beam is aligned through both pinholes.

The second step involves imaging the reflection of the focused beam off a glass slide (see Fig. 3-5). By analyzing the resulting Airy disk at different focal planes, the beam can be adjusted to maximize the homogeneity. A variation of this technique also
allows the 2\textsuperscript{nd} X and 2\textsuperscript{nd} Y deflectors to be translated into the correct position to minimize offset. In this case, three different axial focal positions are generated using the \textit{Point Definition} program (discussed in Sect. 3.5) to rapidly change the focal length of the AOD based lens. Since all three foci are seen simultaneously, with differing levels of defocus depending upon the position of the objective, the Airy patterns of all three can be aligned to match each other by translating the 2\textsuperscript{nd} X and 2\textsuperscript{nd} Y deflectors in their respective directions with the translation stages.

\textit{Collimation}

Tests for collimation are typically done after each telescope to ensure proper separation distances between the lenses. To quantify the level of collimation, the beam is reflected to a distant surface of the room using a mirror. A measurement of beam size is made directly after the second lens and then at the distant surface and the telescope spacing is adjusted to minimize the difference.

\textbf{3.3.2 Lenses and Mirrors}

\textit{Lenses}

The lenses utilized can be subdivided into three types. The first were NIR achromatic doublets from Thorlabs (Newton, NJ), which come in a variety of diameters and focal lengths. Theses include all f50s, f75s and f100s used in the beam expander optics and the scan angle magnifier, which were 25.4 mm in diameter (thus allowing them to fit into lens mounts purchased from Linos for mounting in a 30mm \textit{Microbench} frame). In addition, they include the f200 lenses (diameters of 25.4mm) used in the 2\textsuperscript{nd}
Figure 3-5: Alignment and Collimation procedure  This figure documents the final step for alignment of the 3D scanner through the microscope. (a) Small degrees of misalignment can be do to inexact relay between the two counter-propagating AODs in each dimension (top figure), leading to displacement of the respective focal spots in the lateral direction (bottom figure) (b) Correction for this misalignment is accomplished by translating the 2nd AOD with the positioning stage while monitoring the reflection of the focal spots.
relay telescope and the f150 lenses (diameters of 50mm) used in the 1\textsuperscript{st} and 3\textsuperscript{rd} relay telescopes. Also, they include the f100s (diameters of 30mm) and f200s (diameters of 30mm) used in the microscope mounting optics.

The second type of lens used was an ARB2 NIR coated achromat from Linos. For availability reasons, the only type of this lens was the f120 (diameter of 25.4mm) in scan angle magnifier.

Finally, the last type of lens used was a visible achromatic doublet from Thorlabs. This lens represents both of the f50s (diameters of 25.4mm) used in the detection path to create an effective f25 focal length lens. These lenses are optically identical to the f50s used in the scanning pathway however, since the detected pathway focuses visible light, the visible antireflective coating is used.

\textit{Mirrors}

All mirrors used in the scanner were broadband dielectrics from Thorlabs and either 1 or 2 inches in diameter. These mirrors have reflectivity values over 99.8\% and high damage thresholds (2kW/cm\textsuperscript{2}). The 2 inch diameter versions were used to couple in to and out of the 1\textsuperscript{st} and 3\textsuperscript{rd} relay telescopes while the 1 inch diameter versions were used everywhere else in the scanner.
3.4 Hardware

Specific electronic hardware was needed to perform four separate tasks in the setup, RF generation, data acquisition, power modulation and fluorescence detection. To accomplish this, a combination of application-specific commercial products, multi-function commercial products, and custom-built solutions were used. These will be briefly detailed here.

3.4.1 RF Control

VCOs

As briefly mentioned in Sect. 3.1, the RF generation for driving the acousto-optic deflectors was done by a set of four VCOs (VCO-200A, Isomet; Springfield VA). These specific VCOs are oven-stabilized, which reduces thermal fluctuations and gives them tuning linearity deviations of less than 0.1% over a 50Mhz range (see Fig. 3-6). They also have very high tuning slew rates of greater than 10Mhz/μs which is important for the 3D AOD focusing application since the frequency is continuously changed over time (note that the highest tuning slew rate that would be needed for this application would consist of ramping the entire frequency range of 40Mhz over the aperture time, which is 11μs, corresponding a slew rate of 3.6Mhz/μs, much less than the capable slew rate of the VCOs).
RF Amplification

Regardless of whether the RF signal is generated with a VCO or a DDS based system, the output from such systems are typically less than 1 mW. For example, with the VCO-200A oscillators mentioned above, the maximum output voltage was approximately 260mV pk-pk at 50ohm impedance, which corresponds to 0.34 mW of output power. The diffraction efficiency of an AOD ($\eta$) is dependent upon the input acoustic power based up on the following equation.

$$\eta = \sin^2 \left\{ \frac{\pi}{\lambda \cos \theta} \left[ \frac{M_2 L}{2H} \right] \frac{1}{P_a} \right\}$$

(3-9)

Where $M_2$ is the material dependent acousto-optic figure of merit, $H$ is the height of the transducer, $L$ is the length of the transducer, $\lambda$ is the wavelength, $\theta$ is the incidence angle, and $P_a$ is the acoustic power. In practical acousto-optics, this simplifies to a notion of a saturation power for the deflector, which represents an RF power value after which further increases lead to minimal changes in diffraction efficiency. For the AODs being used here, this value is around 2 Watts. Thus the RF power must be amplified by a factor of approximately 5900 times, which corresponds to an increase in RF voltage by 77 times, or 37dB.

RF Preamplification

To accomplish this level of amplification, a two stage process was used. First an initial 0 to 2.5X (8dB gain) adjustable voltage gain was provided by a first stage amplifier
Figure 3-6: Optical Table Components, RF Generation
See text for details.
"preamplifier" (IA-100-3, Isomet; Springfield, VA). The adjustable gains on each of these preamplifiers are controllable through both a manual potentiometer on the surface of the device and through a DC voltage (0 to 5V) supplied from an external source (see Fig. 3-6). Four of these devices were used, one for each AOD, and modulating voltages for three of the devices were automatically controlled to normalize power across the axial range, while for the last device it was manually controlled to grossly adjust for power.

**Fixed RF amplification**

Since the pre-amplification stage provides a maximum of 2.5X gain, the additional gain must be supplied by a more powerful amplifier (see Fig. 3-6). This extra gain was provided by second stage amplification with a dual channel fixed gain amplifier (DA104-2, Isomet; Springfield, VA). This amplifier provides a 63X (36dB) gain in voltage which, when combined with the preamplifier, increases the VCO output power from 0.34mW to a maximum of 8.4W, which greatly exceeds the saturation power of the AODs. Thus, the maximum gain on the preamplifier was adjusted using the potentiometer to limit the total output power to 2.2W.

**RF normalization**

While individual VCOs themselves are very consistent in terms of voltage to frequency characteristics, there is substantial differences between the different VCOs, as can be seen from Fig. 3-7a. This is a one drawback of using a VCO based system instead of a DDS based system. However, given their high degree of linearity, it is relatively straightforward to compensate for the differences by adjusting the output voltages
accordingly. For example, for the four VCOs utilized in this situation, the 2nd X VCO was arbitrarily taken to be the standard and the rest of the units were normalized to that output, such that a specified output voltage was always adjusted before being sent to the unit. The normalized voltage to frequency curves are shown in Fig. 3-7b and, as can be seen, are now almost exact.

Voltage Generation

To provide a stable DC voltage source for each of the VCOs, a multifunction data acquisition card was used (NI-6259, National Instruments). The capability of the card includes 4 analog output channels, 16 differential (32 single ended) analog input channels, 48 digital input/output channels, and two counter timers [114]. The four analog output channels allow all four VCOs to be controlled by one 6259 board. This card is one of the newer M series models developed by National Instruments that allows for data transfers from the computer to the card via a direct memory access (DMA) channel, rather than interrupts. In essence, this means that large buffers do not need to be stored on the board for fast analog outputs, but, rather, can be stored on the memory in the computer itself. This aspect of the card is crucial for generating voltage ramps necessary for AOD based focusing for three primary reasons. First, generating an accurate ramp requires several incremental changes in voltage values. Indeed, there is an extent to which a voltage ramp that consists of too few discrete elements will optically appear as separate lateral positions in the same objective focal plane versus a single lateral position in a non-objective focal plane. Second, since every position in the scan
Figure 3-7: RF normalization
Plots of voltage versus RF for (a) VCOs under no bias conditions (b) VCOs under voltage bias condition to normalize between oscillators.
pattern is created by chirping all four AODs, four separate voltage ramps are needed per position. Finally, if the structural image is to be created using the AOD based scanning protocol, which is the most direct way to ensure that accompanying functional imaging will be correctly placed, then the number of positions that create the scan pattern can be very large. Thus the memory requirements for even just the analog output task are very demanding. For example, assume a scan pattern requirement of 100X100X100 points. This requirement of a million points requires four million ramps to be generated and output. Now, let's also assume a dwell time per point of 20μs, which allows for the aperture time of the AOD (~15μs) and an imaging time of 5μs (this hypothetical situation is almost identical to the one that is actually used). Given the maximum AO rate of the board to be 1.25Mhz, each ramp would consist of 25 samples. Therefore, the total number of samples to be generated would be approximately 100 million, which is far from the capabilities of the “average” first in first out (FIFO) buffer. While there are boards with enough on board memory to hold approximately a quarter of this many samples, such as arbitrary wave form generators (AWFGs), they are extremely expensive. By storing these samples on the computer itself in the random access memory (RAM), where large quantities of memory are cheaply available, this board, along with other M series boards from National Instruments bypasses this problem.

3.4.2 Data Acquisition

The data acquisition is also done using an analog input channel on the NI-6259. The maximum AI rate specified for this channel is 1Mhz. Given the 5μs imaging time mentioned above, this allows for multiple samples (in this case 5) to be averaged over
this time frame, a process that has been termed oversampling. Like conventional averaging, oversampling has the effect of increasing signal to noise ratios, usually by a factor equivalent to the square root of the oversample count. Note that there are other boards designed specifically for high speed analog input, such as the PCI-6111 S series board from National Instruments, that have much higher AI rates (5Mhz). Using these faster boards could theoretically increase the S/N ratio even further. Indeed, future work on the system involves implementing this board as the primary AI board.

3.4.3 Power Modulation

As noted above, due to reasons of Bragg angle mismatch, the optical power is different at different axial locations. In order to ensure a relatively stable power output across the entire axial scan range, the power to the AODs can be modulated using the voltage control on the RF pre-amplifiers as mentioned above in Sect. 3.4.1. As described in the Sect. 3.3 1st X AOD, the power modulation to the first deflector is provided manually by a user controlled voltage divider. However, the voltage to the other three deflectors is provided directly from the analog output from another D/A card (NI-6040, National instruments). Unlike the M series card mentioned above, this card does not use the DMA channel and thus is limited in the number of analog samples it can hold by the size of its FIFO buffer (2048 samples)[115]. However, for modulating the power, this is not so much an issue since the number of power values is equal to the number of axial locations, which even for structural scans is less than 100.

One fact that must be taken into account when working with the power modulation is that the timing control is different depending upon whether the user is
performing a structural or a functional scan. Indeed, for a structural scan, the specified power modulation level needs to be changed only after each optical section. Assuming an optical section with 200X200 points, where each point consists of a set of 4 20μs frequency ramps, this requires that the power modulation voltage be changed every 0.8 seconds or with a frequency of 1.25Hz. However, for functional imaging, the power level needs to be adjusted for every point, since every point can theoretically be at a different axial location. Thus, assuming a 20μs value for the dwell time at each point, the output frequency is 50KHz. These changes in output frequency are controllable by using a digital output line on the M series board to clock the power modulation board at the frequency deemed necessary by the scan being performed.

3.4.4 Fluorescence Detection

As mentioned above, the detector for the setup was the R6357 PMT from Hamamatsu. This specific PMT is noted for its high cathode sensitivity in the visible wavelength. This unit was part of the H9307-3 PMT module that, in addition to the PMT itself, contained an on board high-voltage power supply, a gain control circuit, and a built in current to voltage converter which produced a 0-1V output signal from the output current range of the PMT. The bandwidth limitation of this IV converter was 200kHz, which caused a shift of approximately 5μs between when the maximal excitation occurred and when it could be sampled. The delay was taken into account in the software (RUF) by shifting the analog input sampling triggers relative to the analog output triggers as will be described in Sect 3.5 RUF.
A custom designed interface circuit for this PMT module was built by electronics hardware engineer Rob Gaddi between the years of 2003 and 2006. Since the implementation has been described in previous work [84] and no changes were made to adapt the system for the fast 3D scanning system, it will not be discussed here except to note basic uses. These were to provide power regulation to the PMT module, create a PMT gain-dependent output voltage from 0-1.2V as means to monitor and control the gain, amplify the 0-1 volt output by a factor of 10 to allow for easier digitization by the D/A board, and clamp the voltage to zero while alerting the user with a red LED light in cases of detector saturation.

3.5 Software

While there were several software developments throughout the course of designing the setup, there are three primary programs that would be used extensively by a user of the fast 3D AOD scanner. The first was developed exclusively by this author and was designed as an initial program to generate the voltage outputs from the M series and E series NI board that would control the VCOs and power modulators, called the Point Definition program. It also serves as the primary program for daily alignment of the system. The second program was a modified version of RUF, a software suite which has previously been used in this lab to control a 2D multi-photon AOD scanner [84] as well as an AOD/DMD based confocal scanner [83]. Both of the above mentioned programs are built using Visual Studios Dot Net 2005 (Microsoft; Seattle, Washington) and the C# language (Microsoft) and both also use the National Instruments methods library for controlling the 6259 and 6040 multifunction data acquisition cards. The last program is
called *Ruffleconverter* and was a graphical interface program written by this author in Matlab 6.5 (Mathworks; Natick, Massachusetts) for displaying and analyzing recorded fluorescent signals.

### 3.5.1 Point Definition

As shown in *Fig. 3-8*, this program consists of a graphical user interface in which the user first inputs the number of scan points desired (*number of points* text box) and then for each of the selected scan points, as chosen from the *point number* drop down menu, enters an *amplitude* parameter, an *offset* parameter, a *physical channel*, a *slope time*, and a *blank time* for each of four different software channels (labeled two X’s and two Y’s). Each of these channels in turn corresponds to one of the four analog outputs used to control the VCOs. The physical connections between the D/A board and the VCOs are made such that the default parameter setting for *physical channel* under each software channel maps the name of the software channel (with the leftmost channel being the 1\(^{st}\) and the rightmost being the 2\(^{nd}\)) to the deflector that the software channel controls. For example, the leftmost channel under the X rubric controls the RF frequency generation for the 1\(^{st}\) X AOD. Additional adjustments available through this program include the ability to adjust the *Modulation Voltage* of the RF wave to increase or decrease the acoustic power and thereby change the efficiency. After a position is defined by filling in the parameters above, the user must then chose to *Store Value*, at which time all the values are fixed for that point and the user can then select another
Figure 3-8: Point Definition Software. This GUI serves as the primary software tool for aligning the fast 3D scanner. See text for details on parameters and use.
point (using the *Point Number* drop down menu) to edit. Once all values for all points are entered, the *Start Button* is used to start the scan, which will run continuously until the *Stop Button* is clicked.

**Amplitude**

This parameter represents the peak to peak voltage of the generated voltage ramp, which will be converted by the VCOs into the RF frequency ramp. Positive values indicate an upchirp and negative values a downchirp. The generated ramp is split equally between the voltage specified in the *offset* parameter channel. For a spherical focus, the *amplitude* parameter to all four software channels must be identical. However, for some tests it is convenient to have the ability to set different values for this parameter on different channels. For example, if the *amplitude* parameters of either both the X or both the Y channels are set to 0 while the other channels are given a value, the result is similar to the case present in Chapter 2, where a cylindrical focus will be generated at the axial location determined by the non-zero channel (and a corresponding orthogonal cylindrical focus will be generated at the focal point of the objective lens). Using two such cylindrical foci, one in the X dimension and one in the Y dimension, allows for convenient assessment and correction (through manipulation of the axial translation stage controls of the AODs) of astigmatism. The default value for this parameter is 0.

**Offset Parameter**

This value controls the absolute voltage offset around which the voltage ramp is generated. If a point with an off-center lateral position is desired, this value must be set
to non-default values for either the X2 or Y2 channel, depending upon whether the lateral positioning is required in the X or Y dimension (Note that due to issues of Bragg angle mismatch, frequency offsets in the first deflection set cannot be used to generate lateral position offsets in the scan pattern since they would correspond to different incident angles to the second set of AODs, thus dropping the diffraction efficiency. However, for alignment and calibrating purposes, it is sometimes helpful to generate a scan pattern (such as a 5 X 5) that can be visualized after the first set of deflectors, which indeed can be done by adjusting the offsets of the X1 and/or Y1 channel). The default value for this parameter of -1.3091V corresponds to the center frequency of the AOD bandwidth.

*Physical Channel*

This parameter, which is chosen from a drop down box, corresponds to the actual analog output channel controlled. As mentioned above, the analog output channels are connected to the VCOs such that with the default values set for this parameter, the individual program channels correspond to the deflectors they control. However, this can be changed by choosing different values under the drop down menu.

*Slope Time*

Intuitively labeled, the *slope time* parameter corresponds to the amount of time in microseconds over which a voltage ramp is generated. Note this value, along with *amplitude* parameter value, determines the slope of the frequency ramp, which determines the focal length of the generated acousto-optic lens as governed by *Eqn. 2-6*. The default value for the *slope time* parameter is 20µs.
**Blank Time**

The *blank time* is the amount of time to wait between ramps. It can be used to generate pulses of light, but in general has little applicability for practical AOD based imaging. The default value of 0 should very rarely be changed.

**Modulation Voltage**

This value corresponds to the output voltage of the E series card. The user has the option to individually set this value or to have the computer automatically set it to normalize the power across the scan range based upon experimental determinations of power drops at different axial positions. These options are switched by using the **Automatic Power Modulation** check box in the top left corner. If the automatic power modulation is chosen, the user must then chose whether to have the program base the power modulating scheme on the selected axial positions for each of the points (in this case, the axial point which would give the least power is selected to have the maximal modulation voltage, and all other points are modulated to normalize to it), or on a specified axial position (in which case, the maximum modulation voltage is chosen for this axial position and all the specified axial points are normalized to it). These options are switched by using the **Defined Max** Check Box, along with the editable text box below it.
Center Point, # by #, and Store Pattern

The three other options on the user interface include the ability to generate only the center point of the scan pattern (Center Point), the ability to generate a discrete array of scan pattern points (# by #) in a lateral plane, and the ability to store user created patterns in order to come back to them at a later time (Store Pattern and Pattern buttons). Each of these have their own areas of usefulness. For example, the Center Point option is very helpful for system aligning and collimation, as is discussed in the Sect. 3.3.1. The # by # option is useful for assessing the homogeneity of the scan pattern of either the second set or first set of deflectors (by clicking the first set check box). Finally, by allowing the user to store previously created scan patterns, the Store Pattern option (A given pattern is stored by first inputting the desired scan pattern, then clicking Store Pattern, then clicking one of the three scan pattern buttons. A previously stored pattern is retrieved on the other hand simply by clicking the scan pattern button under which it was stored) saves enormous amounts of time when the program is used extensively.

Methods

The driving source behind the Point Definition program is a series of NI tasks as defined in the National Instruments library for Visual Studios 2005 Dot Net. There are four different tasks used, a counter/timer task, a digital input/output task, a primary analog output task and a secondary analog output task. The counter/timer task in essence serves as the system clock for the rest of the tasks and operates at a frequency of 2.5Mhz. It is responsible for driving the digital input/output task which, for this program, is itself a system clock for the primary and secondary analog outputs. Of the 32 lines on the primary DIO channel (there are 2 other DIO channels with 8 lines each) of the M series
board, only 2 are utilized in this software program. The first is used to generate a system clock at a frequency of 1.25Mhz (very close to the fastest possible DIO rate of this board), which clocks out the analog output samples from the primary analog output buffer (for VCO control) with each tick. Note that this value can be made smaller by changing the Clock Rate parameter in the graphical user interface. The second line is used to generate a clock frequency of 50Khz, which in turn clocks out the analog output samples from the secondary analog output buffer (for Modulation Voltage control) with each tick. The use of a single DIO channel to drive both of these output buffers allows these two outputs to be synchronized with each other. The analog buffers themselves are generated as an array of doubles. The size of these arrays is determined by the number of points, and for the primary analog output task, the slope time of each point (For example, for a three point scan pattern where each point has a slope time of 20\(\mu\)s, the array size for the primary buffer would be 4X75. The 4 corresponds to the four separate analog output channels, whereas the 75 arises because each of the three points consists of 20\(\mu\)s*1.25Mhz = 25 samples. The secondary analog output buffer in this case however, would consist of only a 1X3 array, with one sample per output position.)
3.5.2 RUF

The RUF software suite is a GUI based generalized system for hardware control and data acquisition that was developed by software engineer Rudy Fink primarily between the years of 2001 and 2007. As it is a comprehensive system and has been written about in previous work [84], it will not be discussed here for documentation purposes but instead will be briefly described with particular attention paid to special modifications that were utilized by Rudy and this author together to adapt the system for the fast 3D imaging.

Description of Use

In terms of user interactions, RUF can be compartmentalized based upon whether the user is performing a structural scan or a functional scan.

Structural Imaging

There are three options available when performing a structural scan in RUF. The user can elect to do a continuous acquisition, to perform a stack image using a stepper motor as the axial scanning mechanism and the AODs for lateral positioning, or to perform a stack image using the fast 3D AOD scanner as the axial and lateral scanning mechanism (see Fig. 3-9). In all cases, the lateral resolution of the image is set by setting the ResolutionX and ResolutionY parameters found under the RUFII subheading (see Fig. 3-10) with the default values being 200 for each, as well as the VCO_Voltage_X_minimum, VCO_Voltage_X_maximum, VCO_Voltage_Y_minimum and VCO_Voltage_Y_maximum parameters, which are found under the Setup subheading (see
Figure 3-9: RUF Software. This software package is the primary one used during experiments (Top) Continuous image acquisition mode (Bottom) Stack collection mode (AOD stack mode (i.e. Duemani3D) selected)
Fig. 3-10) and change the frequency range over which the number of points are generated, with the default values corresponding to the full deflection range of the AODs. Note that there is an automated way to change the resolution using a zoom factor control, in which the user selects an area of interest, and the program automatically adjusts the maximum and minimum voltages. However at the time of this dissertation, this particular software feature was not functioning.

In continuous acquisition, it is possible to acquire the image at an axial location that is not the focal plane of the objective. Indeed, the axial plane at which to acquire the image can be changed by either changing the `VCO_Voltage_Z_minimum` and `VCO_Voltage_Z_maximum` parameters under the `Setup` submenu to be the same value (which specifies the desired axial plane) or by changing the `Normalized_Voltage_Z` value under the `Setup` submenu. If the first option is used, the `VCO_Voltage_Z_minimum` and `VCO_Voltage_Z_maximum` parameters specify the amplitude of voltage ramp to apply to the VCOs. In this way they are similar to the `Amplitude` parameter of the `Point Definition` program described above, except now they apply to all the points in the frame. If the second option is used, the effective amplitude of the voltage ramp applied to the VCOs is the equal to the value specified by the `VCO_Voltage_Z_minimum` parameter plus the `Normalized_Voltage_Z` value times the range determined by `VCO_Voltage_Z_maximum` minus the `VCO_Voltage_Z_minimum` value.

In stack acquisition cases, the resolution in the axial dimension is determined by adjusting the total number of axial images to acquire and the axial scan range. For the stepper motor cases the axial scan range is determined by values read from stepper motor control box. These values are directly input into the `Slice Top` and `Slice Bottom` parameter
settings, under the RUF II subheading. Similarly, the number of axial steps to acquire is determined by adjusting the Slice_Count parameter, which is also under the RUF II subheading. For the 3D AOD case, the axial range is determined by the setting the VCO_Voltage_Z_maximum and VCO_Voltage_Z_minimum parameters in the Setup subheading, and the number of axial steps to acquire is determined by setting the ResolutionZ parameter in the RUF II subheading. As will be shown in the section on predicted versus experimental axial depths, these VCO_Voltage_Z values conveniently map almost directly to the actual distance (i.e., a value of -0.20 corresponds to approximately 20μm below the focal plane of the objective whereas a value of 0.20 corresponds to approximately 20μm above the focal plane).

The basic sequence in acquiring a fast 3D stack is to first use the continuous acquisition feature and the stepper motor to scan up and down the desired object. After estimating the maximum and minimum axial locations, the user can then park the stepper motor at the center of the axial scan range. Then, the user switches over from single frame acquisition to stack acquisition and initiates a 3D fast AOD based scan (this is practically accomplished by checking the Collect Slices check box (see Fig. 3-9), selecting the Duemani3D option in the pull down menu and changing the ResolutionZ parameter from 1 to the desired number of axial steps). If the ImageWriter checkbox is selected, the images are not only displayed, but also stored in a directory specified in the Data submenu. Once the stack is generated and/or stored the program does not automatically stop scanning, but does stop acquiring data, at which point the user can elect to manually stop the program.
Figure 3-10: Important RUF Software Submenus (Left) RUFII submenu (Right) Setup submenu
It should be noted that the speed of the structural scan provided by the AOD is determined by the total resolution of the scan multiplied by the dwell time per point, which for most purposes was fixed at 20μs. Thus, for 250X250 resolution 2D scan, the total time to acquire the image is 1.25 seconds (or 0.8Hz). Likewise for a 200X200X20 resolution 3D scan, the total time to acquire the image is 16 seconds (or 0.063 Hz).

**Functional Imaging**

Once a structural image is obtained, the user can subsequently go back and select points on the structural image at which they want visit at the higher frame rates, by selecting the *Multisite Record* button. The frame rate for this scan is determined solely by the number of points and can be expressed using *Eqn. 3-3* below,

\[
frame\ rate = \frac{1}{(dwell\ time) \times number\ of\ points}
\]  

(3-10)

Where dwell time is adjustable, but usually set to be 20μs. Thus, for a single point, the available frame rate is 50kHz, while for 50 points, the frame rate is 1kHz. Note that this is regardless of whether the user is selecting the points within a 2D section or from a 3D volume.

For the latter case however, RUF allows the user to selected points at different axial sections by providing a scroll bar on the left side of the screen that appears once a 3D structural stack has been acquired (In practice, to get this scroll bar to appear, the user must first click on the *Mark* button and then on the *Clear* button. This should be done after the 3D based structural image is obtained. In order to setup the system for 3D
functional imaging the next sequence of commands should be to unclick the Collect Slices box, then to click the Multisite Record button, and finally to change the ResolutionZ parameter in the RUF subheading from 1 to a value greater than 1. For software reasons, if this exact sequence of commands is not followed, the functional points will not be registered correctly to the structural scan). Note that the scroll bar will display all images since the last time the image cache was cleared (by pressing the X button underneath the scroll bar). As a consequence, if the cache is not cleared before beginning a scan, the functional point assignments will be off. The data is written to text files which are labeled with a .ruf extension. For the experiments performed here, these files are subsequently read using a software program written in Matlab that will be discussed in the next section.

Methods (Overview)

There are two primary “lower level” components to the RUF system, the first deals with hardware control and the second with data generation/acquisition. Each of these systems is themselves designed using interfaces, in which different lower level components can be connected to the same “higher level” processing components, with the hopes that in adapting the software for different optical setups, only the lower level components need to be changed. In terms of hardware control, the lower level components consist of specific information about the data acquisition cards that are used, while in regards to data generation/acquisition, the lower level components describe the protocols to generate the correct buffers for the different analog and digital input/output tasks. The higher processing levels are utilized to create buffers of normalized points in
3D space, demultiplex and process analog input data based upon specifications described in the lower level data acquisition control subsystem, and either display and/or store it. The higher processing levels will not be discussed here.

Hardware Control

This system consists of initiating the NI tasks to be used. Similar to the situation for the Point Definition program, for the fast 3D imaging hardware control component of RUF, there is a counter/timer task, a digital input/output task, a primary analog output task and a secondary analog output task. However, unlike the Point Definition program, there is an additional analog input task here that is used to sample data from the PMT. This task is asynchronously programmed to use a call back function that performs a specific set of instructions once a given number of input samples are acquired.

Data Generation/Acquisition

The data generation/acquisition system is similar to the Point Definition program. In fact the methods used to create the primary analog output and secondary analog output buffers are derived entirely from it. In addition, the method to create the digital input/output buffer is also very similar. For example, it also utilizes two lines from the main DIO channel to drive the primary and secondary analog outputs. However, in addition, it uses a third line from this port to clock the analog input.

The primary difference between the Point Definition program and the RUF program lies in the ability of the later to acquire, display, store and analyze analog input data. As mentioned above, this process requires a specific number of samples to be set
for the analog input task, after which the callback function is called. For the structural imaging case, this specified number of samples to acquire is equivalent to the number of samples in a frame (note that for fast 3D based structural image, a frame is actually the entire scan pattern, which consists of more than a single axial section). For functional imaging, the number of input samples to acquire is equivalent to the number of samples that would be collected in one second (i.e., since samples are collected every 20μs, this implies 500,000 (times the number of oversamples)) Examples of the instructions that are preformed once the data is acquired include demultiplexing it, (Note that demultiplexing the data is necessary since the analog input samples are, in essence, a stream of voltage values. To be able to isolate what values correspond to what position in the scan pattern, this stream of values is broken up based upon the known number of points selected to be imaged as well as the anticipated number of oversamples) displaying the demultiplexed data as an image file, and/or saving the demultiplexed data as an image file or a text file.
3.5.3 Ruffleconverter

Functional imaging results were analyzed via a Matlab (Mathworks) graphical user interface called *Ruffleconverter*, which was written by this author to convert the output text files from RUF into a useable data structure (see Fig. 3-11). For documenting purposes, it will briefly be described here.

When placed into the *Multisite Record* mode, the output from RUF is a series of text files, each of which contains one second worth of data. Note that since RUF is manually stopped, the number of text files for a given scan usually exceeds one (since the user typically blocks the beam and then clicks the stop button, which typically takes longer than one second). The data in these files are organized such that each row corresponds to a single sweep through all the points in the scan pattern and each point in each row is separated by a RUF defined delimiter, usually a space.

When a user clicks the *Open Directory* button in *Ruffleconverter* and chooses a directory where the desired files are located, the program vertically concatenates all the *ruf* extension files in the selected directory, parsing the data into columns which correspond to the individual points selected by the user. There are two ways to read the input RUF files. If the *Average* checkbox is selected, the program assumes that several scans were taken for averaging and labeled in numbered subdirectories under a single parent directory. Then, when the user elects to open the parent directory, the program will subsequently average the selected single scans, stored in each subdirectory, over the amount of time specified by the shortest scan in the average. If this option is left unchecked, the program will open only the *ruf* files in the parent directory.
Figure 3-11: Ruffleconverter. This MATLAB interface is the primary software package used for analyzing and displaying recorded data. See text for details on description and use.
In addition, there are two options available to determine how the data in each column is evaluated. If the *oversample* option is left unselected, each data point in every *.ruf* file is read in sequentially and the program assumes that the sampling rate for the acquisition is the frame rate specified by *Eqn. 3-10*. However, the user also has the option of selecting the *oversample* option and specifying an oversampling frequency, in which case the program sums together a specified number of samples $n$ in each column, where

$$n = \frac{\text{frame rate}}{\text{oversampling frequency}}$$  \hspace{1cm} (3-11)

Note that since in 3D scans the scanner cannot stay indefinitely at a given location, this option allows the user the increase the number of oversamples in much the same way that simply staying longer at a location would in a 2D scan. Regardless of which option the user selects, the concatenated and parsed data is stored as an array which is accessible by the user in the main command window of Matlab.

When the user clicks *Convert*, the program subsequently analyzes and displays the data as separate channels as shown in *Fig. 3-11b*. There are two options for how to analyze the data as well as two options for how to display it. If the user has selected the *filter* checkbox, the program passes the data through a constrained least-squares low pass filter, with a cutoff frequency of 50Hz. This filter is designed in Matlab using the *fircls1* function with an order of 150, a passband deviation value of 0.02, and a stopband deviation value of 0.008. The data is passed twice through this filter using the *filtfilt* function. This filter option was found to preserve any sharp transients, which are
important for the neuronal studies discussed in the next chapter, while still eliminating
the high frequency noise as much as possible. Once the data is filtered, the background
value, which is determined as the average over the last 500 points (this assumes that the
user has blocked the beam before stopping the scan) is subtracted. If the filter checkbox
is not selected, no filtering of the data is performed, but the background is still subtracted.

For displaying the data, the user can either leave the delta/f checkbox unchecked,
which is typically done for non-neuronal random access experiments to display the output
voltage from the PMT, or can check the this box, which serves to normalize the signals
acquired during functional neuronal experiments. This normalization is done using the
following equation.

$$\frac{\Delta F}{F} = \frac{F - F_o}{F_o} \times 100\%$$  \hspace{1cm} \text{(3-12)}

Where $F_o$ is the mean background subtracted fluorescence value over the first 50 data
points of the scan and $F$ is the fluorescence value at the current time point. $\Delta F/F$ values
are typically used in optical experimental neuroscience as a representative for calcium
changes [116].

The final option available in the Ruffle converter program is to selectively plot
one of the data channels on a larger scale. This is accomplished by typing the number of
the desired plot in the text box next to the Plot push button, and then clicking the Plot
button. This option is useful for re-graphing the data for presentation purposes.
3.6 Fluorescent Test Images

To initially test certain aspects of the fast 3D AOD based scanner system, we used two basic types of fluorescent test images, fluorescent beads and pollen grains. The primary aspects of the system that were tested include the positioning ability, the optical sectioning ability, the structural imaging ability and the random access ability.

3.6.1 Positioning Ability

To test how well the actual changes in focal position around the objective focus plane matched the predicated changes, a slide containing 200nm fluorescent beads (T14792, Molecular Probes; Eugene, OR) was used in combination with an objective stepper motor. By collecting stacks of the beads with the stepper motor at different acousto-optic lens (AOL) determined focal positions, the position of the focus relative to the inherent focal position of the objective lens could be measured.

While the effective focal length the AOL can be theoretically calculated using Eqn. 2-6 from Chapter 2, the predicted focal position of the system is determined only through a variation of Eqn. 2-7. This is because, unlike the situation presented in Chapter 2 where the output from the AOL is optically relayed to the back focal aperture of the objective lens with 1:1 telescopes, in this situation, the scan angle is magnified to avoid excess overfilling of the back focal aperture. This changes the effective focal length of the combined system. A simple way to determine the new focal length is to take the paraxial ray assumption and calculate the ray transfer matrix [117] of the entire optical system (with a secondary assumption that the system consists of only the AOL, the scan magnifier, and the objective lens), which is equal to
Figure 3-12: Position Verification. Comparison between predicted focal positions and measured focal positions.
\[
\begin{bmatrix}
    -\nu & 0 \\
    \nu + \frac{1}{F_{obj}} - \frac{1}{\nu} & -\nu & 0 \\

\end{bmatrix} = \begin{bmatrix}
    1 & 0 & 0 \\
    -\frac{1}{F_{obj}} & 1 & 0 \\
    0 & -\frac{1}{\nu} & 1 - \frac{1}{F_{AOL}} & 1
\end{bmatrix}
\]

(3-13)

The effective focal length of the total system can now be set equal to the [1,1] term in this matrix divided by the negative [2,1] term (since the [1,1] term will determine the position dependent size of the beam after the system and the [2,1] term will determine the position dependent angle), which gives

\[
F_{Total} = \frac{F_{obj}F_{AOL}}{\left(\frac{1}{\nu}\right)^2 \frac{F_{obj}}{F_{AOL}} + \frac{F_{obj}}{F_{AOL}}}
\]

(3-14)

Where \(\nu\) is the effective beam magnification value, or 1/1.2 in this situation. Fig. 3-12 compares the predicted versus the measured focal position, and as can be seen, the values are in close agreement throughout the scan range.

### 3.6.2 Fluorescent Beads

To initially test the optical sectioning ability of the scanner, we utilized 10\(\mu\)m fluorescent beads (Molecular Probes) which have a maximal excitation at 505nm and a maximal emission at 515nm. Images were taken using either 2D AOD scanning in combination with axial positioning by an objective lens stepper motor or by all-AOD-
Figure 3-13: 10 Micron Fluorescent Bead (A) Projection views of a fluorescent 10μm bead image stack acquired using an objective lens stepper motor for axial positioning and AODs for lateral positioning. (B) Projection views of a 10μm bead image stack acquired using the 3D AOD scanner for both axial and lateral positioning.
based 3D scanning. Axial step sizes in both cases were approximately 250nm. Both sets of images were subsequently reconstructed using visualization software (AMIRA) and axial, sagittal, and coronal projections were generated. As can be seen in Fig. 3-13, the differences between the stepper motor and AOD-based axial scan cases were minimal.

3.6.3 Pollen Grains

To further test the system on larger and more intricate structures, we performed a similar analysis using fluorescently labeled spiny pollen grains. These structures are approximately 35μm in size, but notably have very fine processes which extend to smaller than a 1μm in diameter. Once again, images were taken either by 2D AOD scanning together with an objective lens stepper motor for axial positioning, or by all-AOD-based 3D scanning. In both cases, the axial step size was approximately 400nm. Image stacks were subsequently collated and a 3D reconstruction (AMIRA, Voltex) was generated (Fig. 3-14). In addition, the image from every 10th axial step is shown to allow for direct comparisons at each individual level. As can be seen from the figure, once again, the differences between the stepper motor based axial scan case and the AOD based axial scan case were minimal.

Higher Magnification Structural Scan

To get an assessment of the fundamental resolution of the system, a higher magnification scan was performed on a single spine of the pollen grain at the objective focus as well as the at the extremes of the axial scan ranges used for the biological experiments that will be described in Chapter 4 (± 25μm). Note that to get images of the
Figure 3-14: Pollen Grain (A1) Top down view of a reconstructed fluorescent pollen grain image created from a stack taken using a stepper motor for axial positioning and AODs for lateral positioning (A2) Every 10th individual section from the stack used to create A1. (B1) Top down view of a reconstructed pollen grain image created from a stack taken using the 3D AOD scanner for both axial and lateral positioning. (B2) Every 10th individual section from the stack used to create B1.
same spine, the objective was moved in the appropriate direction to keep the imaged spine in focus (i.e., if the 3D AOD system positioned the focus 25μm below the inherent focus of the objective lens, the objective was moved 25μm up to compensate and keep the image in the field of view). The results are shown in Fig. 3-15. From this figure, it can be seen that while there are changes in resolution, particularly in the converging beam situation, a sub-micron resolution is maintained. How these changes in resolution will affect the visualization of small neuronal process such as spines will be discussed in Chapter 4.

3.6.4 Random Access Tests

While the structural image differences between the all AOD based axial scan versus the stepper based axial scan are minimal, it is important to note that the primary purpose for the system is not for structural imaging, but rather for fast functional imaging where the advantage provided by the scanner is the ability to perform random access scans in an entire volume, rather than just a single optical section. To test this ability, the 3D scanner was first used to image a cluster of 1μm beads suspended in KOH solution (It was an interesting finding of a previous member of this lab, that when these beads are placed on a slide and a coverslip is placed on top, they tend to form stable 3D structures. On replication of this technique, we find that the beads tend to align along two primary axial planes. This is probably due to them either sticking to the top coverslip or the bottom slide. In any case, the situation allowed us to image 1μm beads that were in separate axial dimensions).
Figure 3-15: Magnified Pollen Grain Comparison
Single section images of a pollen grain spine acquired at higher magnification using the all AOD based scanner. (b) Image at the inherent focus of the objective lens. (c) Image of the same structure acquired by changing the collimation to a diverging beam, which shifts the focus below the objective focal plane (~25μm), and moving the objective up approximately 25μm to compensate. (a) Image acquired by changing the collimation to a converging beam and moving the objective down to compensate.
After the initial structural image was obtained, a random access scan was performed by selecting positions that were either on or off the bead in both the axial and lateral dimensions. As can be seen from Fig. 3-16, if the selected point was on the bead, the result was a fluorescence level that nearly maximized the PMT voltage. Whereas selected positions that were off the bead resulted in fluorescence levels that were practically zero.
Figure 3-16: 3D Random Access Imaging (a-b) 1μm beads were separated in different axial and lateral dimensions using the procedure described in the text and random access imaging points were selected at specified axial planes for quick 3D imaging (c) The resultant fluorescence either nearly maximized the PMT voltage if the selected point was on the bead (green) or gave minimal signal if the selected point was off the bead (red).
Chapter 4: Single Neuron Studies

This chapter is dedicated to demonstrating the fast 3D random access capabilities of the scanner by imaging important biological parameters of neuronal physiology. In this regard, the signal that was chosen as the variable for study was calcium. The reason for this is four fold. First, calcium influx through voltage gated calcium channels can be triggered by initiating back propagating action potentials, thus experimentally eliciting it only requires a single electrode to be placed in the soma of the neuron being studied. Second, this influx occurs throughout the entire dendritic arbor and, as a result, the changes can be monitored at a variety of different locations within the volume of space occupied by the neuron. Third, using calcium sensitive dyes with moderate $K_d$ values (~150nM), the speed of the calcium transients is on the order of milliseconds, which allows the 3D scanner, with frame rates of up to 50kHz, to selectively image multiple sites near simultaneously. Finally, the amount of calcium influx through during a bAP can be estimated with a single optical measurement known as the fractional change of fluorescence or $\Delta F/F$.

4.1 Methods

Hippocampal brain slice preparation

Three hundred and fifty micron hippocampal brain slices were obtained from 4-6 wk old Sprague Dawley rats and prepared in accordance with the guidelines of the National Institutes of Health as approved by the animal care and use committee of Baylor College of Medicine. Before decapitation, rats were anesthetized with a mixture of
ketamine, xylazine, and acepromazine, and perfused trans-cardially with a 2-4°C solution consisting of 2.5mM KCl, 1.25mM Na2H2PO4, 25mM NaHCO3, 0.5mM CaCl2, 7mM MgCl2, 7mM dextrose, 100mM choline chloride, 1.3mM ascorbate and 3mM pyruvate. The brain was then dissected, hemisected and mounted in the holding chamber of a vibratome (Vibratome 100, Ted Pella; Redding, CA) in this same solution. Hippocampal slices of 350um thickness were cut and subsequently transferred to a holding chamber containing an artificial cerebrospinal fluid solution containing 125mM NaCl, 2.5mM KCl, 1.25mM Na2H2PO4, 25mM NaHCO3, 2mM CaCl2, 2mM MgCl2, and 10mM dextrose at 35°C. Slices were held at this temperature for 45 minutes before movement to room temperature for 20min before use.

Experimental procedures

The experimental procedure involved first identifying and patching onto a cell of interest. In this regard, hippocampal slices were perfused in an artificial cerebrospinal fluid solution containing 125mM NaCl, 2.5mM KCl, 1.25mM Na2H2PO4, 25mM NaHCO3, 4mM CaCl2, 2mM MgCl2, and 10mM dextrose. Note that the extra calcium (as opposed to a standard of 2mM) served to enhance the transient signals. The solution was bubbled with a 95% O2-5%CO2 mixture. Patch pipettes were pulled using a pipette puller (Model P-97, Sutter Instruments) to tips with resistance values between 3-5MΩs. This was confirmed by measuring voltage values on the seal test of the patch clamp amplifier (Axo-Patch 200A, Axon Instruments). These pipettes were filled with solution containing 120mM K-Gluconate, 20mM KCl, 10mM HEPES, 2mM MgCl2, 4mM Mg2GTP, 0.3NaGTP and indicators at concentrations specified below. Pyramidal cells of
interest from the CA1 region of the hippocampus were identified and patched using widefield IR-DIC imaging.

**Indicators**

A combination of two separate dyes (Alexa-594 (50 μM) as the structural indicator and Oregon Green Bapta-1 (200μM) as the functional indicator) were also included in the pipettes. Both dyes are available from Molecular Probes (Eugene, OR) and a brief description of their properties is given below.

**Alexa-594**

Alexa-594 (Alexa-594 labeled cadaverine) is a member of the new Alexa Flour family of fluorescent dyes and conjugating indicators which has been highly cited for their enhanced brightness and photostability [118]. Alexa 594, as the name implies, is excited with a maximum single photon wavelength of 594nm. For multi-photon purposes, the peak two photon excitation wavelength has been measured at 1074nm [119], however, due to its broad excitation spectra, different types of studies have utilized it with an excitation wavelength of 800nm as is done here [12,120]. The peak emission wavelength of this dye, regardless of whether single photon or multi-photon excitation is used, is approximately 617nm [121], which is conveniently red-shifted from the excitation spectra of many functional indicators. As a result, this dye has been extensively implemented as the multi-photon structural label for experiments involving many different types of neurons[12,122,123].
The functional label used for the neuron studies is Oregon green Bapta-1 (OGB-1)[124]. This is a calcium sensitive dye that has also been extensively utilized in mapping calcium transients in a variety of neurobiology experiments [116,125,126]. The peak single photon excitation wavelength of this indicator is 494nm and while most multi-photon studies usually utilize a wavelength around 880nm with this indicator, it has been shown that there is a localized two photon excitation peak of this dye at 760nm [127], which is very close to 800nm power peak of the MIRA HP laser. The peak emission wavelength of this dye is 523nm, which is far enough away from the peak emission wavelength of Alexa-594 to ensure minimum cross talk between the dyes with different emission filter sets. Upon binding calcium, the fluorescence of OGB-1 increases approximately 14 times, which makes it very useful for measurements of fractional changes in fluorescence ($\Delta F/F$). The $K_d$, or disassociation constant for this dye is 170nM at 22°C, which is lower than other well used calcium indicators, such as Fura-2, which has a $K_d$ of approximately 266nM [128] under similar conditions.

### 4.2 Structural Imaging

To assess the capabilities of the all AOD based 3D imaging system in generating a structural image, a pyramidal neuron was patched and filled as mentioned in the methods section. A fast 3D imaging stack was acquired over approximately a 50µm axial range using the RUF acquisition software as described in Section 3.5 of Chapter 3. The resolution for this scan was set at 300X300X40 (note that, as mentioned in Chapter 3 Section 3.5, since the total number of points in this scan pattern exceeded 800,000, the
total stack had to be acquired using a series of smaller stacks. In this case, a series of 8 separate stacks of 300x300x5 each were used. A top down maximum projection image of the result is shown in Fig. 4-1a. As a comparison, a stack of the entire neuron was acquired using a stepper motor (Remote Focusing Accessory 99643, Nikon) for axial positioning and AODs for lateral positioning, with a resolution of 300X300X80. A top down maximum projection of this image is shown in Fig. 4-1c. For a fair comparison between the all-AOD based case and the AOD plus stepper motor case, the center 50μm of the stepper motor based scan were isolated and a separate top down maximum projection image of this set was created. This result is shown in Fig. 4-1b. As can be seen from comparing Fig. 4-1a and Fig. 4-1b, the differences between the all AOD based scan and the AOD plus stepper motor based scan are negligible over a 50μm axial range. More importantly for functional imaging, oblique dendritic branches are still visible using either method. This supports notion that the structural scan on which the points for functional imaging will be selected can be performed with the all AOD based scan, which is the most direct way of ensuring proper registration between the two scans.

4.2.1 Magnified Structural Imaging

Since there is anticipated reduction of resolution as the focused is moved away from the focal plane of the objective, there remains a question as to how much this resolution reduction will affect the ability to visualize small neuronal structures, such as thin dendrites and spines. In order to assess this, a higher magnification structural scan comparison was performed. In this regard, a section of a dendrite was identified by manually moving the stepper motor to an optimal section while the performing a full 2D
Figure 4-1: Neuron Structural Comparison Maximum projection image of a CA1 pyramidal neuron acquired using either (a) the all AOD based 3D scanner with a user specified resolution of 300X300X40 or (b) AOD based lateral positioning with stepper motor based axial positioning at a user specified resolution of 300X300X50. Both images were taken over an axial range of 50μm that was based around the center of the neuron. For completeness, a complete stack of over the entire 85μm axial range of the neuron was also acquired with a resolution of 300X300X85 and (c) shows the maximum projection image.
scan. When a dendritic branch was identified, the scan range was adjusted in RUF to reduce the field of view. A single image was then taken. This is shown Fig. 4-2b. The effective axial plane of imaging was subsequently changed to a value that corresponded to approximately 25µm below the focal plane of the objective. The objective was then raised by the same amount such that the dendritic segment came into focus again. This image is shown in Fig. 4-2a. Finally the axial plane of imaging was changed again in the same manner to approximately 25µm above the focal plane of the objective and the objective was lowered by approximately 50µm (which put it 25µm below the objective focal plane) at which point the dendritic branch once again came into focus. This image is shown in Fig. 4-2c. Comparison between Figs. 4-2a, 4-2b, and 4-2c, show that while there are differences between the different axial sections, indeed individual structures are noticeably bigger in Fig. 4-2c, the thin dendritic branches as well as the spine heads are visible in all three images.

4.3 Functional Imaging

To test the ability of the scanner to track changes in calcium, localized calcium transients were monitored in response to stimulated back-propagating action potentials. To accomplish this, an all-AOD based structural scan of an identified CA1 pyramidal neuron was performed over an axial scan range of approximately 25µm. The resolution of this structural scan was 200X200X20. Note that since the total number of points in this case was intentionally kept to 800,000 to avoid having to acquire the full scan with a series of smaller stacks since this would throw off the point placement for the functional scan. The maximum projection image of this scan is shown in Fig. 4-3a. From the
**Figure 4-2: Magnified Neuronal Structural Comparison** Single section images of the same dendritic segment acquired at higher magnification using the all AOD based scanner (b) dendrite segment visualized at the inherent focus of the objective lens (a) image of the same dendritic segment acquired by changing the collimation to a diverging beam, which shifts the focus below the objective focal plane (~25μm), and moving the objective up approximately 25μm to compensate (c) image of the same dendritic segment acquired by changing the collimation to a converging beam and moving the objective down to compensate.
acquired structural stack, different points along the main dendritic branch were selected for functional imaging in the RUF software as described in Chapter 3, Section 3.5. The functional scan was subsequently performed by visiting only these selected sites at the elevated frame rate determined by Eqn. 3-3. The only change made in the optical path between the structural scan and the functional scan was to exchange to 600/200 emission filter with the 550/100 and 535/50 emission filter set combination, to isolate the functional indicator (OGB-1). Note that as opposed to previous AOD based scanning microscopes [12,13], the points selected for the functional scan in this situation were from different lateral and axial locations. Indeed, as can be seen from the color coded axial location map on the bottom right corner of Fig. 4-3a, the axial range defined by the functional scan points selected from this neuron is approximately 10μm, with all points visited being below the focal plane of the objective. The signals from each of the identified points were processed and analyzed using the RuffleConverter program and the results are shown in Fig. 4-3b. As can be seen, the change in normalized fluorescence signal (ΔF/F) caused by the influx of calcium in response to a train of 3 back propagating action potentials (bAPs), shown as the bottom trace of Fig. 4-3b, is clearly visible at the five different locations sampled.

4.3.1 Large Axial Range Functional Imaging

While the data in Fig. 4-3 does show the ability of the scanner to perform random access functional imaging on a neuron, the extent of the scan in the axial dimension was rather limited. To demonstrate the scanners random access ability over a larger axial range, another functional scan was performed on a different cell. Once again a CA1
Figure 4-3: Neuron Functional Imaging  (a) Maximum projection image of a CA1 pyramidal cell with five different axio-lateral locations identified for functional imaging. Note that the color code for the relative axial position is in the bottom right corner of the image. (b) Top four traces, calcium influx in response to three stimulated bAPs (bottom trace) at the different locations identified on the neuron in (a).
pyramidal neuron was identified, patched, filled and structurally scanned as described above. However, in this case, the structural scan was taken over a axial range of ~50μm, with the resolution set at 200X200X20. As in the previous situation, this structural scan served as the framework on which specific points were selected throughout the dendritic arbor for functional imaging. The top down maximum projection of the structural scan is shown in Fig. 4-4a, with the user selected functional imaging points overlaid. Once again, the axial location of the user selected points relative to the focal plane of the objective are mapped via a color code depicted in the bottom right corner of Fig. 4-4a. As can be seen from the color map, the full range of the scan in this case extends 30μm in the axial dimension, and image points are taken from both above and below the objective focal plane. The calcium transients at the different locations in response to the stimulated bAP train shown in Fig. 4-4b are displayed next to the identified points and are also color coded for axial position.
Figure 4-4: Neuron Functional Imaging  
(a) Calcium transients in response to 3 stimulated bAPs (shown in (b)) at 20 different axio-lateral locations on a CA1 pyramidal cell. All traces were acquired in a single scan. Color code indicates approximate axial location relative to the focal position of the objective lens. Total axial scan range of ~30μm. (b) Current injection stimulated bAP train.
Chapter 5: Summary and Future Directions

The essence of this work can be compartmentalized into three parts, corresponding to the three previous chapters. First a laser beam scanning mechanism was demonstrated that utilized a unique arrangement of AODs and an objective lens to scan in both the lateral and the axial dimensions with transition times on the order of 20μs. Second, a multi-photon microscope was designed and built using this scanning scheme. Finally, this multi-photon microscope was utilized to functionally record several calcium transients from different axial and lateral positions on a neuronal dendritic arbor, a task that can be accomplished with no other system.

5.1 Potential Improvements

5.1.1 Phased Transducer Array AODs with Acoustic Beam Steering

The biggest limitation to how far the beam can be scanned in the axial dimension is the drop in diffraction efficiency that results from the small incident aperture of the OAD-1121 AODs. Indeed, it can be calculated that the available axial scan range afforded by the bandwidth of the deflectors and a 60x objective lens is approximately 200μm [129], whereas for the experiments performed here, the total axial scan range was only 50μm. A similar situation exists for the lateral scan range as well, where the bandwidth provided by two AODs per dimension allows for twice the resolution that was utilized in this work (note that resolution here refers to the number of resolvable spots as described in Chapter 2).
Conceptually, there are two ways to increase the effective incident aperture of a deflector. The first is to increase the divergence of the acoustic wave. However, for the OAD-1121 deflectors, this approach would result in a drop in the overall diffraction efficiency and is not feasible from a manufacturing standpoint [130]. The second option is to utilize an array of transducers instead of a single transducer. With this scheme, it is possible to steer the acoustic beam by changing the phase between the RF waves at each transducer. While this has been shown to increase the interaction bandwidth and diffraction efficiencies of isotropic AODs and AOMs to values comparable with anisotropic deflectors while preserving larger incident apertures [131,132], it is unclear whether one could continuously change the phase of a chirped RF wave, which is what would be needed for this application. In addition, if it is possible, the RF generation mechanism would have to become much more sophisticated since there would be more channels (one for each transducer instead of one for each deflector) involved and the phase condition for each channel would have to be taken into account. This, in essence, would require a DDS based system.

5.1.2 Spherical Aberration Correction

Spherical aberration is the result of the light rays propagating through the edge of lens traveling a different optical path than paraxial light rays. In normal microscopy, objective lenses are well corrected for this situation, with the result that, if the incident beam is well collimated, the majority of the rays are in phase at the focal plane of the objective. However, if this situation is not upheld, as in is the case with the AOD based focusing situation where the collimation is changed, then the focal positions of the
different rays will be not be the same. While the amount of spherical aberration introduced in this work did not significantly effect the imaging resolution over the axial range used, indeed other groups have shown minimal changes in spherical aberration in water immersion lenses over a similar axial range [133], with larger axial ranges it is anticipated that spherical aberration will have a larger role. In such cases, it might prove useful to utilize a reverse Gaussian aperture [134] to preserve the resolution over the entire axial range at the expense of the resolution at the focal plane.

5.2 Biological Directions

Even if no changes to the system are made, it still has functional applicability in several areas of neurobiology. These include experiments on the single neuron level as well as experiments at the systems level.

In regards to single neuron physiology, numerous studies looking at the calcium response to back propagating action potentials throughout extensive regions of dendrites should now be achievable with much less experimental effort. By utilizing pharmacological blocking techniques with these experiments, it will be possible to discern how specific channels affect the efficacy with which the bAP is effectively transmitted to different segments of the dendrite. While it can be argued that these types of experiments have been done before with much less sophisticated systems by simply increasing the number of experiments, the abilities provided by this new system will not only serve to improve the efficiency with which the results can be gathered, but also shed insights into dynamic neuronal processes that were hidden in previous experiments. Indeed, given the well documented ability of neurons to transform in response to different
situations, there is a strong indication that repetitive experiments are studying a dynamic system while simultaneously changing it.

Possibly the most significant advantages this system will offer however will be gained from studies of populations of cells. In such experiments, the primary goal is to analyze the response from the multiple distinct somas, which are filled with a bath loaded dye. Thus, the resolution requirements for these experiments drop substantially and the field of view requirements increase drastically. As a result, lower magnification objective lenses are used. The axial range of the 3D AOD imaging system is based primarily on the objective lens used and, as was seen in Chapter 2, with a 10X objective, the effective axial range is on the order of millimeters. This gives an extensive volume over which cells can be sampled at frame rates of 50Khz, opening the door for studies of neuronal interactions in slices and even live animals that are currently unapproachable.
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