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A Dual Acousto-Optic Laser Scanning Microscope System for the Study of Dendritic Integration: Design, Construction, and Preliminary Results

by

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ABSTRACT

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Recent research has highlighted the vital role played by dendrites in effecting the computational properties of single neurons in the central nervous system (CNS). An ultraviolet (UV) acousto-optic laser scanning microscope system was developed that enables UV laser pulses to be delivered to multiple user-selected sites in the microscope's specimen plane with high spatial (<10μm) and temporal (<20μs) resolution. By employing “caged” neurotransmitters, the system can effect physiologically realistic spatio-temporal patterns of “synaptic” stimulation to the dendrites of a single cultured neuron. This system was combined with a previously developed acousto-optic laser scanning system for fast, multi-site optical recording of electrical activity (Bullen et al. 1999). This combination—the “Dual Scanner”—allows the study of important dendritic questions such as the underlying mechanisms of spatial and temporal summation. This thesis describes several current outstanding questions of dendritic integration, the design and construction of the system, and some promising preliminary results.
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This thesis was written with several objectives, and audiences, in mind. First was a need to try to document much of the know-how that I/we have gained over the last few years in order to allow future members of the Saggau lab to get up to speed quickly in projects involving acousto-optic scanning. Second was a need to provide some documentation that is suitable for others, outside our lab, who may be interested in also developing an acousto-optic scanner, whether for the study of neurobiology, or for other applications (analytical chemistry and certain microlithography applications come to mind). As such, the thesis should serve as a core to an eventual book chapter that I/we will eventually write after the technique has “matured” a little more.

For these reasons, the bulk of the thesis (Chapters 3-5) consists of documenting the system known as the Dual Scanner, and might be termed the “engineering” section. The remaining chapters, arguably, comprise the “science” section.

The motivation for constructing the Dual Scanner—to provide a unique apparatus suited for the study of dendritic integration—is discussed generally in Chapter 1. Chapter 2 provides deeper motivations by proposing types of experiments the Dual Scanner could carry out that would be valuable and natural extensions to the tremendous strides in understanding dendritic integration that have been unearthed over the past decade. This chapter was/is intended in part as a springboard, both perhaps for myself and certainly for others in the lab, to a PhD proposal.
Chapter 6 summarizes preliminary results that point generally to the viability of the UV acousto-optic scanning system developed. And, finally, Chapter 7 plots a future trajectory of work to be done with the Dual Scanner.

After some time now, the Dual Scanner is ready to move from the “engineering” phase to the “science” phase. I look forward to this next “volume” of this work—my PhD thesis.
CHAPTER 1

INTRODUCTION

Interest in the role that dendrites play in the computation performed by a single neuron has never been greater. New techniques, most notably dendritic patch-clamp recording and two-photon microscopy, have produced a veritable renaissance over the past decade. These techniques have allowed the detection of electrical and chemical signals, respectively, at previously unattainable spatial and temporal resolution--down even to the level of the tiny (~1μm) protuberances that adorn the cell's dendrites, known as dendritic spines.

The physiological role of dendrites is, to oversimplify, twofold. First, they offer ample surface area to allow the neuron to receive thousands, to tens of thousands, synaptic inputs, mostly at the spines; therefore, the post-synaptic machinery, from receptors to Ca$^{2+}$-signalling complexes, can be found repeated at many locations in the dendritic arbor. Second, dendrites are also the transmission line by which postsynaptic potentials, both inhibitory and excitatory (IPSPs and EPSPs, respectively), are conveyed to the soma, and to the axonal hillock where the neuron "decides" whether to fire. Recently, clear evidence has been found that the dendrites-as-cable also carry two other important types of signals: back-propagating action potentials (BPAPs) (Spruston et al., 1995b) and dendritic spikes (Golding and Spruston, 1998). BPAPs are the reverse ("antidromic") flow of action potentials from the axonal hillock back into the dendritic arbor. Dendritic spikes are local action potential-like spikes supported by voltage-gated Na$^+$ and Ca$^{2+}$ channels. The definitive finding that dendrites contain voltage-gated conductances, which give rise to "active" (i.e. nonlinear)
electrical properties, and that these support important mechanisms of physiological signaling, has been arguably the principal breakthrough of the past decade (Johnston et al., 1996).

In both their roles as synaptic targets and as electrical cables, dendritic properties change dynamically on a variety of time scales, in response to both activity and modulatory inputs. These changes can be fast, reflecting the sophisticated biochemical machinery that is local to the dendrites, such as that for dendritic protein synthesis (Steward and Schuman, 2001). The properties of a neuron's dendritic tree and the rules by which they change, if they can be gleaned, could conceivably provide a complete specification of the computational role that a neuron plays. This computational role is commonly referred to as dendritic integration. Alternatively, to highlight the fact that these computations are occurring at the cellular rather than the network level, the term single neuron computation is also used.

Current Technical Limitations

Theoretical considerations of dendritic integration (Mel, 1994) raise many interesting questions and possibilities about how a single neuron may respond to complex spatio-temporal patterns of input, which the cell surely receives in vivo. However, direct experimental investigation of these ideas is currently hampered by the inability to produce a precisely controlled pattern of multiple synaptic inputs. In short, limitations in controlling the input to the system under study impede the characterization of that system. Fundamentally, two classes of
methods are currently employed to stimulate neurons experimentally: electrical
stimulation and direct application of neurotransmitter.

Electrical stimulation is the most commonly employed experimental
technique, and generally takes one of three forms: 1) stimulation of presynaptic
cells or afferents (axonal bundles targeting the neuron under study), 2) direct
stimulation of a neuron’s dendrites by injecting current via a dendritic patch
clamp electrode, and 3) indirect stimulation of the dendrites via an extracellular
electrode.

Each of these has significant limitations. Local stimulation methods using
patch or extracellular electrodes provide good control of the synaptic inputs, but
stimulation at more than one or two sites is impractical to impossible.
Furthermore, these techniques are ultimately not physiological. In vivo, cells are
excited by the application of neurotransmitter that, through the activation of
postsynaptic receptors, gives rise to a cellular depolarization. These approaches
bypass the dendrite’s complex synaptic machinery in producing an artificial
depolarization.

In contrast, the stimulation of afferents leads to true synaptic stimulation
that is very physiological, and it can lead to multi-site stimulation of the neuron
under study. This technique suffers however, from the inability to precisely
control the timing, strength, and location of the synaptic inputs.

Because of these limitations, an important alternative to electrical
stimulation techniques is the direct application of neurotransmitter. This class of
techniques has several variants. The simplest approach is to use a puffer pipette;
application of a pressure pulse forces the release of contents from this
micropipette filled with agonist. Alternatively, using the technique of
iontophoresis, somewhat finer temporal control is obtained by using a current
pulse to generate fluid flow of the charged solution.

Both of these techniques are limited, however, in the spatio-temporal
resolution they can provide: ~100ms temporally and ~100μm spatially are
optimistic estimates (Katz and Dalva, 1994)\(^1\). Furthermore, stimulation at multiple
sites is difficult; two sites is a common upper limit (Cash and Yuste, 1998; Magee
and Cook, 2000).

**Optical Stimulation: Photolysis of Caged Compounds**

A third method for direct delivery of neurotransmitter—photolysis of caged
compounds—overcomes the spatio-temporal resolution limitations of these
techniques. In this technique, photolabile derivatives of neurotransmitters, such
as glutamate, are synthesized. These derivatives are, ideally, completely inert
and biocompatible (i.e. nontoxic) prior to activation by light, generally in the
ultraviolet (UV) range. Following illumination, the protecting, or “caging”, group is
cleaved, effectively releasing the neurotransmitter at the site of illumination. This
is illustrated for the case of caged glutamate in Figure 1-1.

Photolysis offers compelling advantages: it is less invasive (no
microprobes are required) and allows the delivery of compound to be faster

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\(^1\) The estimates of (Katz and Dalva, 1994) are likely somewhat too negative. Some authors
certainly seem to do better using puffer pipette (Magee and Cook, 2000) or iontophoresis (Cash
and Yuste, 1998) techniques. Nonetheless, photolysis clearly offers much greater spatio-temporal
resolution.
(<1μs) and more localized (<1μm). Given that the synaptic cleft can be estimated as ~.5μm² in area and that the concentration of neurotransmitter in the cleft is estimated to mostly decay within 200μs (Clements, 1996), it is clear that photolysis provides a superior physiological mimic of synaptic transmission than is possible using either iontophoresis or puffer pipette techniques.

Because of these advantages, photolysis of caged compounds has become widely used over the past decade. Examples can be found in the literature of the use of relatively wide-field illumination (~20-100 μm) using flash-lamp illumination (Kotter et al., 1998; Katz and Dalva, 1994), to more localized (~10μm) multimode and single-mode fiber illumination (Parpura and Haydon, 1999; Margulis and Tang, 1998), to highly focal laser photolysis using high-NA objective lenses (Schiller et al., 1998), which can be made diffraction limited (<1μm) with proper optics. Commercially available caging groups employed range in their peak absorption wavelength from 200-350nm (Haugland, 1996), but all are somewhat absorptive at the 337-364nm wavelength range of common laser sources (nitrogen at 337 to solid-state sources at 351-355 to argon ion at 351-364nm). These near-UV wavelengths are favored for their biocompatibility.
Photolysis of caged compounds, in short, allows physiologically realistic optical stimulation of neurons at high spatio-temporal resolution. This tool has been applied in several recent studies of dendritic integration. Kandler, Katz, and Kauer demonstrated that long-term depression (LTD) of a synapse could be elicited using photolysis (Kandler et al., 1998). Because no pre-synaptic neuron was involved, this allows an important conclusion to be drawn—the mechanism underlying the onset of LTD must be post-synaptic. In another work, Margulis and Tang leveraged the precise control over the timing of "synaptic" stimulation that photolysis allows to investigate directly the important problem of temporal summation at a single synaptic site (Margulis and Tang, 1998).

**Acousto-Optic Scanning**

Photolysis of caged compounds overcomes spatio-temporal resolution limitations of other direct neurotransmitter application techniques, and allows more precise control over synaptic inputs than can be achieved with electrical stimulation techniques. However, a thorough study of dendritic integration would require the ability to stimulate at multiple sites in a controllable spatio-temporal pattern. To achieve this, a laser can be used for photolysis and laser-scanning techniques can be employed to provide control over the location, timings, and strengths of synaptic inputs to a single neuron under study.

The most common laser-scanning technique used today consists simply of galvanometer-driven mirrors. This approach is straightforward, but its speed and flexibility are limited. Inertia limits the speed at which the laser can be repositioned, as each mirror (one for the X- and one for the Y-dimension) is
forced to traverse a linear path between the selected sites of interest, which, in
the study of dendritic integration, may be at nonadjacent locations. The fastest
available systems employ resonant galvanometers, but they only operate well
when continuously scanning in a linear pattern; they are not suited for “hopping”
to particular points in the field-of-view and remaining there for a programmable
time before hopping to the next site.

Acousto-optic (AO) scanning overcomes these limitations of galvanometer
based scanning by employing acoustic waves to steer a laser beam. In this
technique, an ultrasonic wave propagating through an optical quality crystal
effectively forms a phase grating: the alternating regions of compression and
extension in the wave give rise to a periodic modulation of the optical index-of-
refraction. By varying the frequency of the acoustic wave, the “grating constant”
can be tuned, which changes the angle of the laser light diffracted into the first
diffraction order. As shown in Figure 1-2a, the intensity of the light in the first
order can also be modulated, by tuning the amplitude of the applied acoustic
wave.

By employing AO laser scanning in microscopy, a focused laser spot can
be repositioned from any arbitrary location in the field-of-view to another in
<10μs. Thus, the technique is sometimes referred to as random-access
scanning, to contrast it with the techniques of frame scanning or line scanning
that are currently employed with galvanometer-driven mirrors, as shown in Figure
1-2b.
Figure 1-2 Acousto-optic Laser Scanning Microscopy a) Acousto-optic deflectors (AODs) steer a laser beam using an acoustic wave as a tunable phase grating. This “grating” deflects most of the incident light into the first diffraction order. The deflection angle is directly proportional to the acoustic wave frequency, while the intensity of the deflected light is controlled (nonlinearly) by the acoustic wave amplitude. The acoustic wave's frequency and amplitude are directly proportional to those of the applied RF wave. b) Raster scanning wastes time illuminating uninteresting sites. AODs support random-access scanning in which only sites of interest are illuminated, enabling much faster frame rates.

Bullen, Patel, and Saggau have employed random-access, acousto-optic laser scanning as a technique for multi-site optical recording of membrane potential in cultured neurons (Bullen et al., 1997; Bullen and Saggau, 1999). In their work, a visible wavelength laser (Argon) was used to excite a fluorescent voltage-sensitive dye (VSD) that stained the membrane of the cells. Random-access, AO scanning allowed a few sites of interest to be illuminated, and fluorescence simultaneously detected, at very high frame rates, where “frame” is taken to mean the time spent “sampling” each site of interest once before repeating the cycle again in the next frame. The frame rates achieved (1-10kHz) were sufficiently fast to allow physiological membrane potential transients, in the form of excitatory post-synaptic potentials (EPSPs) and action potentials (APs), to be followed. By judicious selection of the sites of interest, say several sites along a dendrite and perhaps its parent dendrites, these membrane potential transients can be traced as they propagate towards the soma. This approach is
thus a powerful means for investigating how electrical signals propagate under various conditions in the dendrites of a neuron—a central question in the field of dendritic integration.

*Fast, Multi-site, Graded Optical Stimulation*

The subject of this thesis is the recent work done to extend the capabilities of the existing visible AO laser scanning microscopy system for optical *recording* of living neurons, to include also a UV AO laser scanner for optical *stimulation* of neurons via photolysis of caged neurotransmitter. A Q-switched (pulsed) UV laser is employed as the light source. The UV AO scanner allows the spatial location and intensity of the laser pulses arriving at the specimen to be controlled. The repetition rate of the laser is 50kHz and the pulses are of sufficient energy such that a single pulse can elicit a wide range of physiologically significant levels of activation. With this repetition rate, up to 25 sites can be activated in 500μs; these stimuli can, from a physiological standpoint, be considered “quasi-simultaneous.” This, therefore, allows for the straightforward investigation of a neuron integrates multiple simultaneous synaptic inputs throughout the dendritic arbor—the fundamental problem of spatial summation that has hitherto been difficult to study.

The spatial resolution of the UV scanner is determined by the optics for focusing the laser beam and ultimately by the diffraction limit of light. Currently, the resolution achieved is <10μm. The temporal resolution of the photostimulation can be considered as <10μs, which is determined by the very fast pulse duration of the laser (~25ns) and the time constant governing the
uncaging reaction (<10μs for the caging group employed). Meanwhile, the 50kHz laser repetition rate, as mentioned, allows multiple sites to be quasi-simultaneously stimulated from a physiological point of view, and allows the timing between pulses at various sites to be adjusted with 20μs resolution.

Summarizing, the UV AO scanner that has been developed allows for fast, multi-site, graded physiological optical stimulation at high spatio-temporal resolution. This allows complex spatio-temporal patterns of synaptic activation to be programmed by the user, enabling a direct investigation of the many questions pertaining to dendritic integration, including, as examples, spatial summation, the location dependence of temporal summation, and the location dependence of neural excitability.

The Dual Scanner

The system that has been developed is referred to in this thesis, and in the author's laboratory, as the "Dual Scanner." This reflects the fact that it is an extension of the previously developed system for fast, multi-site optical recording. It contains two AO scanners, one for a visible Argon laser, and another for the Q-switched UV laser. The two sub-systems are referred to herein as the "UV scanner" and the "visible scanner." In sum, the system is suited for fast, multi-site optical recording and stimulation. Because of the light scattering of these laser sources, the system is currently best suited for the study of cultured neurons. In particular, the work done here has focused on cultured neurons of the hippocampal brain region.
This system developed will ultimately enable all-optical investigation of dendritic integration in cultured neurons, using both voltage-sensitive dyes and caged compounds in the specimen bath. The need for the various microprobes commonly used for electrical stimulation, electrical recording, and direct delivery of neurotransmitter is obviated. The experimenter can visualize the cell under study with the microscope, and select multiple sites for both recording and stimulation separately. In this way, the electrical activity at various sites in the neuron can be "imaged" in response to a complex "challenge" of realistic simulated synaptic stimulation. No other comparable approach for the study of dendritic integration is available today.

In addition to these strengths in investigating the physiology of neurons, the Dual Scanner was also designed to enable investigation of neurons' biochemistry as well. As such, the Dual Scanner was also designed to allow wide-field fluorescence imaging with a cooled CCD camera. Employing appropriate fluorescent markers, various biochemical, as well as morphological, processes can be dynamically visualized. Modifications to the Dual Scanner underway will allow simultaneous wide-field imaging and optical stimulation/recording. This will provide a powerful means of correlating the wealth of physiological data the Dual Scanner will provide with the underlying biochemical processes in the cells.

To date, preliminary tests have been done demonstrating the viability of the recently developed UV scanner. The temporal resolution and ability to grade the UV stimulation are excellent, while the spatial resolution achieved (<10μm) is
comparable to that in the existing literature. Efforts are underway to reduce it to  
<2μm, where the stimulus can be considered nearly monosynaptic (i.e. affecting the receptors of only a single synapse).

*Outline of this Thesis*

The next chapter will provide an overview of dendritic integration in the hippocampus, discussing some of the remarkable findings of the past decade as well as the considerable need and opportunity for the Dual Scanner to resolve some outstanding issues. Chapter 3 will provide an overview of the system apparatus, while Chapters 4 and 5 will further discuss the optical and electronic components of the system, respectively. Attention will mostly be focused on the UV scanner, with some discussion as well of the visible scanner. Chapter 6 presents preliminary data demonstrating the viability of the UV scanner. Chapter 7 offers concluding remarks and some discussion of future directions.
CHAPTER 2  
DENDRITIC INTEGRATION IN THE HIPPOCAMPUS

The purpose of the Dual Scanner is to investigate dendritic integration in hippocampal pyramidal nerve cells. In this chapter, an overview is provided of the progress in understanding the computational functions of single neurons and the important role that dendrites play. Introductions to the anatomy and the physiology of the hippocampus are provided. This enables a discussion of recent advances in understanding dendritic integration in the hippocampus as well as the role that the Dual Scanner can play in addressing important unresolved issues.

2.1 SINGLE NEURON COMPUTATION (DENDRITIC INTEGRATION)

In 1943, McCullough and Pitts showed that a neural network comprised of "simple" neurons that simply added their weighted inputs and performed a thresholding operation could be used to perform any computational task, given enough such neurons. This powerful notion—the neural equivalent of a Turing machine—combined with the experimental inaccessibility of the elaborate dendritic trees of central neurons led to the view that dendrites were "uninteresting"—they merely served to increase the surface area of a neuron, much like the arborization of trees. All the computation of the cell, the hypothesis went, occurred in the soma, and it was liable to be simple. In 1957, Rall pointed out that even the most "uninteresting" dendrites—those that could be simply viewed as a linear circuit consisting of constant capacitances and resistances—would have significant effects on the process of dendritic integration. Specifically, the "cable theory" he developed for neurons showed that more distal inputs
would be attenuated and filtered relative to more proximal inputs. This simple finding did much to increase interest in the role of dendrites, but experimental progress was still, comparatively at least, slow over the next decades.

Subsequent ideas, such as “passive normalization” (Rall and Rinzel, 1973) or the “boosting” of inputs by active (i.e. voltage- and/or time-gated) channels (Miller et al., 1985), were often directed at finding mechanisms to counteract the disparity in synaptic inputs across the dendritic tree, in order to restore the “simple” neuron paradigm.

2.1.1 Ideas and Evidence of Single Neuron Computation

Simultaneously, a countervailing notion appeared that single neurons themselves could carry out more complex computations than simply adding and thresholding their varied inputs. Noting the widely varying dendritic geometries present in different regions of the CNS, many authors stressed the role of dendritic geometry in mediating a neuron’s function. Since the 1970s, anatomical data has revealed patterns of synaptic organization that further support this notion: different regions of dendritic trees often receive their synapses from functionally distinct inputs (for review by brain region, see: Shepherd 1998). As an example, a hippocampal pyramidal neuron in the CA1 region receives inputs from the CA3 region, from the entorhinal cortex, and recurrent connections from other CA1 neurons; inputs from each of these areas targets a specific region in the dendritic arbor. Such patterns have given rise to ideas that individual branches performed local computations; each branch could form a local AND or OR gate, for example, depending on the strength of the synapses there. The
same could be said of two daughter branches of a parent dendrite. Adding the role of shunting inhibition—inhibitory inputs with large conductances that effectively damp out depolarizing activity—to this model produces a NOT operation that can veto inputs on a particular branch or on daughter branches (Figure 2-1). Under this dendrite-as-computer metaphor, each branching level of the dendritic tree corresponds to a layer of logical gates. Besides the passive effects of dendritic geometry and the patterns of synaptic placement, the possible importance of active conductances has long been appreciated (Rall and Shepherd, 1968), and it was noted as early as 1971 that dendrites might serve to compartmentalize local “nonlinear processing” (Llinas and Nicholson, 1971). The local nonlinearities could serve to produce multiplicative operations or to produce local thresholding that would render the AND/OR/NOT operations described more “digital” (Mel, 1999).

Taking a step back, it’s clear that the idea that individual neurons may subserve complex computational functions has been closely intertwined with the
growing sense that the role of dendrites is more complex than originally imagined. Thus, the emergent fields of “single neuron computation” and of “dendritic integration” are practically one and the same. What evidence, if any, is there to support any of these notions?

There are examples in which synaptic placement and dendritic geometry are known or suspected to belie neuronal function. The directional selectivity (DS) of retinal ganglion cells has been proposed to arise from a specific alternating placement pattern of excitatory and inhibitory synapses on the same dendrite (Koch et al., 1982). Experimental evidence in support of this hypothesized dendritic processing was just recently obtained (Taylor et al., 2000). In the auditory brainstem, meanwhile, models based on experimental evidence show that dendrites act as high-quality temporal coincidence detectors to allow the delay in hearing between the two ears to be detected, a mechanism likely employed for auditory direction sensing (Agmon-Snir et al., 1998).

Meanwhile, it is suggested that properly placed modulatory synaptic inputs could effect the multiplicative gain factor seen in a variety of systems, including the boosting of the orientation-tuning curve of V4 neurons of visual cortex by attentive gazing (McAdams and Maunsell, 1999).

Fewer concrete examples exist of the role that active conductances can play in effecting local “nonlinear processing.” Binocular disparity tuning in the visual cortex of cats exhibits great spatial resolution, much finer than the size of the receptive fields that has been mapped in the retinal ganglion. It has been proposed that individual dendritic branches in VC cells combine the inputs from
the left and right lateral geniculate nuclei (LGN) to create local binocular disparity sensors in individual pyramidal cells; the mechanism proposed is a local "energy" sensor (a multiplier) dependent on active conductances (Mel et al., 1998). In the locust, a multiplicative relation is shown to accurately characterize the response of the lobular giant motion detector (LGMD) neuron that acts as a "looming" (danger) sensor (Gabbiani et al., 1999). What biophysical form might underlie such multiplicative operations? One author has suggested that a combination of "synaptic clustering"—the preferential grouping of oft-correlated inputs onto a particular dendritic branch—and the presence of local active conductances in such a combination to effect a soft or hard threshold operation could provide the explanation (Gabbiani et al., 1999).

In spite of these many examples, there has not yet been, to this author's knowledge, any definitive evidence of active conductances promoting local nonlinear processing essential to a cell's function. However, active conductances definitely promote local nonlinear activity in the form of dendritic spikes—strong, transient depolarizations that are initiated in the dendritic arbor. Evidence of this phenomenon came as early as the 1950s using extracellular field recordings (Cragg and Hamlyn, 1955), and was among the first indications of the presence of active conductances in dendrites. More recently, they have been directly observed using dendritic patch clamp recordings (for example: Stuart et al., 1997). Interestingly, it is found that the spike itself does not propagate efficiently into the soma/axon. The depolarization achieved there is, however, greater than it would have been without the spike generation; thus, the dendritic spike may
represent a significant boosting of the localized synaptic inputs that caused it. In other words, dendritic spiking may represent a localized nonlinear thresholding operation that combines with other computational subunits to produce the neuron's final output.

Finally, there is another important thread of evidence supporting the hypothesis that dendrites are more than merely passive integrators: the recent direct observations of back propagating action potentials (BPAPs) (Stuart and Sakmann, 1994). The active conductances of dendrites in many neuron types support the propagation of this information that the cell has just fired. The BPAP may then act to suppress synaptic inputs (and thus limit further firing) by shunting them (Stuart and Hausser, 1998), but it also could provide a Hebbian-style associativity mechanism that enables persistent synaptic modifications (Magee and Johnston, 1997) (to be discussed below). Furthermore, the frequency-dependence of BPAP propagation and on the Ca$^{2+}$ transients it produces locally at synapses adds frequency tuning and filtering to the myriad computational roles that single neurons can play.

2.1.2 The "Year of the Dendrite"

The past decade can reasonably be considered as a renaissance in the field of dendritic integration. The introduction of infrared differential interference contrast (IR-DIC) microscopy enabled the development of dendritic patch clamp recording techniques (Stuart et al., 1993); this has permitted direct electrical access to the dendrites, in spite of their small size. Meanwhile, the growing popularity of confocal microscopy (1995) and the advent of two-photon
microscopy (Denk et al., 1990) have allowed unprecedented optical access to the dendrites, enabling high-resolution of Ca$^{2+}$ dynamics and morphological studies at the level of dendritic spines—i.e. at the level of single synapses! This combination of techniques has led to an explosion of results about dendrites in all regions of the brain.

After many years of speculation, a great deal has been learned in a very short time regarding the active conductances in dendrites, with detailed findings regarding the densities and locations of Na$^+$, K$^+$, and Ca$^{2+}$ conductances throughout the dendritic trees of various central neurons. As previously mentioned, the presence of dendritic spikes has been directly measured, as has the propagation of EPSPs through the dendritic tree using dual patch recordings. The role of dendritic spines as biochemical compartments for localizing Ca$^{2+}$ transients has been largely established by optical imaging of diffusion time constants (Svoboda et al., 1996). And finally, as mentioned, there has been the direct observation of BPAPs and their likely role in synaptic plasticity.

This wealth of data has done much to support the notion of single neurons as complex computers, but has done little to help ascertain the nature of the computational role of any particular neuron type. The cases where hypotheses can be ventured, many of which were described above, largely pertain to sensory systems where synaptic inputs can be controlled by changing the external stimuli.
2.1.3 Dendritic Integration in the Hippocampus

In the case of non-sensory neurons, such as those in the hippocampus, elucidating computational roles is severely hampered by the lack of techniques for directly manipulating synaptic inputs. Furthermore, gathering evidence to advance the localized non-linear computation hypothesis is greatly hampered by the current difficulty of recording from multiple locations within a neuron. The aim of the Dual Scanner project is to address these two serious limitations to aid in the investigation of dendritic integration in hippocampal pyramidal cells by allowing for multi-site stimulation and recording at high spatio-temporal resolution.

As will be discussed next, the hippocampus is an attractive area for study due to its well-established role in learning in memory, its well-mapped anatomy, and recently, over the past decade, the wealth of information available about the detailed physiology of both its principal cells and its interneurons. The accumulation of data has allowed some striking (but tentative) conclusions to be drawn, like the apparent linearity of spatial and temporal summation observed throughout the dendritic tree of CA1 pyramidal neurons and the seeming elucidation of a sharp temporal coincidence criterion underlying synaptic modification. These findings will be discussed in some detail, as well as some of their limitations.

Possible experiments with the Dual Scanner could refine these findings as well as offer access to new experimental regimes. In particular, the multi-site photolysis capability is well-suited for the study of spatio-temporal summation—
the integration of inputs that need not be either spatially or temporally coincident. An understanding of the physiology of the hippocampus shows that this intermediate regime between pure spatial and temporal summation may be particularly relevant. Meanwhile, the multi-site recording capability permits the visualization of localized activity such as dendritic spiking and BPAPs, which may have significant bearing on the computational function of the neuron. Putting these together, the ability to simultaneously control the spatio-temporal stimulus and to record activity locally in the dendritic arbor offers exceptional flexibility in investigating the increasingly apparent and complex synergism between local activity and synaptic activation.

2.2 OVERVIEW OF THE HIPPOCAMPUS

The hippocampus is among the most widely studied regions of the brain. It is conveniently identified and dissected, and has a fascinating, well-characterized laminar structure that dates to the work of Ramon y Cajal at the end of the 19th century. It is also known to play a pivotal role in the area of learning and memory, as evidenced by the well-known case of patient HM who has been unable to form new long-term memories since undergoing a bilateral hippocampectomy in the 1950s (Scoville and Milner, 1957). Furthermore, the hippocampus has been identified as the principal or at least a significant locus of various disease states. Most epileptic seizures occur in the hippocampus, which is thought to have the lowest seizure threshold of any brain region. Meanwhile, Alzheimer's disease typically involves significant neurodegeneration in the hippocampus and
hippocampal formation. Finally, the hippocampus is of further interest clinically because it is especially vulnerable to ischemia and anoxia.

2.2.1 Anatomy of the Hippocampus

The hippocampus is considered part of the limbic system, which generally speaking is concerned with emotion-laden or drive-related behaviors and responses to sensory inputs. The limbic structures are interposed between the hypothalamus, the seat of involuntary responses to stimuli, and the neocortex, the seat of voluntary responses. As shown in Figure 2-2, the hippocampus itself consists of two “cashew” shaped structures—one in each brain hemisphere. The lower tip is located near the temporal lobe and is referred to as the temporal pole, while the upper tip resides near the septal nuclei and is called the septal pole. The curved line connecting these poles is deemed the septotemporal axis.

Although there are variations in the structure and cell behavior along this

![Figure 2-2 The Hippocampal Formation](image)

*Figure 2-2 The Hippocampal Formation* The hippocampal formation in each hemisphere is ashew-shaped, following a C trajectory from the lower temporal lobe along the cingulate gyrus towards the septal pole. The thin extension beyond the septal pole is the *fornix*. Sketch from (Amaral,D & Witter, MP; 1995); photograph courtesy of G. Faas.
axis, to a large extent, sections cut normal to the septotemporal axis all show similar structure and behavior. The characteristic interlocking-C structure of these “slices” is shown in Figure 2-3. As seen here, there are three main fields of principal neurons, those of the dentate gyrus (DG) and those from two areas of the cornu ammonis (CA), namely CA1 and CA3. Beyond the CA region is a diffuse area of neurons referred to as the subiculum (S).

Roughly speaking, information flows one-way through the hippocampus. Fibers enter the hippocampus from the entorhinal cortex (a region of the neocortex). They cross (perforate) the subiculum to reach the cells of the dentate gyrus. The axons of these cells collect to form a tract known as the mossy fibers. These in turn project to innervate the more proximal area of the CA region, known as the CA3 region. The efferents (i.e. outgoing bundle of axons) of the CA3 region collect to form the Schaffer collaterals, which innervate the more distal cells of the CA1 region. Summarizing, information loosely flows through a "trisynaptic pathway" consisting of the peforant path, the mossy fibers, and finally

![Figure 2-3 Hippocampal Slice](image)

Transverse cut along septotemporal axis reveals characteristic "interlocking C" structure of the hippocampus. Cell bodies are in dark regions. The "trisynaptic pathway" consists of (1) the perforant pathway (PP) from the entorhinal cortex (EC) to the granule cells of the dentate gyrus (DG), (2) the mossy fibers (MF) from the DG cells to the pyramidal cells of the CA3 region, and (3) the Schaffer collaterals (SC) from the CA3 pyramidal neurons to the CA1 pyramidal neurons. These finally project, in large part, back to the entorhinal cortex—thus a large "loop" is formed from the neocortex to the hippocampus and back to the neocortex.
the Schaffer collaterals.

The “final” CA1 neurons project into the diffuse subiculum, which sends some projections back to the source—the entorhinal cortex—and others into a collection of fibers known as the fimbria that propagates along the septotemporal axis and then beyond the septal pole into the **fornix**, which can be seen in Figure 2-2. The fornix is often considered the primary output site of the hippocampus, and it projects to a variety of areas including the hypothalamus, thalamus, and the basal ganglia.

Beyond the highly linear pathway described, there are a large number of other connections in the hippocampus, including **associational** projections that connects cells at various locations along the septotemporal axis, **commissural** projections that connect the two hemispheres, and **recurrent** connections which refer to projections from one area back onto itself.

In addition, interspersed throughout the dendritic arbors of the principal cells in each region (the “pyramidal cells in the CA regions, and the “granule cells” in the DG), are a large number of **interneurons**. These neurons provide inhibition, often synchronized (see below) throughout the hippocampus.

Taking a step back, it can be seen that the pathway through the hippocampus and through the regions it targets leads back to the entorhinal cortex where the pathway began. Thus, the entire system can be considered to form a loop—known as the **Papez circuit**. In sum, it can be loosely though that the hippocampus receives information from the neocortex, transforms it in some way, and then sends it back.
2.2.2 Plasticity and Memory Formation in the Hippocampus

Since the pioneering work of Lomo and Bliss, the phenomenon of long-term potentiation (LTP) in the hippocampus has drawn considerable attention (Bliss and Lømo, 1973). In particular, it has been studied extensively as a possible validation of the hypothesis of Donald Hebb—namely that “when...cell A...excite[s] cell B or repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased” (Hebb, 1949).

LTP is characterized by a persistent modification in the strength of a particular synapse, lasting hours to days. It is sometimes divided into “early-LTP” (also known as short-term potentiation (STP)) and “late-LTP” referring to changes that last for under an hour, and those that last much longer, and it can certainly be further subdivided. Both are long-term poteniations in relation to other, much shorter-lived, potentiation phenomena such as facilitation or post-tetanic potentiation (PTP). Induction of LTP is generally achieved by a burst or a train of bursts of high-frequency stimulation (~50-100Hz in most cases). More recently, so-called pairing protocols have been employed in which lower frequency (and arguably more physiological) pre-synaptic stimulation of a cell is paired with post-synaptic depolarization or spiking.

There has long been evidence to suggest that LTP occurs in vivo during the presence of theta oscillations, which are known to occur in the hippocampus during exploratory behavior in rodents (Buzsaki et al., 1983), during memory tasks in humans (Kahana et al., 1999), and during the REM sleep state (Lancel,
1993). Theta rhythms are generally classified as occurring in the narrow 6-8Hz frequency range. During theta activity, there are periodic bursts of high-frequency gamma activity (30-100Hz), which often appears phase-locked to the slower theta rhythm. It is generally thought that the pyramidal cells and interneurons are externally induced to operate on a theta rhythm, by an incompletely understood mechanism that appears to hinge on cholinergic activation (i.e. release of the neuromodulator acetylcholine) from the septal nuclei (Buzsaki, 2002).

A powerful hypothesis then is that, during theta activity, the hippocampus receives information from the neocortex and forms temporary memories. It is supposed, then, that during periods not of theta activity, referred to as “slow wave” activity, the hippocampus sends outputs to the neocortex that serve to consolidate the memories there for “permanent” storage. This hypothesis receives compelling support from experiments suggesting that temporal patterns of bursting activity observed during theta rhythms are “re-played” at a faster rate during slow wave activity (Nadasdy et al., 1999).

2.3 DENDRITIC INTEGRATION IN THE HIPPOCAMPUS

The significance of the hippocampus in learning and memory is, thus, the motivations for investigating the role of single neurons in the hippocampal network. Moreover, the various phenomena of persistent synaptic modification, including LTP, argue for a significant role for individual neurons. Focusing exclusively on the question of how the hundreds to thousands of excitatory inputs at a given cell are integrated (i.e. not considering the role of the extensive inhibitory network of interneurons), there are three important factors: passive
mechanisms due to dendritic morphology/geometry, active mechanisms arising from the various voltage-gated conductances, and mechanisms at the synapses themselves including receptor distribution and pre-synaptic factors. Research into each of these areas has greatly accelerated this past decade with the advent of improved experimental techniques, leading to considerable advances in our understanding of dendritic integration. Brief summaries of some important recent findings are given here to provide a flavor for the “state of the art” of understanding dendritic integration in the hippocampus.

2.3.1 Location Dependence of Synaptic Inputs

Taken all together, much of the work of the past decade strongly suggests that the post-synaptic response of CA1 pyramidal neurons to its many (thousands) of synaptic inputs, is independent of synapse location. These findings were recently reviewed (Magee, 2000). Three facets of location independence of synaptic input can be distinguished. First, it is found that the EPSP evoked at the soma by unitary synaptic transmission (i.e. single site, single event stimulation) is the same regardless of where in the dendritic tree the stimulus occurs (Pettit and Augustine, 2000). Second, the time-course of the EPSPs evoked at the soma is also the same, regardless of synaptic location. As a consequence of this, no effect of temporal summation (either supra- or sublinear) is found throughout the dendritic tree (Magee, 1999). Finally, the summation of spatially distributed synaptic inputs (spatial summation) is also found to be, to a great extent, location independent (Cash and Yuste, 1999).
The normalization of synaptic inputs depends on several dendritic properties—passive properties, active properties, and synaptic properties. Using "cable theory," it is found that a purely passive cell—one with no voltage-gated conductances—would cause distal inputs to be significantly attenuated and broadened upon reaching the soma. On the other hand, the large input resistance $R_{in}$ at distal dendrites owing to the smaller branch sizes, could conceivably compensate for the cable effects by promoting large, local EPSPs at distal dendrites; this "passive normalization," however, is not found to be sufficient to compensate for cable effects (Rall and Rinzel, 1973; Rinzel and Rall, 1974; although, see Jaffe and Carnevale, 1999). It had long been presumed that the role of voltage-gated conductances in the dendrites was to boost distal inputs (Anderson et al., 1987); but the recent findings that the primary changes in channel distribution are an increased density of hyperpolarizing currents such as $I_h$ and A-type $K^+$ channels in the distal arbor argue against this possibility. An increase in synaptic weight, it was recently found, appears to cause normalization of unitary EPSPs (Magee and Cook, 2000); this effect, at least in CA1 pyramidal neurons, is likely to be mediated at the post-synaptic cell (Pettit and Augustine, 2000). Meanwhile, voltage-gated conductances—in particular the $I_h$ conductance—appear to play an important role in normalizing the decay kinetics, and consequently the temporal summation, throughout the dendritic tree (Magee, 1998). Finally, a delicate balance between voltage-gated conductances and receptor types appears to underlie an observed linearity of spatial summation; nearby inputs do not appear to significantly "shunt" one another, at
least to the extent cable theory predicts (Cash and Yuste, 1998; Cash and Yuste, 1999).

A picture that is now gaining currency, then, is that the many hundreds to thousands of excitatory inputs to a hippocampal pyramidal neuron (and perhaps also to neocortical pyramidal cells, for that matter) all have an equal ability to cause the cell to fire. Of course, the neuron’s plasticity allows the strengths of individual synapses to be altered; but this spatial distribution of synaptic weights does not, it appears, include any “pre-existing” pattern of synaptic strengths. Some simple modeling studies have suggested that such a location-independent synaptic topology would best support a high-fidelity associative memory network, which may be a functional role of the hippocampal network (Cook and Johnston, 1997). Furthermore, the normalization of EPSP decay times is consistent with the notion that spike timing is the means by which neurons encode information (Mainen and Sejnowski, 1995).

As tantalizing as the notion may be, the evidence for location independence is not entirely definitive. The idea that local computations may occur on individual dendritic branches has hardly been eclipsed, particularly in other cortical areas such as visual cortex (for review, see: Mel, 1999).

2.3.2 Locus of Long-Term Potentiation

The chemical or physical locus of persistent synaptic modification has long been a source of controversy; in particular, it was asked, is the change in synaptic weight effected pre- or post-synaptically? This debate, it is argued, may be coming to a close with the unifying notion of a “silent synapse.” The idea is
quite simply that the primary mechanism for modifying synaptic strength is controlling the number of AMPA receptors at that synapse. The term “silent synapse” arises because it is found that some identified synapses (via tell-tale protein markers) show no apparent response to stimulation. This is readily explained: they possess only NMDA receptors, which are inactive at small depolarizations, and no AMPA receptors. Persistent activity coupled with a BPAP can produce a depolarization sufficiently large to release the Mg\textsuperscript{2+}-block on NMDA receptors, allowing a sharp Ca\textsuperscript{2+} influx that triggers the insertion of new AMPA receptors. In summary, then, the mechanism for LTP is post-synaptic; the “silent synapse” concept can also, however, conveniently explain the reductions in synaptic transmission failures that have previously been used to argue that pre-synaptic modifications (i.e. changes in transmission probability or quantal content) underlie LTP (Kullmann and Nicoll, 1992; Larkman et al., 1992).

Encouragingly, the simple idea that potentiation arises simply from the insertion of new AMPA receptors provides a straightforward explanation of the complementary phenomenon of long-term depression (LTD). Evoked in the lab through either low-frequency (~5Hz) bursts of activity or pairing protocols with a particular timing relationship, LTD represents a persistent down-regulation of a synaptic weight. The existence of LTD as a counterweight to LTP presumably gives synapses considerable dynamic range to alter their strengths free of saturation effects, although it has been observed that depressing a synapse following the onset of late-LTP can be difficult (Staubli and Scafidi, 1999).
2.3.3 Temporal Window of Activation

Another recent finding that appears to synthesize years of research is the apparently narrow temporal window (~20ms) of activation that determines whether particular synaptic connections will be potentiated or depressed. This finding grew out of the finding that LTP could be induced using a novel protocol (pairing protocol) in which synaptic activation of a cell was paired with an induced BPAP in that cell. This was observed both in hippocampal CA1 neurons (Magee and Johnston, 1997) and in neocortical pyramidal cells (Markram et al., 1997).

Subsequent work by Bi and Poo in dissociated cultures of hippocampal neurons uncovered the sharp temporal coincidence requirement that apparently governs this potentiation (Bi and Poo, 1998). They identified interconnected glutamatergic cells in their culture and subjected both the pre- and post-synaptic cell to a low-frequency (1Hz) train of spikes, and varied the relative phase of the two spike trains. When the pre-synaptic spikes preceded the post-synaptic spikes by <20ms, LTP was induced; when the post-synaptic spikes came first, however, again by <20ms, LTD was induced instead. Thus synaptic modification requires that the pre- and post-synaptic spiking be temporally coincident to within ~20ms, with the precise order of the spiking determining whether the change is an increase or decrease in the synaptic strength. The particular order observed is remarkably consistent with the Hebbian hypothesis for plasticity: when the EPSP precedes the BPAP (i.e. the pre-synaptic spike precedes the post-synaptic one), it conceivably helped to induce the post-synaptic cell to fire. Indeed, the data of Bi and Poo includes supra-threshold synaptic connections—those which caused
the post-synaptic cell to fire, without requiring the post-synaptic current injection—among the “positively correlated” spiking patterns that produced LTP.

This remarkable finding, like that of the “silent synapse” concept that has led to emergence of AMPA receptor number as the controlling mechanism of synaptic strength, does much to unify the phenomena of LTP and LTD. Because of the difficulty of precisely controlling the timing of both the EPSP and the BPAP in a slice preparation, further work confirming the existence of this temporal window in living hippocampal tissue has yet to appear. However, a strikingly similar window was observed in one living tissue preparation: the developing visual system of the frog at the converging retinotectal synapses (Zhang et al., 1998).

In spite of the lack of definitive evidence for such a “temporal window” in a hippocampal brain slice, the idea that LTP induction is dependent on coincident pre- and post-synaptic spiking with a strict timing requirement is gaining widespread acceptance. In particular, it appears that LTP in some hippocampal regions only arises during bursting behavior (i.e. multiple spiking events) in adult animals, whereas single correlated spiking events might be sufficient in developing animals (Paulsen and Sejnowski, 2000). This observation, along with the fact that bursting is seen to occur during both theta and slow-wave activity, lends credence to the notion that firing patterns may comprise temporal codes.

There is ample evidence that LTP induction is, at least in some cases, associative or cooperative. In conjunction with the notion that a simultaneous BPAP is required for LTP, it is presumably required that several synapses onto a
particular neuron must conspire to cause that cell to fire in order to induce potentiation (or depression) in some or all of the participating synapses. This phenomenon is termed cooperativity; meanwhile, the term associativity has been used to refer to a restricted subset of cases where potentiation of weaker synaptic input was dependent on nearly coincident activation of stronger synaptic inputs (Barrionuevo and Brown, 1983). This conception may now be dated by the notion that the BPAP acts as a “global associative signal” (Paulsen and Sejnowski, 2000). In other words, there may be no preference for the simultaneous activation of any particular pairing of synaptic inputs (or groups of inputs to, say, a particular dendritic branch) given the emerging notion that all synaptic inputs are “created equal.”

2.4 INVESTIGATING DENDRITIC INTEGRATION IN THE HIPPOCAMPUS WITH THE DUAL SCANNER

Experimental advances of the past decade have allowed questions regarding dendritic integration and single neuron computation in the hippocampus to be addressed much more directly. The Dual Scanner is designed to complement these approaches, providing exceptional flexibility in eliciting and recording neural activity in dissociated hippocampal cultures. Here a discussion is provided about the strengths and limitations of dissociated hippocampal cultures. Then, a smorgasbord of experiments envisioned with the Dual Scanner are discussed.
2.4.1 Dissociated Hippocampal Cultures

Both the voltage-sensitive dye imaging and the UV photolysis aspects of the Dual Scanner are best carried out using a "thin" preparation that does not scatter much light. Dissociated hippocampal cultures consist of a monolayer (or sometimes, a few layers) of cells grown directly on optical cover glass. The cells are optically accessible, in contrast to brain slices in which both imaging and photolysis would need to occur through >100μm of highly light-scattering tissue. Because of this significant advantage, with the present configuration of the Dual Scanner, hippocampal cultures are employed almost exclusively. Some discussion of this model system is provided here.

The cultures employed are raised by a methodology close to that developed by Brewer et al (Brewer et al., 1993). Cells are obtained by dissociation from a developing but clearly formed hippocampus in late-embryonic rats. They are then plated and raised in a serum-free medium optimized for the survival of cultured cells. Eliminating serum, a common component of many cell cultures, provides three advantages: 1) the growth of glial cells (neuron support cells) is suppressed, 2) the density of cells can be kept low, and, most importantly, 3) the strong background fluorescence due to the uptake of voltage-sensitive dye by serum debris is avoided. The result is a "clean" system of mostly neurons, spaced sufficiently so that individual neurons can be easily identified and studied. The cultures employed with the Dual Scanner are typically plated at an even lower density than that of Brewer et al, down to <50 cells/mm². By reducing the cell density, the number of processes (esp. axons) is considerably
reduced, thereby limiting the amount of non-specific fluorescence that can contaminate attempts to optically record from specific processes.

Once plated, the cells retain their in vivo phenotype—the cells had already differentiated into pyramidal neurons or interneurons and develop the appropriate morphologies and other properties (e.g. glutamatergic or GABAergic transmission). They also form new synaptic connections, and do so in a predictable stages following plating (Benson and Cohen, 1996), suggesting that developmental regulation mechanisms controlling the growth of the dendritic and axonal arbors remain in place. Voltage-gated conductances including Na\(^+\), Ca\(^{2+}\), and K\(^+\) conductances all appear throughout the dendritic arbor (Masukawa et al., 1991), so fundamental patterns of channel expression remain unaltered.

It is important to note some of the limitations of dissociated cultures as a model system. First, the cells no longer participate in the well-defined hippocampal network—pyramidal cells can be identified, but cannot be identified as either CA3 or CA1 cells, for instance. Furthermore, the cells are restricted to a two-dimensional substrate, in contrast to the three-dimensional arborizations present in vivo. Consequently, the cell morphology and patterns of receptor and channel expression do not exactly match the in vivo hippocampus.

Nonetheless, there are several indications that cultured hippocampal neurons remain an appropriate experimental model. Although the channel distributions employed were slightly different, studies in both cultures and brain slices showed that a remarkable balance between active and passive effects gives rise to linear spatial summation (Cash and Yuste, 1998; Cash and Yuste,
1999). It has been found that the induction of LTP or LTD is possible in cultured neurons, following the same rules for temporal coincidence of pre- and post-synaptic activity that appear to apply in brain slices (Bi and Poo, 1998). Other significant physiological phenomenon, such as depolarization-induced suppression of inhibition (DSI), likely mediated by the release of endogenous cannabinoids have also been documented and studied in cultures (Ohno-Shosaku et al., 1998; Ohno-Shosaku et al., 2001). Morphological changes such as spine formation in response to conditions favoring plasticity (NMDA receptor activation) have also been observed (Goldin et al., 2001).

In summary, dissociated hippocampal cultures are clearly only a model system, but a still-growing body of evidence suggests that they possess most of the salient features of their in vivo counterparts. They are indispensable for the work here, as they are especially suited for single neuron studies with the Dual Scanner. Cultured neurons are also especially suited for the study of biochemical and molecular process within living cells. Recently, for example, mRNA transport and dendritic protein synthesis was dynamically visualized in cultured hippocampal neurons (Smith et al., 2001). Indeed, the combination of the Dual Scanner’s strength in investigating physiology with the host of techniques for investigating molecular biology in cultured neurons constitutes a compelling experimental system for investigating the function of single neurons.

2.4.2 Proposed Experiments for the Dual Scanner

Approaches used to date for investigating Single Neuron Computation have often been hampered by limitations in spatial or temporal resolution, or the
number of sites that can be recorded and/or stimulated simultaneously. The Dual Scanner addresses these limitations, by allowing complex spatio-temporal patterns of activation at high resolution, as well as the recording of activity, particularly spiking activity such as BPAPs and dendritic spikes, at multiple sites throughout the dendritic arbor. The Dual Scanner can serve to address limitations in prior studies of spatial and temporal summation by virtue of its greater resolution of stimulation (<2μm spatial (expected, after modification), ~20μs temporal). Furthermore, the Dual Scanner, rather than investigating the processes of spatial and temporal summation as separate phenomenon, enables the study of the more general problem of spatio-temporal integration. This includes, for example, the combination of spatially distributed inputs at slightly offset times or the combination of temporally displaced stimuli at slightly offset positions.

By combining a fine control of synaptic stimulation with the ability to record local summation and local spiking events, the compelling hypothesis of compartmentalized computation in the dendritic arbor (Mel, 1999) can be directly investigated. Finally, by employing pharmacological approaches to eliminate the effect of active conductances, the Dual Scanner enables the direct measurement of a neuron’s passive properties, and the verification of the tenets of the “cable theory” used to describe passive neurons. Brief descriptions of several specific aims are provided here.
Revisiting Temporal Summation

Passive cable theory predicts sublinear temporal summation: an input shortly following a prior depolarization should be less effective owing to shunting and a reduced driving force. How does this temporal interaction depend on spatial parameters? Passive cable theory suggests that nearly coincident inputs to distal sites would interact more when summing at the soma than those originating from more proximal sites. This is a simple consequence of the filtering (broadening) of distal inputs. On the other hand, the input resistance at distal sites is much greater, thus reducing the effective membrane time constant there by as much as ten times (Segev and London, 1999); this has the effect of significantly decreasing the interaction between inputs by shortening the EPSPs themselves. Summarizing, passive cable theory predicts that interaction between inputs is minimal at the dendritic site of initiation, but that the interaction grows as the EPSPs propagate toward the soma. The interaction itself, following passive cable theory, should always be sublinear.

A recent study which stimulated hippocampal cultures via photolysis through a single mode optical fiber found such a sublinear relationship when two inputs were separated by ~10-50ms, but also found that when the inputs were more coincident (<10ms), the summation was supralinear (Margulis and Tang, 1998). In other words, temporal summation appears to follow a tri-phasic relationship, in which active nonlinearity (boosting) dominates over short time scales, and passive nonlinearities (suppression by shunting and reduced driving
force) dominate over longer time scales. At time scales beyond ~50ms, there was no significant interaction between the two inputs.

This study, however, did not investigate the dependence of this temporal summation on the location of the input. Nor did it examine the local temporal summation at the site of stimulation. The study by Magee discussed earlier investigated these issues using dendritic patch clamp techniques, revealing temporal summation in CA1 pyramidal neurons to be location-independent (Magee, 1999). While in some cases it was sublinear and others supralinear (depending on stimulation frequency), the summation at the soma (and, for the most part, locally at the dendrites) was invariant with respect to stimulation site. This surprising result was attributed to the gradient of $I_h$ channels. The dominant interaction, when it was revealed by an $I_h$ blocker, was the active interaction—high frequency stimulation (>50Hz) yielded strong supralinear summation, whereas lower frequency stimulation produced only a mild sublinear summation.

The Dual Scanner would allow the strengths of both these studies to be combined. The uncaging stimulus would be as physiologically realistic as in the Margulis/Tang study, while the multi-site stimulation/recording capabilities would match the strengths of the Magee study. Does the location-independence of temporal summation still hold true for synaptic, rather than current injection, inputs? The visible scanner would allow the visualization of activity throughout the dendritic arbor, allowing the experimenter to see at exactly which points the two EPSPs are interacting—at the dendrite, at the soma, or somewhere in between? Furthermore, the Dual Scanner would enable an enable an important
extension of these works: the study of temporal summation for inputs that are slightly spatially offset. Specifically, across what spatial length scale does the Margulis/Tang tri-phasic relationship hold?

Revisiting Spatial Summation

The high spatial and temporal resolution of the Dual Scanner should allow the critical “break-points” in the various nonlinear mechanisms governing dendritic excitability to be discerned. For example, it can be supposed that since a tri-phasic relationship exists for temporal summation, as just discussed, a similar relationship could exist for spatial summation: at small inter-stimulus distances, active mechanisms might act to provide boosting, while at larger inter-stimulus distances, passive mechanisms might dominate and cause inputs to suppress each other.

As discussed earlier, recent work by Cash and Yuste points to the linearity of spatial summation throughout the dendritic tree (Cash and Yuste, 1998; Cash and Yuste, 1999). These studies employed iontophoresis to directly apply glutamate to multiple sites (using multiple pipettes) for direct application of neurotransmitter (e.g. glutamate) with a spatial resolution only sufficient to allow inputs to be spaced >10μm. At these distances, they found that spatial summation was largely linear: there was no interaction, either positive or negative, between the two inputs. However, it’s possible that either or both passive and active nonlinear mechanisms only come into play on shorter scales. While, as the authors argued, the electrotonic length of the cells is known to be much greater than 10μm (Cash and Yuste, 1998), this quantity generally refers to
the DC electrotonic length. However, the isopotentiality of transient (AC) signals, such as EPSPs, may be far more restricted; only in this small range could either passive or active effects manifest themselves.

Improvements (in progress) to the Dual Scanner should provide a spatial resolution of <2μm that would enable effects at these length scales to be discerned. That interesting phenomena may occur at these scales would be consistent with the notion of “synaptic clustering” that’s been advanced as a mechanism by which local computation may occur in dendrites (Koch and Segev, 2000). In addition to looking for break-points with respect to spatial scale, one can look for break-points with respect to excitation strength. It might be imagined that passive (attenuating) effects dominate at lower excitation, while active (boosting) effects dominate at higher excitation—forming a classic thresholding nonlinearity.

In short, it can be argued that the work of Cash and Yuste demonstrates only the linear summation of outputs from distinct computational compartments, but may have lacked the spatial resolution to effectively investigate the interesting nonlinear phenomenon within those compartments.

Direction Selectivity

A functionally compelling form of spatio-temporal integration is direction selectivity—the differential excitability of a neuron for different directions of spreading activity. As discussed above, such mechanisms have been found, proposed, and/or explained in a variety of sensory neurons, such as the retinal ganglion. In these cases, the role of direction selectivity is clear-cut. One
mechanism that can underlie such selectivity is an alternating pattern of excitatory and inhibitory synaptic input (Taylor et al., 2000). Might there be direction-selectivity in hippocampal pyramidal cells? What role might it play? What mechanisms could explain it?

In hippocampal pyramidal cells, there is often a clear pattern of innervation. Various layers can be identified in the dendritic field of CA1 pyramidal neurons—stratum oriens, stratum radiatum, and stratum lacunosum-moleculare—each of which receives its inputs from a different region of the hippocampal formation. Thus, direction selectivity could conceivably play a role in the network properties of the hippocampal formation. The highly linear arrangement of pyramidal neurons also argues for the existence of such a mechanism. A straightforward mechanism would simply be the temporal summation of inputs arriving first at distal locations and later at proximal ones. If timed to match the forward conduction velocity of the neuron, then the inputs could coincide, adding together (either sub- or supralinearly). Such a mechanism for input summation might be very strict, requiring a precise timing relationship between the inputs, or it might be more lax due to filtering of dendritic inputs.

The Dual Scanner is well suited to identify and characterize any direction selectivity in the responsiveness of hippocampal cells. Patterns of stimulation in both the “preferred” and “null” directions can be programmed. Furthermore, by employing multi-site recording to measure conduction velocities, for example, the hypothesized mechanism of direction selectivity can be tested directly against the selectivity that is observed.
Spiking Activity in the Dendritic Tree: Back Propagating Action Potentials and Dendritic Spikes

The presence of Na\(^+\) and Ca\(^{2+}\) channels allows spiking activity to arise in the dendritic arbor, both in the form of dendritic spikes and back-propagating action potentials (BPAPs). Evidence for this activity dates back to extracellular recordings (Cragg and Hamlyn, 1955) and has been more recently confirmed in dendritic patch clamp recordings (for review, see: Stuart et al., 1997). The Dual Scanner allows direct observation of spiking activity throughout the dendritic tree, as well as the observation of how it spreads throughout the dendritic arbor.

Dendritic spikes are action potential-like waveforms, usually due to Na\(^+\) channel activation. They may simply be a sharp, localized activation that leads to an opening of Ca\(^{2+}\) channels, triggering a host of biochemical processes. But they may also propagate towards the soma in a regenerative manner; however, it is found that they often do not reliably propagate to the soma (Golding and Spruston, 1998). The response at the soma, however, is still boosted relative to that which would be produced by the EPSPs that gave rise to the spike. Dendritic spikes represent a "saturating nonlinearity"—the initiation of such a spike suppresses further synaptic integration. It would seem wasteful of the cell's potential computational power for a dendritic spike to have much global effect in the dendritic arbor; thus, it is proposed that they likely represent a local nonlinear summation that can combine with other dendritic spikes in the arbor at the soma. In other words, dendritic spiking represents the thresholded output of a localized "simple cell" within the neuron.
The Dual Scanner allows dendritic spikes to be observed dynamically in a neuron. The problem of their propagation to the soma could be studied more directly than has been done to date with patch clamp techniques. Furthermore, the Dual Scanner enables a significant extension of the study of spatial summation discussed above. Whereas previous studies have investigated the summation of EPSPs generated throughout the dendritic arbor, the Dual Scanner allows the summation of dendritic spikes to be considered. Such a study would be impossible with patch clamp techniques owing to the difficulty of causing and observing spike generation at multiple locations. In short, the Dual Scanner should offer direct visualization and experimental control of this likely important aspect of compartmentalized computations in the dendritic arbor.

BPAPs, meanwhile, appear to play many roles in neuronal processing. Fundamentally, they are a signal to the dendrites that the cell has just fired. Several effects of this signal have been proposed and/or observed. First, they can serve to attenuate ongoing synaptic integration by providing a large shunting current; this effect has been observed (Stuart and Hausser, 1998). Second, they appear to contribute to burst firing of neurons by activating dendritic Ca\textsuperscript{2+} conductances, thereby increasing the cell’s excitability (by increasing the likelihood of dendritic spike generation, for example); this effect has been documented in the neocortex (Williams et al., 1998). Finally, and most importantly, they appear to provide the critical signal that leads to long-term synaptic modifications for nearly coincident synaptic inputs (Magee and Johnston, 1997).
How effectively do BPAPs propagate into the dendritic arbor? In hippocampal neurons, it is generally found that the Na⁺ conductances actively support the propagation, but that it is nonetheless decremental, likely due to the increasing density of K⁺ conductances (Spruston et al., 1995b). Following the cable theory of Rall, it is predicted that the amplitude of the BPAP is changed at branch points in a manner dependent on the impedance mismatch there (Goldstein and Rall, 1974). When $d_i^{3/2} < \sum_o d_o^{3/2}$, where $d_i$ is the diameter of the parent branch and $d_o$ are the diameters of the daughter branches, the BPAP should propagate as if it encountered an increased cable size and is thus attenuated. Conversely, when $d_i^{3/2} > \sum_o d_o^{3/2}$, the BPAP should be boosted.

Using the Dual Scanner, such changes in the strength of a BPAP would be evident and could be correlated to dendritic geometry. It would also enable the observation of branch point conduction failures, which have been observed (Spruston et al., 1995b). Although the Rall theory is based on a passive cell model, it may still be an accurate predictor of AP shape changes at a branch point, which, given the long space constants for transient signals (like BPAPs), may be based more on local geometric (i.e. passive) factors than on the relative distributions of active conductances throughout each of the branches. Following this hypothesis, it would be important to measure the AP shape changes immediately on either side of a branch point. This is feasible with the Dual Scanner, while impractical to impossible with patch clamp techniques.

Finally, a significant parameter regarding spikes and BPAPs in the dendritic arbor is their conduction velocity—this sets the optimal delay between
synaptic inputs from different regions of the dendritic arbor. Considering the regionalized innervation of principal cells in the hippocampus, this could constitute a significant network parameter. Another important question is whether there are significant changes in conduction velocity at branch points. The Dual Scanner allows these measurements to be taken directly.

Temporal Window of Activation

Recent evidence suggests that long-term synaptic modifications arise from the temporally coincident arrival of a BPAP and synaptic activation (i.e. an EPSP), with a sharp temporal window determining whether the modification is a potentiation or a depression (Magee and Johnston, 1997; Bi and Poo, 1998; Markram et al., 1997). This exciting development provides a satisfying picture consistent with the Hebbian learning hypothesis. The Dual Scanner can help address several remaining questions about this process.

Does this temporal window act simultaneously at many synapses? The Dual Scanner should permit direct observation of “learning” at multiple synapses, ideally after a single BPAP-EPSP pairing at each, although trains of BPAPs and EPSPs might be required. This would validate the notion that the BPAP acts as a “global” associative signal, with both potentiation and depression possible at each synapse. The conduction velocity of the BPAP could be observed to ascertain the optimal delay between spatially removed synaptic inputs to achieve simultaneous potentiation (or depression).

Does a dendritic spike always accompany synaptic modification? It’s known that the coincidence of the BPAP and EPSP causes a localized \( \text{Ca}^{2+} \)}
increase that likely causes the persistent modification. It's also known that such a coincidence can give rise to dendritic spikes. It's conceivable that the significant Ca\textsuperscript{2+} changes required to induce plasticity only arise during dendritic spike events.

Is LTP/LTD a purely postsynaptic phenomenon? All studies done to date of this Hebbian plasticity mechanism have employed true synaptic stimulation to generate the EPSP—in other words, they activated the presynaptic cell as well as the postsynaptic one. Because the Dual Scanner uses photolysis to stimulate the postsynaptic cell, it is suited to investigate the hypothesis that long-term modifications can be induced through postsynaptic mechanisms alone. That photolysis can cause LTD has been reported at least once (Dodt et al., 1999).

Is there a correlation between EPSP and/or BPAP strength and the strength of modification? By allowing the strength of the BPAP to be observed and the strength of the EPSP to be controlled, the Dual Scanner is well-suited to making our understanding of plasticity more quantitative.

*Examining Passive Cable Theory*

Though widely accepted, many of the fundamental tenets of the cable theory that describes the spread of electrical activity in passive neurons have never been directly confirmed experimentally. By employing a cocktail of drugs to eliminate the most significant voltage-gated conductances, cells can be “passivated.” The Dual Scanner then enables the investigation of these passive cells from several different perspectives, which should, if passive cable theory is accurate, provide a consistent picture.
The three key parameters describing a passive neuron are $R_i$, $R_m$, and $C_m$—the internal and membrane resistivities, and the membrane capacitance. $C_m$ and $R_i$ should be relatively constant throughout a cell (estimated at $\sim 1 \mu F/cm^2$ and $\sim 100 \Omega \cdot cm$, respectively), while $R_m$ may vary considerably due to non-uniform channel distribution—in this case, perhaps, voltage-independent “leak” channels. Based on these parameters, and the geometry of the cell, a wide variety of cell properties can be predicted, including the “space constant” over which an EPSP decays by $1/e$, the time constant governing EPSP decay times, the input resistance at particular synaptic sites, and the conduction velocity of EPSPs.

Given the $5mV$ resolution of optical recording (Bullen and Saggau, 1999) and the ability to synaptically stimulate any portion of the dendritic tree, the Dual Scanner is capable of measuring each of these passive properties directly. From these fundamental parameters, several important functional consequences arise that could also be tested. For example, cable-theory predicts a location-dependence for EPSP decay times—it should be as much as ten times shorter at distal sites where the input resistance is generally increased, owing to the smaller size of the dendrites there (Segev and London, 1999). This should produce an observable local reduction in temporal summation. It is also predicted that the locus of sublinear (i.e passive) spatial summation should be directly related to the measured space constant.
CHAPTER 3

OVERVIEW OF THE DUAL SCANNER

This chapter is intended to provide an overview of the design and construction of the Dual Scanner. Figure 3-1 shows a photograph of the Dual Scanner system. The two lasers, visible and UV, are arranged near the center of the table, and are each directed into optical paths that follow the edge of the table, constructed with an optical rod prototyping system. These two parallel optical paths comprise the UV scanner and visible scanner, which are combined just before entering the microscope. Each path contains two orthogonally oriented acousto-optic deflectors (AODs), which together provide X-Y scanning. Subsequent optics expand the angular range of the X-Y scan pattern, so that the pattern, upon entering the objective lens in the microscope, nearly fills the objective’s field-of-view. Beyond the microscope is the detection optical pathway,

Figure 3-1 Photographs of the Dual Scanner a) Optical table. Lasers near center of table lead into AO scanner paths, along the table perimeter. These are combined before entering the inverted microscope. The stainless steel specimen stage assembly built around the microscope is seen. Beyond the microscope (not visible) is the detection optical assembly containing a video camera, CCD camera, and photodiode detectors. b) Electronics instrumentation rack. Control computer containing Dual Scanner DAQ boards is above the rack, with interface circuitry in Eurocard style card racks below it. Oscilloscope and patch clamp amplifier below are used for patch clamp recordings.
which consists of pathways for imaging with both a video and CCD camera, as well as a photodiode detection path for optical recording. The computer seen above the instrumentation rack runs the system control software, which allows the experimenter to select sites-of-interest for both recording and stimulation after visualizing the specimen with the microscope. This computer handles both the control of the scanners as well as the acquisition of both optical and electrical recording data, using a set of commercial data acquisition (DAQ) boards. In the instrumentation rack below are two Eurocard (DIN41612) style card racks that contain custom interface circuits that provide an interface between the DAQ cards in the scan computer and the various devices on the optical table, including the deflector drivers, photodetectors, and electrophysiology apparatus.

The Dual Scanner system can be broadly divided into four sub-systems: 1) optics, 2) electronics, 3) software, and 4) mechanics. In this chapter, further orientation to the considerations of each of these subsystems is provided in turn, with greater emphasis placed on the newly developed UV scanner. The following two chapters discuss the optical and electronic subsystems in greater detail.

3.1 OPTICS

The “optics” of the Dual Scanner consists of the UV and visible laser scanners, the microscope itself, and the detection optical pathway. Each of these is described in turn here.

3.1.1 Acousto-Optic Laser Scanners

The term “acousto-optic scanner” here refers to the combination of a laser source, a pair of acousto-optic deflectors (AODs), and the “scanner optics.”
Designing the AO scanners, then, consists of selecting the appropriate laser and AODs from commercial sources as well as designing the scanner optics. The considerations involved in each of these are discussed here.

**Lasers**

The lasers chosen for the visible and UV scanners were designed with the applications of optical recording and stimulation, respectively, in mind. The visible laser scanner employs a continuous-wave (CW) argon gas laser (*T532R-AP-A01*; Melles Griot), which provides more than adequate power (~40mW) specifically at the 488 and 514nm wavelengths that are near the absorption peak of the di-8-ANEPPS voltage-sensitive dye, which is favored for the fast, ratiometric measurements of membrane potential intended, and described elsewhere (Bullen and Saggau, 1999).

For the UV scanner, the goal of multi-site optical stimulation requires that significant amounts of UV energy be delivered in periods short enough that successive doses to multiple sites on the specimen can be considered quasi-simultaneous; this is a central requirement for the study of dendritic integration. The best option is to employ a pulsed laser that concentrates its energy periodically into short powerful bursts; a suitable CW laser, in contrast, would need to be of very high power to deliver the same energy in the same time interval. In addition, emission in the biocompatible 340-360nm wavelength range was required.

A diode-pumped solid-state Q-switched laser was elected (modified 3505; DPSS Lasers) that offered a favorable combination of high average power
(750mW) and high repetition rate (50kHz). The model is a frequency-tripled Neodymium Vanadate (Nd:YVO₄) laser, which emits ~33ns pulses at 354.7nm. The repetition rate was specified to the manufacturer, and factory-set; the 50kHz figure was chosen to allow 10 sites to be activated within 200μs, which is a rather strict requirement for what constitutes “quasi-simultaneous” activation physiologically. This assumes that a single pulse contains sufficient energy to allow significant (ideally complete) uncaging of neurotransmitter in the focal volume. The energy delivered by a single pulse is simply 750mW/50kHz, or 15μJ. Accounting for expected losses in the scanning system (as well as the observed ~20% throughput), this should lead to pulses that somewhat exceed the energies delivered by others (generally <2μJ) over much longer periods using non-pulsed sources (i.e. CW lasers or arc lamps) for the photolysis of caged glutamate (for example: Schiller et al., 2000). It should be stressed, however, that it cannot be stated *a priori* whether delivering the energy in a concentrated ~33ns pulse might affect, either positively or negatively, the absolute energy requirement for uncaging. Fortunately, as shown later in Chapter 6, preliminary tests have shown the energy delivered by one pulse to be sufficient to elicit a considerable physiological response. This implies that the full 50kHz “bandwidth” can be employed in creating temporal patterns of activation.

The specifications of the UV laser are provided in Table 3-1.
Acousto-Optic Deflectors

Each scanner system consists of two orthogonally oriented AODs, which together allow two-dimensional (X-Y) scanning. AODs consist of a crystalline material fused to a piezoelectric transducer. The transducer applies an acoustic wave to the crystal, typically in the 50-200MHz frequency range for visible to near-visible optical wavelengths. Varying the frequency of the applied RF wave changes the angle by which the light in the first diffraction order is “deflected”—this is referred to here as the scan angle\(^1\); varying the amplitude of the applied RF wave changes the \textit{diffraction efficiency}—the fraction of the incident light deflected into the first diffraction order.

In selecting an AOD for laser scanning microscopy, two important parameters must be considered: 1) the time-bandwidth product \(N\), and 2) the “access time” \(\tau\). Its name notwithstanding, the time-bandwidth product actually reflects the \textit{spatial resolution} of the deflector, while the access time reflects the \textit{temporal resolution}.

\[\begin{array}{|c|c|}
\hline
\text{Wavelength} & 354.7\text{nm} \\
\hline
\text{Average Power} & 750\text{mW} \\
\hline
\text{Repetition Rate} & 50\text{kHz} \\
\hline
\text{Average Pulse Energy} & 15\mu\text{J} \\
\hline
\text{Pulse-to-pulse Energy Variation} & <10\% \\
\hline
\text{Beam Diameter (1/e\(^2\))} & 1.4\text{mm} \\
\hline
\text{Pulse Duration (FWHM)} & \sim 33\text{ns} \\
\hline
\end{array}\]

Table 3-1 Specifications of Q-Switched Nd:YVO\(_4\) Laser Values are manufacturer-specified and reflect the specific configuration employed in the Dual Scanner.

\(^1\) Ultimately the frequency applied determines the “scan position”—the position of the focused laser spot in the final specimen plane, but this only occurs after the subsequent scanner optics. The term “scan angle” reflects the fact that the AOD itself produces an angular scan pattern.
Figure 3-2 Effective Optical Aperture Determines AOD Spatio-Temporal Resolution

a) Effective optical aperture $D$ formed by the laser beam diameter or the AOD itself. Larger $D$ leads to a greater time-bandwidth product $N$—more angular positions can be resolved due to reduced optical diffraction of the beam. Smaller $D$ leads to a faster access time—the acoustic wave crosses the optical wave more quickly. b) Objective lens transforms resolvable angular positions into resolvable focused spots in the specimen plane. Thus, the value of $N$ directly determines the system’s spatial resolution.

The access time $\tau$ represents the time required to reposition from one scan angle to another after the RF frequency applied is changed. This is simply given by

$$\tau = \frac{D}{v} \quad (3.1)$$

where $D$ is the “optical aperture” size, and $v$ is the acoustic velocity in the AOD.

Figure 3-2a illustrates the meaning of the optical aperture: the parameter $D$ is the smaller of the laser beam diameter, or the physical aperture of the AOD. Thus the access time is, intuitively, just the time required for the acoustic wave to propagate across the incident optical wave.

Meanwhile, the time-bandwidth product $N$ represents the number of angular positions that can be deemed as resolved, generally using the classic Rayleigh criterion as a definition. The parameter $N$ is given by
\[ N = \frac{\Delta f \cdot D}{v}, \]  

(3.2)

where \( \Delta f \) is the acoustic frequency bandwidth\(^2\). The derivation of this is discussed in Chapter 4. As shown in Figure 3-2b, the time-bandwidth product \( N \) directly determines the ultimate spatial resolution of the laser scanner. The number of resolved angular directions in the back focal plane of the objective lens determines the number of spatially resolvable focal spots in the specimen plane\(^3\).

Given the field-of-view \( \Delta x_{\text{FOV}} \) occupied by the scan pattern in the specimen plane, which is determined by the combination of the scan angle magnification and the objective magnification, the spatial resolution of the laser scanner—simply the size of the focal volume \( \Delta x_{\text{spot}} \) per resolvable scan position—can be determined:

\[ \Delta x_{\text{spot}} = \frac{\Delta x_{\text{FOV}}}{N} \]  

(3.3)

Examining together Equations (3.1) and (3.2), it is clear why \( N \) is referred to as the time-bandwidth product, since it is the product of the access time \( \tau \) and the acoustic frequency bandwidth \( \Delta f \). It is apparent also that the AOD user has some flexibility to trade-off between the parameters \( \tau \) and \( N \), by varying the laser beam diameter \( D \). In other words, the user must select the optimal trade-off between spatial and temporal resolution. Most commercial devices have a rectangular aperture, which is elongated along the axis of the acoustic wave, but

\(^2\) A multiplicative factor can be added to the expression for \( N \) to reflect the particular resolution criterion employed.
is shorter along the perpendicular axis (that normal to the page in Figure 3-2a).

Cylindrical optics can be used to fill this asymmetric aperture for maximal spatial resolution $N$.

Commercial devices, including those employed in the Dual Scanner, typically operate in the Bragg mode, further discussed in Chapter 4, in which a majority of the incident light is deflected into the first order, much like is achieved with "blazed" diffraction gratings. Both the UV and visible deflectors employed are specified for greater than 80% diffraction efficiency, at the maximal acoustic wave amplitude.

**Scanner Optical Layout**

Both the UV and visible scanners follow a similar design; a generalized layout for each scanner is shown in Figure 3-3. As seen, each scanner's optical pathway can be divided into four components, termed the Beam Expander, AO

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$^3$ It would be more accurate to say that the time-bandwidth product $N$ is an upper limit on the number of resolvable spots in the specimen plane since other factors can conspire to worsen the
Scan Optics, Scan Magnification Optics, and Relay Optics, respectively. Each of these components is based on the concept of a relay telescope, which is depicted in Figure 3-4. A relay telescope is simply a pair of lenses spaced by the sum of their focal lengths. It can be shown that such a configuration allows light to propagate without divergence due to diffraction, save for a scaling factor $M^4$,

where $M = \frac{f_2}{f_1}$.

The four components of the AO scanner each consist of one or more relay telescopes. Their functions are briefly described here; more complete explanations are provided in Chapter 4.

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For the laser beams employed, accurately described in the paraxial approximation as Gaussian beams, the beam waist at (or near) the input and output planes of the relay telescope are equivalent, save for a scaling factor $M$, given by the is to transform the input Gaussian beam waist parameter $\omega_o$ and beam divergence $\theta_o$. The transformations are given by,

$\omega_o' = M\omega_o$

$\theta_o' = \frac{1}{M}\theta_o$

where $\omega_o'$ and $\theta_o'$ are the output beam waist and divergence parameters, respectively.
1. **Beam Expander**: A relay telescope that expands the laser beam (i.e. $M>1$) so that the beam fills (or slightly overfills) the aperture of the AO deflector, which serves to maximize the scanner's spatial resolution\(^5\).

2. **AO Scan Optics**: Two orthogonally oriented AODs are employed here, providing angular deflection in the X and Y dimensions respectively. A relay telescope follows each AOD. These telescopes serve two purposes. First, the plane in the middle of each telescope is a good location for an adjustable slit that serves to select the first diffraction order from each AOD, and reject all other orders. Second, the relay telescope between the two AODs ensures that the two deflectors reside in equivalent optical planes. In this way, the X and Y scanning ultimately appear to originate from a common "pivot point."\(^6\)

3. **Scan Magnification**: A series of one or more relay telescopes that "compress" the laser beam (i.e. $M < 1$) to increase the angular range of the scan pattern. The scan magnification factor is chosen so that the final angular scan range nearly fills the microscope objective's field-of-view.

4. **Relay Optics**: A final telescope, generally with no magnification (i.e. $M = 1$), is employed, with sufficiently long focal lengths (~100mm) to allow the pivot point of the scanner to be relayed to the back focal plane of the objective, which is generally located some distance within the microscope.

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\( ^5 \) In the current configuration, this beam expander only fills the shorter axis of the rectangular aperture. As discussed, cylindrical optics are required to achieve maximal spatial resolution.

\( ^6 \) In other words, it appears as if the scanning were the result of adjusting a multi-axis gimbal mount—the beam is scanned in both directions, but it originates from a stationary point.
Figure 3-5 Generalized One-Dimensional Acousto-Optic Scanner. An AO scanner consists of a series of relay telescopes. The planes between each telescope and at the back focal plane of the objective, where all the laser paths cross through a common point, are referred to as pivot points. These planes are equivalent to that of the AOD itself. The planes in the shared focal plane within each telescope are image planes. The scan pattern is focused to a sharp image here; these planes are equivalent, save for magnification, to the specimen plane.

Illustrating the role of each of these components, a generalized linear view of a one-dimensional AO scanner is shown in Figure 3-5. An expanded beam enters the AOD and a significant fraction is deflected into the first diffraction order. By varying the acoustic frequency, a “scan pattern” is created that is comprised of the different beam directions “occupied” over time. In the subsequent telescope, the undeflected “zero order” light is blocked with an adjustable slit located in the focal plane between the telescopes.

At this focal plane, each angular position of the deflected beam is focused to a distinct spatial location; hence, this plane, and all the focal planes within the subsequent relay telescopes, are termed “image planes.” Meanwhile, the shared focal planes between the relay telescopes are termed “pivot points,” because

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7 The small fraction of light in other diffraction orders—the second order, the first order on the other side of the zero order—are also blocked here.

8 As discussed in Chapter 4, the spacing between relay telescopes is also set to the sum of the focal lengths of the lenses involved. This ensures that the output focal plane of the first relay telescope becomes the input focal plane of the second relay telescope.
the path for all scan angles/positions cross through a common point in these planes.

Following the scan telescope, a beam aligner centers the scan pattern on the subsequent scan magnification and relay optics, which image a pivot point to the back focal plane of the objective lens. The objective lens focuses the laser beam to a small focal spot in the specimen plane; each angular scan position at the pivot point in the back focal plane is focused to a different location in the specimen plane.

Spatio-Temporal Resolution of the Dual Scanner

The Dual Scanner employs Tellurium Oxide (TeO$_2$) AODs for the visible scanner, while fused silica AODs are employed in the UV scanner owing to their high UV transmittance (and resistance to damage from the high energy pulses). As currently configured, the Dual Scanner does not employ cylindrical optics to fully exploit their potential spatial resolution. In this state, the former nominally provides $N\sim128$, while the latter offers a more modest $N\sim20$, because fused silica is an inferior acousto-optic material.

In the Dual Scanner, a high numerical aperture (NA), 100X magnification objective lens (Plan Neofluar; Zeiss) is employed in order to allow high-resolution single cell imaging. The field-of-view of this objective lens is approximately 200$\mu$m. The scan magnification optics of the two laser scanners are designed to fill most but not all of this field-of-view; this represents a compromise between achieving the best spatial resolution and having optical access to the more distal regions of a cell during a particular experiment.
In practice, as shown in Chapter 4, these configurations of AODs/objective lens/scan magnification optics yield a resolution of ~2.5\(\mu\)m for the visible scanner and <10\(\mu\)m for the UV scanner. This visible scanner resolution is sufficient to provide the local optical recordings desired. Meanwhile, it would be desirable to improve the resolution of the UV scanner to <2\(\mu\)m to achieve a more nearly monosynaptic locus of activation. Current efforts underway to employ cylindrical optics should enable eventual such performance.

3.1.2 Inverted Microscope

The Dual Scanner is designed primarily for studies employing cultured neurons, which are essentially “two-dimensional” preparations: they are not very thick nor do they scatter much light. With such preparations, it is possible and convenient to employ an inverted microscope—i.e. one in which the objective “points” upwards and looks at the specimen from below. Inverted microscopes are desirable for electrophysiological studies because the objective lens does not extend into the specimen dish, thereby allowing easy access to the specimen from above by one or more microprobes. In sum, the thinness of the specimen permits optical access from the bottom and “electrical” access to the same cell from the top—a configuration that is not possible with thicker, light scattering preparations.

The microscope selected (Axiovert S100TV; Zeiss) contains all the necessary accessories (two polarizers, two prisms) for differential interference microscopy (DIC), which is a technique that allows thin specimens to appear thicker (three-dimensional). In a typical Dual Scanner experiment, DIC trans-
illumination (i.e. illumination from the top via a condenser lens) is used first to visualize the cells’ structure prior to selecting the sites-of-interest for recording and stimulation. By employing DIC, the video image obtained before an experiment is adequate to allow the neuron’s fine processes—dendrites and axons—to be seen.

After the sites are selected, an epi-illumination (i.e. illumination from below via the objective lens) scheme is employed whereby both lasers are coupled in from the side of the microscope and reflected up towards the objective lens, where they are focused onto the specimen. A custom dichroic mirror “slider” was employed to permit the laser illumination to enter from the side.

As discussed below (Mechanics section), the slider allows the microscope to be switched between two epi-illumination modes: side-on illumination with the Dual Scanner lasers or conventional rear-on illumination from an arc lamp (Hg or Xe) located at the microscope’s normal epi-illumination port at the back of the scope. This second option allows high-resolution wide-field fluorescent imaging to be done, which will be useful in experiments such as those correlating structural parameters to physiological ones, where a finer structural image is required than DIC can provide. For these experiments, a suitable fluorescent dye is loaded into the cell; the resultant fluorescent images using this epi-illumination pathway provide structural resolution down to the level of dendritic spines.

The microscope employed contains a video port at the bottom. For the Dual Scanner system, a mirror assembly is attached to this video port, which
directs the light collected by the objective lens to the multi-function custom
detection pathway discussed next.

3.1.3 Detection Optics

A detection optical assembly was constructed that permits easy switching
between two light detection modalities: 1) imaging and 2) photometric detection.

Imaging Detection

Two imaging modes are supported. The first is real-time imaging using a
video camera and a variable zoom lens that allows external magnification to be
added. High magnification is helpful for viewing specifically the cell body during
patch clamp seal formation; after the seal is formed, the magnification is removed
and the whole cell, i.e. the dendritic tree, can be visualized for the experiment.
The second imaging mode is low-light level imaging with a cooled CCD camera.
This is employed for wide-field fluorescence imaging, to acquire high resolution
structural images of the cell under study.

Photometric Detection

The alternative photometric detection pathway consists of optics for
collecting all the light from the field of view to a small focus (<1mm) on the active
area of one or more photodiode detector. These photodiodes are employed to
collect the fluorescence signal generated during optical recording of membrane
potential. As is described elsewhere, typically one detector is used in the
detection pathway for optical recording experiments (Bullen et al., 1997). In this
case, a separate “Reference” photodetector is employed outside the detection
pathway. This detector samples the visible laser illumination just before it enters the microscope; the signal from this detector serves to cancel out intensity fluctuations from the laser source. Alternatively, two detectors can be used in the detection pathway to collect fluorescence in two disjoint wavelength bands; these signals can be used to form a “ratiometric” measurement that allows the membrane potential signal to be calibrated, rather than simply relative (Bullen and Saggau, 1999). The optics of the detection pathway are discussed in further detail in Chapter 4.

3.2 ELECTRONICS

The “electronics” of the Dual Scanner consists of circuitry for the generation and/or measurement of a variety of electrical signals. These include:
1) analog voltages which control the UV and visible AO scanners, 2) digital signals to control the UV laser Q-switch, 3) fast, high-resolution measurement of the analog signals arising from the photodiode during optical recording 4) analog signals, both generated and obtained with electrophysiological probes, and, finally, 5) various other digital signals for timing and control.

In the Dual Scanner, the general flow of the electrical signals is from commercial DAQ boards located in the scanner control computer to custom interface circuitry (as required) to the final control or measurement device on or near the optical table. This flow is reversed for measurement, rather than control, signals. The custom interface circuits were built on prototyping boards and then mounted within Eurocard (DIN41612) style card racks, which in turn were mounted in a standard 19” instrumentation rack. The Eurocard style
instrumentation boxes contain a "front panel" with BNCs that connect to each of the devices on the table, and a "back panel" of connectors leading to the various DAQ boards in the scan computer.

3.2.1 Acousto-optic Scanner Control Electronics

The "control" of an AO scanner refers to the generation of RF waves, ideally pulse sinusoids, which determine the frequency and intensity of the acoustic waves produced in each of the two AODs per scanner. For both the UV and visible scanners, this control is achieved using a voltage-controlled oscillator (VCO) that is termed the "deflector driver."\(^9\) Each driver, one per AOD, inputs an analog tuning voltage that directly (i.e. linearly) controls the frequency of the output RF wave. The UV deflector drivers also have a "modulation" input, which accepts another analog tuning voltage that directly controls the RF wave amplitude. Thus, to control the AO scanners, commercial D/A boards are employed to generate these analog tuning voltages. A total of three pairs of D/A channels are required to variously control the UV scan position, UV intensity, and visible scan position. These are referred to herein as the UV Scan, UV Mod, and Vis Scan D/A channels, respectively.

D/A Converter Boards

For both the UV and visible scanners, fine positioning resolution is desired. This refers to the number of distinct locations that the laser focal spot

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\(^9\) An alternative approach to generating the RF waves required is a direct digital synthesizer (DDS) board. However, a commercial DDS is not readily available for the 100-200MHz tuning range of the UV AODs. For the visible AODs, the only DDS board known which is suited to...
can be centered on in the field-of-view. This is distinct from the spatial resolution of each scanner—the number of positions that can be selected may be considerably greater than the number of (side-by-side) focal spots that can be resolved. For both the UV and visible scanner, a positioning resolution of at least 500 spots would provide ~400nm resolution given the ~200µm field-of-view of the 100X microscope objective.

Nominally, the scan position/intensity values only need to be updated at 50kHz for the UV scanner, and up to 100kHz for the visible scanner\textsuperscript{10}. However, another important consideration is minimizing the time for the tuning voltages to “settle” to stable, steady-state values. For the UV scanner, this ensures that the acoustic frequency and amplitude are at the desired values, to a high precision, when each pulse arrives at the AODs. For the visible scanner, a fast settling time allows the fluorescence value during each “pixel dwell period”—the short time, ~10-100µs, spent at each site of interest—to quickly settle to a DC value. As has been described, this enables “oversampling”—multiple samples are taken per site and per “frame,” and then averaged to improve the signal quality (Bullen et al., 1997).

To meet these requirements of high positioning resolution and fast settling time, a 5MHz, 12-bit resolution D/A board was elected (AWFG; Keithley): 12-bit

\footnote{provide the ~500mW power required (IntraAction) does not allow fast enough frequency updates (only ~5kHz) for multi-site optical recording.}

\footnote{The 100kHz specification for the visible scanner is based on a maximum of 10-20 optical recording sites sampled at 5-10kHz.}
resolution ensures at least 500 spots of positioning resolution\textsuperscript{11}, while a 5MHz update rate ensures fully settled output values at the actual, slower update rates. This board contains two D/A channels, so one each is used for the UV Scan, UV Mod, and Vis Scan control. An additional advantage of this board is the significant on-board memory buffer (32Ksample), which allows a pattern of scan positions to be loaded in prior to the experiment, so the control can run autonomous from the CPU during the experiment.

\textit{Scan Pattern Synchronization}

Using the D/A cards described above, the scan position/intensity of both the UV and visible scanners are periodically updated. For the UV scanner, it is required to synchronize the periodic pulses from the Q-switched laser with the periodic updates of the acoustic wave frequency and amplitude in the two AODs. In order to achieve this synchronization, the laser was modified by the manufacturer to allow the Q-switch to be externally controlled. The modified laser contains two digital control inputs, \textit{Q-Switch Clock} and \textit{Q-Switch Gate}, which, as their names suggest, allow the user to clock and gate the pulsing of the UV laser, respectively. Figure 3-6 shows the relative timing between these two digital signals and the updates to the UV Scan and UV Mod signals. The clock signals make a digital LO→HI transition every 20μs, causing the laser to emit pulses at the designed 50kHz repetition rate. Similarly, the UV Scan/Mod signals are updated every 20μs, but lead the laser pulses by 10μs. Thus a 100kHz clock is

\textsuperscript{11} This author follows the heuristic that one should always buy two bits of resolution in excess of the nominal specification, as these are rarely achieved in practice.
Figure 3-6 Timing of UV Scanner Control Signals Electronic control of the UV scanner consists of 2 digital signals (Q-Switch Gate and Q-Switch Clock) and two pairs of analog signals, UV Scan and UV Mod, to control the scan position and diffraction efficiency, respectively. A 100kHz clock is used to clock the output signals. The scan position and diffraction efficiency tuning voltages are updated ahead of the laser pulses which occur at 50kHz, so the acoustic wave frequency and amplitude are settled when the pulse arrives.

used in updating both the digital and analog output signals that control the UV scanner. The two digital control signals are generated using the set of digital outputs available on the UV Scan D/A board; these digital outputs can be pre-programmed just as the analog outputs are, and are updated by the same clock.

For the visible scanner, the laser position repetitively cycles through a selection of 10-20 sites-of-interest on the specimen at rates of up to 50-100kHz. In order to assign fluorescence measured at the photodiode detector to specific time-slots and specimen sites, it is important to synchronize the A/D converter samples with the scanner control.

Further details about the implementation of timing and synchronization in the Dual Scanner are provided in Chapter 5 and Appendix 4.
DAQ Board Interface Circuits

The analog and digital control signals described cannot be directly connected to their target devices—the deflector drivers and the UV laser, respectively. Custom interface circuitry must be employed, generally for two reasons:

1. To match the output voltage range of the D/A boards to the input tuning voltage range of the deflector drivers, in order to utilize the boards' 12-bit resolution, and,
2. To supply ample current to the various low-impedance (50Ω) inputs, including the UV deflector driver inputs and the UV laser digital control inputs.

Each D/A board has its own interface circuit located in the Eurocard card racks in the instrumentation panel.

3.2.2 Optical Recording Electronics

The detection apparatus for the visible scanner consists of a photodiode (S1226-18BK; Hamamatsu), a custom photodiode amplifier which converts the generated photocurrent to a measurable voltage, a fast, high-resolution “oversampling” A/D converter (Chico/AIX; Innovative Integrations), and a custom interface circuit which transforms the photodetector amplifier output to match the A/D input voltage range.
The methodology is essentially unchanged from that described previously by Bullen, Saggau, and Patel (Bullen et al., 1997). A flowchart of the signals acquired and processed to achieve fast optical recording is given in Figure 3-7a. As shown, the ratio between the signal measured at the Signal and Reference photodiodes is calculated, $F = \frac{S}{R}$, compensating for the significant (~5-10%) fluctuations of the Argon laser. The membrane potential $V_m$ is proportional to the normalized change in fluorescence $\Delta F/F$ arising from the voltage-sensitive dye at
each recording site. The fluorescence sample must be digitized with at least a true 14-bit resolution, because the changes in fluorescence to track the smallest $\Delta V_m$ of interest are quite small: $\Delta F/F \sim 0.1\%$ for $\Delta V_m = 1\text{mV}$. Furthermore, the sampling rate of the A/D converter must be as high as possible; likewise, the settling time of the photodiode detector amplifier and the A/D interface circuit must be as fast as possible. Fast A/D conversion rates allow multiple samples to be acquired during the 10-100$\mu$s period that the laser dwells at each site, during which the fluorescence signal is nominally stationary. These samples are then averaged to improve the signal quality$^{13}$. This technique of “oversampling,” depicted in Figure 3-7b, gives the experimenter the flexibility to vary the sampling rate and the number of sites, while always achieving the best signal-to-noise ratio.

The simultaneous requirement of high-resolution (>14 bits) and fast A/D conversion is exacting. The combined system comprised of the A/D converter itself and the custom interface circuit must achieve this performance.

### 3.2.3 Other Dual Scanner Electronics

Ideally, the optical recording and stimulation capabilities of the Dual Scanner system would be entirely sufficient for substantial investigation of...

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$^{12}$ The two key differences are: 1) the present A/D converter samples at a much faster rate (2.5MHz), and, 2) the present A/D converter board is multi-channel, so multiplexing is no longer required.

$^{13}$ This noise reduction is easily described for normal (Gaussian) noise distributions. The expected value of the variance of the average of an ensemble of independent observations of a
dendritic integration in cultured neurons. However, for the purposes of control experiments\textsuperscript{14}, and because, at this time, electrical recordings can offer greater resolution than optical recordings, the use of conventional electrophysiological techniques has been incorporated into the Dual Scanner. The electrophysiology apparatus consists of a commercial patch-clamp amplifier system (BVC-700A; Dagan) and a combined A/D and D/A board (CIO-DAS08-AOL; Computer Boards). The A/D digitizes the current and voltage measurements obtained in a patch-clamp recording (1993) with 12-bit resolution and up to a 20kHz sampling rate. Concurrently, the D/A converter can be used to generate 12-bit control voltages to stimulate or “clamp” the cell under study by applying voltage or current, in “voltage-clamp” and “current-clamp” recording modes, respectively.

Another important aspect of the Dual Scanner system is the capability for real-time visualization of the specimen on the computer monitor; this allows the specimen visualization to be tightly integrated with the software used to select sites of interest for both optical recording and stimulation. To achieve this, a PCI frame-grabber card is employed (IV-400; µTech) that has the capability for “non-destructive overlay”: this permits the image acquired from the video imaging pathway to be displayed in real-time on the computer monitor while the software

\begin{equation}
E\left[\left(\frac{1}{N}\sum_{i}(y_i - \mu)^2 - E\left[\frac{1}{N}\sum_{i}(y_i - \mu)^2\right]\right)^2\right] = \frac{\sigma^2}{N}.
\end{equation}

\textsuperscript{14} In addition, patch clamp recordings are required in order to achieve calibrated optical measurements of membrane potential employing ratiometric recording (Bullen and Saggau, 1999).
simultaneously can add graphics at the same pixels, i.e. to mark the sites of interest.

Finally, the scanner control computer parallel port is used to generate up to eight digital signals during the course of the experiment. These are used 1) to control a variety of devices including, for example, mechanical shutters for the laser and an arc lamp, and, 2) for synchronization of the various system DAQ devices, as is discussed further in Chapter 5.

3.2.4 Dual Scanner Electronics Summary

Table 3-2 recapitulates the commercial DAQ boards employed in the Dual Scanner. The design and construction of the interface circuitry required for these boards, as well as their performance relative to the specifications just discussed, is the subject of Chapter 5.

<table>
<thead>
<tr>
<th></th>
<th>Part Name (Vendor)</th>
<th>Resolution (bits)</th>
<th>Num Channels</th>
<th>Update Rate (Capability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visible Scanner Control</td>
<td>AWFG (Keithley)</td>
<td>12</td>
<td>2</td>
<td>Variable, to ~100kHz/Chan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1MHz/Chan)</td>
</tr>
<tr>
<td>UV Scanner Control</td>
<td>AWFG (Keithley)</td>
<td>12</td>
<td>2</td>
<td>100kHz/Chan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1MHz/Chan)</td>
</tr>
<tr>
<td>UV Modulation Control</td>
<td>AWFG (Keithley)</td>
<td>12</td>
<td>2</td>
<td>100kHz/Chan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1MHz/Chan)</td>
</tr>
<tr>
<td>Electrophysiological Recording</td>
<td>CIO-DAS08-AOL (Computer Boards)</td>
<td>12</td>
<td>8</td>
<td>15kHz total*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(20kHz/Chan)</td>
</tr>
<tr>
<td>Electrophysiological Stimulation</td>
<td>CIO-DAS08-AOL (Computer Boards)</td>
<td>12</td>
<td>2</td>
<td>15kHz total*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(20kHz/Chan)</td>
</tr>
<tr>
<td>Optical Recording</td>
<td>AIX Module/Chico Baseboard (Innovative Integrations)</td>
<td>16</td>
<td>4</td>
<td>2.5MHz/Chan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2.5MHz/Chan)</td>
</tr>
</tbody>
</table>

Table 3-2 Summary of Dual Scanner Data Acquisition (DAQ) Boards Number of channel and vendor specified resolution of each board is given. The D/A or A/D update rate actually employed in the Dual Scanner is given, with the board's maximal update rate capability given in parentheses. (*) 15 kHz figure reflects the aggregate update rate for all the channels employed. Thus, if 2 A/D channels are employed, then each samples at 15kHz/2 = 7.5kHz
3.3 SOFTWARE

In order to be useful as a scientific instrument, the Dual Scanner employs a computer interface that allows all the parameters of an experiment to be specified—the sites for recording and stimulation, the sampling rate for optical recording, the specification of a command voltage pattern for electrical stimulation, etc. Subsequently, considering the live nature of the specimen, the software quickly (within 10s) displays the data collected during the experiment, both the optical and electrical recordings, so the user can determine whether to continue the experiment, abort it, or change the experimental parameters (i.e. vary the strength or locations of stimulation). For the optical recordings, the steps depicted in Figure 3-7—"demultiplexing" of the data into traces for each site of interest, ratio formation, filtering, and bleaching correction—are all carried out so that a trace for each recording site specified can be displayed. The software also provides provision to average the results of multiple experiments, as required to improve the recording signal quality. Finally, it provides a means to archive data collected and to export the data to external data analysis packages.

![Figure 3-8 Screen Shot of Dual Scanner Control Software](image)

*Figure 3-8 Screen Shot of Dual Scanner Control Software The Modify/Scanning Pattern... dialog box is shown, where sites-of-interest for optical stimulation (darker) and optical recording (lighter) are shown. The video image shown was live. The recording patch clamp pipette is visible from the right.*
The software that has been developed runs under the Windows operating system. A view of the computer screen during the selection of sites of interest is given in Figure 3-8. A description of the features of this software is provided in Appendix 2.

3.4 MECHANICS

The optical layout of the Dual Scanner outlined above requires a mechanical implementation. An optical rod prototyping system (*Microbench*; Linos Photonics) was employed. A significant advantage of this system is the self-alignment of the many lenses employed along the central axis of the rod system. Another is that each section of the "optical bench" is mounted to the optical table at a fixed optical height; in this way, sections of the bench (e.g. a particular relay telescope) can be removed or reinserted with minimal or no realignment. Finally, the prototyping system vendor\(^{15}\) provides a large number of accessories that can easily be incorporated into the optical path.

Using the rod prototyping system, most of the Dual Scanner is comprised of commercially available parts. Several components, however, were custom designed. Among these include a variety of optical mounts for odd-sized optics and an adaptation system that allows the optics constructed with the prototyping system to adapt to the many available components of the microscope manufacturer. Here, a brief description is provided of three significant custom

\(^{15}\) In addition, its competitors (AF Optical; ThorLabs) provide a large assortment of useful accessories.
mechanical designs: 1) the AOD tilt unit, 2) the custom dichroic mirror slider, and 3) the specimen stage.

_Acousto-optic Deflector Tilt Unit(s)_

Custom assemblies were developed that allow the commercial AODs employed in the Dual Scanner to be adapted into the optical rod system. These assemblies, termed here “tilt units,” allow the AOD to be rotated about either of the two axes orthogonal to the optical axis (i.e. parallel to the optical rods). This is required in order to achieve the Bragg angle condition required for maximum diffraction efficiency (see Chapter 4).

The tilt units were designed to be used in an “X-Y positioner” accessory of the optical rod system (_06 5040; Linos Photonics_). The tilt unit consists of two rigid assemblies that are free to rotate with respect to each other about the desired axis. One assembly is stationary—simply attached to the X-Y positioner and is thus constrained. The other is attached to the AOD and is rotatable. The two assemblies are attached via a threaded stainless steel shaft that contains a smooth bearing surface about which the AOD assembly is free to turn. A micrometer is rigidly attached to the stationary assembly and pushes against a protrusion on the rotatable assembly that extends directly from the bearing shaft. This produces fine control over a pure rotation.

Ultimately, each deflector is both positionable and rotatable within the optical rail assembly. In this way, the optical intensity throughput of the AOD can be maximized using three “knobs”: X-position, Y-position, and the tilt angle.
Custom Epi-Illumination Dichroic Mirror Slider

In designing the optical paths for the laser scanners, it is desirable to avoid all microscope optics, due to poor UV transmission as well as incompatibility with the relay optical imaging approach, and create a “straight shot” to the objective lens. For this reason, a custom dichroic mirror “slider” was constructed that replaces the commercial slider in the scope and allows side-illumination\textsuperscript{16}. This is shown in Figure 3-9. While commercial sliders contain two (or more) positions to allow the user to switch between different dichroic mirrors for conventional rear illumination via an arc lamp (epi-illumination), the custom slider also allows two dichroic mirrors to be mounted, but one is rotated 90 degrees to allow for side-on illumination. Thus, by “sliding” between the two dirhroic mirrors,

\textbf{Figure 3-9 Custom Dichroic Mirror Slider} Dovetail at bottom allows assembly to “slide” in the microscope (\textit{Axiocvert S100TV}; Zeiss) between two positions: one for rear-on illumination (far mirror mount) and one for side-on illumination (near mirror mount). Note the aperture in the foreground for mounting the final (shared) lens in the AO scanner pathways. Threaded holes on right side allow the mounts to be adjusted (done once), and then clamped in place with large screw. The notches near the bottom allow the slider to “click” into place in each of the two mirror positions.

\textsuperscript{16} In the meanwhile, some microscope manufacturers (Olympus) have developed new designs that allow for such “straight shot” laser illumination, useful in many applications.
the user can switch between focal laser scanning illumination by the UV and
visible lasers and wide-field illumination with an arc lamp for structural imaging.

The slider is situated just below the objective lens; the dichroic mirrors
reflect the shorter-wavelength illumination sources, while transmitting the longer-
wavelength fluorescence that is collected by the objective, allowing it to pass
below to the detection optical pathway.

*Specimen Stage*

Many microscope systems designed for physiology applications employ
the perhaps counterintuitive design principle of translating the microscope
relative to a fixed specimen stage. This is considered advantageous owing to the
number of delicate microprobes that are used, some of which become “attached”
to the specimen (e.g. patch clamp pipettes). However, in the Dual Scanner, laser
illumination from a stationary optical assembly is coupled into the microscope;
hence, the microscope must remain stationary as well. As a result, it was
necessary to design a stable specimen stage, which was large enough to
accommodate one or more micromanipulators, and which could be smoothly
translated relative to the fixed microscope.

The stage, partially visible and labeled in Figure 3-1, consists of two large
plates that are in actuality “sandwiches” consisting of a thick plate of aluminum
with thinner (1/16”) plates of stainless steel on either side. The specimen stage
itself is a smaller aluminum plate that is connected as a “bridge” between the
plates, and contains cutouts to accept the specimen dish. The two plates are
joined at the rear with a much thicker aluminum plate to form a solid mechanical
attachment. Thus, the bulk of the stage consists of a "U" shaped assembly that wraps around the microscope. The large stainless steel sandwiches provide ample area for micromanipulators that can be attached magnetically on the top side; on the underside, the steel plates provide a smooth bearing surface.

The whole assembly is lifted to the correct height by a set of three columns. On two of these columns a large stainless steel ball bearing forms the topmost surface that contacts the stage at two points; on the third, an assembly of two orthogonally mounted translation stages is found, whose top smooth stainless steel bearing surface contacts the stage. This pair of translation stages allows the stage to be positioned relative to the microscope.

This design was carefully undertaken to avoid mechanical obstructions with the AO scanner and detection optical assemblies, which reside partially underneath the stage assembly.
CHAPTER 4  

DUAL SCANNER OPTICS

In this chapter the "optics" of the Dual Scanner are discussed. This includes a discussion of the theory of AO deflection, the important parameters of AO materials, the design and alignment of the laser scanners, the performance of the laser scanners, and the design of the detection optical assembly.

4.1 THEORY OF ACOUSTO-OPTIC DEFLECTION

4.1.1 Raman-Nath Interaction

As discussed in the last chapter, it is appealing to view an AOD as a phase grating created by the alternating regions of compression and extension in the acoustic wave. Assuming the simple case of an isotropic acoustic medium and a longitudinal (i.e. non-shear) acoustic wave, this can be described by a scalar equation coupling the displacement (strain) in the crystal to the index of refraction,

$$\Delta n(x,t) = -\frac{1}{2}pn^3s(x,t),$$

(4.1)

where \( p \) is a phenomenological constant known as the photoelastic coefficient and \( s(x,t) \) is the longitudinal strain in the crystal. If the AOD is driven by a sinusoidal wave, an optical wave encountering the crystal sees a frozen-in-time index of refraction given by \( n(x) = n_o + \Delta n_o \cos(Kx) \), where \( K \) is the acoustic wavenumber, and a pure plane-wave is assumed. The acoustic wave gives rise to a variation in the phase the optical wave accumulates in propagating through the crystal, described at a particular instant by
\[ \Delta \phi(x) = \frac{2\pi}{\lambda_o} L \Delta n_o \cos(Kx) \]  \hspace{1cm} (4.2)

where \( \lambda_o \) is the vacuum wavelength of the optical wave, and \( L \) is the length traversed through the crystal. At this instant in time, the emerging optical plane wave can then simply be described by

\[ E(x) = \tilde{E}_0 e^{i(k_0 n L + k_0 L \Delta n_o \cos(Kx))} \]  \hspace{1cm} (4.3)

where \( \tilde{E}_0 \) is the complex incident amplitude of the optical wave and \( k_0 \) is the vacuum wavenumber \((2\pi/\lambda_o)\). Diffraction theory informs us that the far-field intensity distribution can be determined by taking a Fourier transform of this field-distribution at the grating. It is well known from communication theory that sinusoidal phase modulation gives rise to a set of Bessel functions (of the first kind) centered on the carrier frequency (Proakis and Salehi, 2002). Using this insight, it is seen that the resulting electric field can be described by the infinite set of Bessel functions

\[ E(x) = \sum_{m=-\infty}^{\infty} J_m(\Delta \phi) e^{i(k_0 n L + m K x)} \hspace{1cm} (4.4) \]

The \( e^{imKx} \) terms give rise to angular deflections that can be shown to be approximately \( \theta_m = m(\lambda/\Lambda) \). As shown in Figure 4-1, the resulting far-field intensity distribution is a set of diffraction orders, each containing the fraction \( J_m^2(\Delta \phi) \) of the incident optical intensity.

\[^1\] This employs the small angle approximation \( \sin \theta = \theta \)
\[ n = n_0 + \Delta n \cos(Kx) \Rightarrow \]

\[ \Delta \phi(x) = \frac{2\pi}{\lambda_0} \Delta n \cos(Kx) \cdot L \]

\[ I_2(x) = J_2^2(\Delta \phi) \]
\[ I_1(x) = J_1^2(\Delta \phi) \]
\[ I_0(x) = J_0^2(\Delta \phi) \]
\[ I_{-1}(x) = J_{-1}^2(\Delta \phi) \]
\[ I_{-2}(x) = J_{-2}^2(\Delta \phi) \]

Figure 4-1 Raman-Nath Acousto-optic Interaction In the thin grating approximation, the incident beam (approximated as a plane wave) is diffracted into an infinite set of diffraction orders.

This straightforward, first-approximation view of the AO deflection process was unsurprisingly the earliest to be described, first by Debye and Sears, and later more analytically by Raman and Nath. It is generally referred to as Raman-Nath diffraction. As we have seen, this process gives rise to an infinite set of diffraction orders. Typically, however, the user is interested only in scanning in one of the orders, namely one or the other first order (i.e. \( I_1 \) or \( I_{-1} \)). A typical performance parameter of an AO device is the diffraction efficiency, given by

\[ \eta = \frac{I_1}{I_{\text{incident}}} , \quad (4.5) \]

reflects the fraction of the incident intensity that is coupled into the (useful) first-order. Simply examining the Bessel functions, it is found that the largest possible diffraction efficiency is given by \( \eta_{\text{max}} = .339 \). Both the large number of diffraction
orders and this low diffraction efficiency render the Raman-Nath interaction impractical for most purposes.

4.1.2 Inadequacy of the Raman-Nath Picture

Given the poor characteristics of the Raman-Nath interaction, it can be viewed as fortunate that this simplistic description of the AOD as a phase grating fails to properly describe the AO interaction in most devices. It is found that the Raman-Nath picture applies only in the limit of small $L$, i.e. for thin devices; practically, devices are typically somewhat to considerably larger than this length scale. Examining the literature, one finds several different-sounding descriptions of why the Raman-Nath description breaks down for increasing device thicknesses:

1. **Acoustic Diffraction** The acoustic wave is not a pure plane wave. Thinner devices give rise to more “diffraction” of the acoustic wave, so that it is actually comprised of several plane waves traveling in a range of directions.

2. **Optical Diffraction** In the Raman-Nath picture, the amount of energy diffracted into higher orders increases without bound as the length $L$ increases. However, the incident optical wave is diffracted (made less planar) by the effective apertures formed by the alternating regions of compression and extension. With the optical wave smeared across the acoustic wave, each successive infinitesimally thin grating in a real thick grating is made less effective, so little energy actually reaches the higher orders.

3. **Momentum Mismatch** The acousto-optic interaction requires strong coupling between the optical and acoustic waves; in quantum mechanical terms, this implies large probabilities for the coincidence of photons and phonons. This physical intuition is captured by a coupled wave equation
solution that shows ultimately that energy coupling to higher diffraction orders is unlikely due to phase mismatch.

All of these intuitions are informative, but the third is (arguably) the most fundamental. Using the concept of phase matching, it can be shown how the AO interaction transitions from the Raman-Nath regime for small $L$ to a new regime—the Bragg interaction—at longer $L$. This unifying picture for AO interactions was developed in 1967 by Klein and Cook, and its insights hold even under the simplifying assumptions that the optical and acoustic waves are pure plane waves (Klein and Cook, 1967). The Klein-Cook formulation serves as the foundation that describes the fundamental physics; the effects of optical and acoustic diffraction could later be incorporated as perturbations to this basic picture.

### 4.1.3 Klein-Cook Formulation

The unifying Klein-Cook formulation is based on a coupled-wave formulation of the heterogeneous Helmholtz equation used to approximate paraxial plane waves,

\[
(\nabla^2 + k^2)E = -\mu_0 \frac{\partial^2 \Delta P}{\partial t^2},
\]

where $\Delta P$ reflects the variation in the electronic polarizability caused by the acoustic wave (because the index-of-refraction is related to the electronic density). The term on the right-hand-side represents a source term, which encapsulates the AO interaction. Because $P = \varepsilon_0 (n^2 - 1) E$, it can be written, in a scalar form, as
\[(\nabla^2 + k^2)E = -2\mu_0\varepsilon_0 n \frac{\partial^2}{\partial t^2} (\Delta n E) \quad (4.7)\]

where \(E\) is the electric-field. In the Klein-Cook formulation, it is simply postulated that the total optical wave consists of an infinite superposition of plane waves embodying each of the diffraction orders,

\[E(r,t) = \sum_{m=-\infty}^{\infty} E_m(z) \hat{e}_m e^{i(\omega_m t - k_m \cdot r)} \quad (4.8)\]

where \(\hat{e}_m\) is the unit vector for the direction of the \(E\)-field in the \(m\)'th diffracted order, and \(k_m\) and \(\omega_m\) are the wavevector and frequency of the \(m\)'th order, respectively. These are given by the relations

\[
\begin{align*}
\omega_m &= \omega_0 + m\Omega \\
k_m &= k_0 + mK
\end{align*}
\quad m = \pm 1, \pm 2, \pm 3, \ldots \quad (4.9)
\]

where \(\Omega\) and \(K\) are the acoustic frequency and wavevector, respectively. This requirement is inspired by an underlying assumption that the higher order plane waves are born from "collisions" of photons and phonons, and are therefore constrained by the conservation of energy and momentum, respectively.

Two other requirements circumscribe the Klein-Cook formulation. First, the AO interaction is "linear"—it only arises from collisions with a single phonon (i.e. there are no "multi-phonon" effects). Second, the commonly employed slowly-varying envelope approximation is invoked in which the field envelopes \(E_m(z)\) are assumed to vary slowly so that the second derivative \(\frac{\partial E_m^2}{\partial z^2}\) can be neglected.
Applying these considerations in an isotropic medium, one arrives at an infinite set of coupled differential equations (Xu and Stroud, 1992):

\[
\frac{\partial E_m}{\partial z} - j\Delta k_mE_m(z) = \frac{\Delta \phi}{2L} [E_{m-1}(z) - E_{m+1}(z)]
\]  

(4.10)

where \( \Delta \phi = \frac{2\pi\Delta n L}{\lambda_0 \cos \theta_0} \) is the phase shift induced by the AO interaction as above, corrected for the incident angle \( \theta_0 \) relative to the propagation direction Z. From the form of the equation, it’s clear that the term \( \Delta \phi/2L \) is a per-unit-length measure of the strength of the AO coupling between orders.

Meanwhile, the factor \( \Delta k_m \) reflects the momentum mismatch\(^2\)

\[
\Delta k_m = k'_m - k_m
\]

(4.11)

where \( k'_m \) is the modulus of the resultant wavevector, and \( k_m \) is the modulus expected for a wavevector of the resultant frequency and direction in that medium\(^3\). The concept of momentum mismatch is depicted in Figure 4-2. Given a certain acoustic wavevector and a particular diffraction order \( m \), only at one particular incident angle is the phase-matching condition met, where \( \Delta k_m = 0 \). In this situation, the acoustic and optical waves interact strongly across time and space; seen another way, this allows the regions of high photon and phonon

\(^2\) Actually, the factor abbreviated by \( \Delta k_m \) in Eqn. (4.10) is only an approximation, but a good one, of the true momentum mismatch specified in Eqn. (4.11).

\(^3\) The expected modulus is given by \( k_m = \frac{n_m \omega_m}{c} \). The index \( n_m \) varies for different orders because, generally, the material may be anisotropic, and thus may vary for different propagation directions. Practically, in an isotropic material, \( k_m \) is constant for all \( m \) because changes to \( \omega_m \) are very small (~10\(^{-6}\)).
probability in the optical and acoustic waves to coincide throughout the entire device.

Examining Eqn. (4.10), the effect of the mismatch term is to reduce the effective coupling to (and from) the \( m' \)th order when \( \Delta k_m \neq 0 \). Eqn. (4.10) cannot be solved analytically, but the numerical analysis by Klein and Cook revealed two regimes where analytic solutions could be applied. In the Raman-Nath regime, where the device thickness \( L \) is thin, the effect of the mismatch term \( \Delta k_m \) can be ignored. In this approximation, an analytic solution is found\(^4\), \( E_m(z) = E_0 J_m(\Delta \phi) \).

This gives rise to the intensity distribution described above in Eqn. (4.4). This is a rather academic result, of course, because the small \( L \), which allows this multiple-order analytic form to be written, also prevents much energy from ever reaching the higher orders.

\(^4\) This solution holds for the case of normal incidence, i.e. \( \theta_0 = 0 \). It is slightly modified for non-normal incidence.
At longer device thicknesses, coupling between orders is significantly curtailed by the momentum mismatch. If the angle of incidence is adjusted so that $\Delta k_1 = 0$, the so-called Bragg angle, then Klein and Cook found that it is reasonable to ignore all other orders, and only consider $E_0$ and $E_1$. In this situation, termed the Bragg regime, you simply have a pair of first-order coupled differential equations

$$\frac{dE_0}{dz} = -\frac{\Delta \phi}{2L} E_1(z)$$
$$\frac{dE_1}{dz} = \frac{\Delta \phi}{2L} E_0(z)$$

(4.12)

It can easily be seen that a pair of sinusoidal functions form the solution. In the typical case where $E_0(z) = E_0$ and $E_1(z) = 0$, you have

$$E_0(z) = E_0 \cos \left( \frac{\Delta \phi z}{2L} \right)$$
$$E_1(z) = E_0 \sin \left( \frac{\Delta \phi z}{2L} \right)$$

(4.13)

The field builds up in the first order $E_1(z)$ at the expense of the zeroth order $E_0(z)$. The diffraction efficiency $\eta$ can be easily determined, since

$$\eta = \frac{l_1}{l_0} = \frac{E_1^2(L)}{E_0^2(0)}.$$  Thus, we have,

$$\eta = \sin^2 \left( \frac{\Delta \phi}{2} \right)$$

(4.14)

If the device is made a particular length, such that $\Delta \phi = \pi$, then the diffraction efficiency $\eta = 1$. Thus, the diffraction efficiency of a Bragg device theoretically reaches 100%. Hence, most AODs are designed for use in the Bragg regime. In
practice, such diffraction efficiencies are not reached because real devices are in fact a combination of the Bragg and Raman-Nath regimes. Thus, some energy is coupled into the higher orders, but, by arranging for the optical wave to be incident at the Bragg angle, most (up to 90%) of the light can be coupled into a single first order.

4.2 ACOUSTO-OPTIC DEFLECTORS FOR LASER SCANNING

The theory of Bragg diffraction described enables an understanding of all the practically important parameters of acousto-optic devices used for laser scanning, which are depicted in Figure 4-3. This section describes these parameters and how they impact laser scanner performance, leading to a discussion of the specific AODs chosen for the and the system parameters, such as scan angle magnification and beam diameter, that determine the ultimate spatio-temporal resolution of the Dual Scanner system. Finally, optical polarization considerations for AODs are discussed, both generally and in the context of the Dual Scanner.

![Figure 4-3 Bragg Acousto-optic Interaction](image.png) AOD is tilted relative to incident optical axis by an angle $\theta_B$, known as the Bragg angle. At the central acoustic frequency, the incident beam is deflected by an angle $2\theta_B$. Practically, the scan angle range over the acoustic frequency bandwidth is generally $\sim \theta_B$. 

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4.2.1 AODs for Laser Scanning Applications

*Acoustic Optic Materials: Figure of Merit*

An important parameter describing an AO material is referred to as the *acousto-optic figure of merit*, and denoted $M_2$. Referring to Equation (4.14), it is clearly desirable to maximize the phase change per unit length ($\Delta \phi/L$) in order to minimize the thickness of material required for maximal diffraction efficiency. The parameter $M_2$ reflects the extent to which the optical index of refraction is modulated by an acoustic wave of intensity $I_s$,

$$\Delta n_o = \left( \frac{1}{2} M_2 I_s \right)^{1/2}.$$  \hspace{1cm} (4.15)

Commonly used AO media are those w/ large figures of merit, such as Tellurium Oxide ($\text{TeO}_2$) or Lead Molybdenate ($\text{PbMoO}_4$). These give rise to large diffraction efficiencies with modest acoustic power and moderate device thicknesses.

*Bragg Angle and Associated Parameters*

As the vector diagram in Figure 4-3 demonstrates, it is straightforward to determine the Bragg angle of an AOD. In order to satisfy the phase-matching (momentum conservation) condition, the incident optical wavevector $\vec{k}$ is added to the acoustic wavevector $\vec{k}$ to yield a deflected optical wavevector $\vec{k}'$. Because the magnitude of the optical wavevector is much greater than that of the acoustic wavevector, this can be considered an isosceles triangle. The Bragg angle,
defined as the angle the incident wavevector must be off-axis to achieve the optimal diffraction efficiency\(^5\), is simply half the apex angle,

\[
\sin \theta_b = \frac{|\mathbf{k}|}{2} = \frac{\lambda}{2\Lambda}
\]  

(4.16)

where \(\lambda\) and \(\Lambda\) are the optical and acoustic wavelengths, respectively. It is apparent that the deflection angle \(\theta_{\text{def}}\) — the angle by which the first diffraction order is separated from the zero order — is given by \(2\theta_b\).

The Bragg angle \(\theta_b\) and the deflection angle \(2\theta_b\) are fundamental parameters of an AOD, given on the device specification sheets. It is clearly dependent on the optical wavelength. Furthermore, it is also apparent that the deflection angle is dependent on the acoustic velocity \(v\) and the acoustic frequency \(f\), given that \(\Lambda = v/f\). Under the small-angle approximation, which is generally valid for practical values of the Bragg angle, \(\sin \theta_b \approx \theta_b\) and Equation (4.16) can be rewritten

\[
\theta_b = \frac{\lambda f}{2v}.
\]  

(4.17)

Using this formulation it is easy to derive another key practical parameter of an AO device — the scan angle range \(\theta_{\text{scan}}\). This is the range of deflection angles that can be scanned to across the acoustic frequency bandwidth of the deflector, and is simply given by

\[
\theta_{\text{scan}} = \Delta \theta_{\text{def}} = \frac{\lambda \Delta f}{v}.
\]  

(4.18)

\(^5\) Or, equivalently, the angle by which the AOD needs to be tilted. This is the approach used in
It is apparent, then, that maximizing the scan angle range is tantamount to maximizing the acoustic frequency bandwidth $\Delta f$. However, note that varying the acoustic frequency also causes the phase-matching condition to be violated, causing a decrease in diffraction efficiency that increases as the frequency is varied further from the center frequency. To counteract this, practical devices employ a phased array of transducers that generate acoustic waves over a small range of directions. As the acoustic frequency is varied, the incident optical wave "selects" the acoustic wavevector that satisfies the phase-matching condition.

Practical limitations in designing these transducers generally limit the acoustic frequency bandwidth $\Delta f$ to $\sim 50\%$ of the central acoustic frequency $f$ (J. Lekavich; personal communication). Thus, following Equations (4.17) and (4.18), it is evident that, practically, the scan angle range $\theta_{\text{scan}} = \theta_b$, as is shown in Figure 4-3.

*Time-Bandwidth Product*

Figure 4-3 also depicts the effective optical aperture $D$. It is straightforward to see that $\tau = D/v$, where $v$ is the acoustic wave velocity. Meanwhile, as discussed in the last chapter, the time-bandwidth product $N = \tau \Delta f$ determines the spatial resolution of the laser scanner. This can now also be easily understood. The number of angularly resolvable "spots" can be obtained by dividing the total angular scan range of the AOD by the angular "spreading" (divergence) of the

\[\text{practice.}\]

\[\text{While the this transducer based limit on the scan angle range must be construed as a limitation, it does confer one advantage: it ensures that there is no "cross-talk" from other diffraction orders, which can never be fully eliminated, into the selected first diffraction order.}\]
incident optical wave (i.e. the laser beam) due to diffraction. Using the approximation that this diffractive divergence \( \theta_{\text{beam}} \approx \frac{\lambda}{D} \), we show

\[ N = \frac{\theta_{\text{scan}}}{\theta_{\text{beam}}} = \frac{\lambda \Delta f / \nu}{\lambda / D} = \frac{\Delta f D}{\nu} = \tau \Delta f. \]  

(4.19)

While this equation makes it apparent that varying the optical aperture \( D \) can be used by the user of an AOD to trade off spatial and temporal resolution, as was discussed in Chapter 3, it is also apparent that the acoustic velocity \( \nu \) of the AO medium determines the spatio-temporal resolution of the AOD for a given \( D \). Slower acoustic waves increase the spatial resolution \( N \) at the proportional expense of temporal resolution \( \tau \).

**Acousto-Optic Deflectors for the Dual Scanner**

The acoustic velocity is therefore a significant parameter of interest in specifying an AO material. This intrinsic property of the AO medium, along with three “extrinsic” properties of the AO scanning system—1) the effective optical aperture \( D \), 2) the scan angle magnification factor, and 3) the field-of-view of the objective lens—ultimately determine the spatio-temporal resolution achieved by the laser scanners. Table 4-1 shows the properties of the AODs employed in the Dual Scanner. The properties in white are the nominal device specifications, provided by the vendors; the shaded properties incorporate the extrinsic parameters of the current Dual Scanner optical layout (discussed further below).

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\(^7\) A multiplicative factor can be added to this expression, which is tantamount to specifying the angular resolution criterion (i.e. Rayleigh, Sparrow) and the shape of the optical aperture \( D \) (i.e. rectangular, circular, Gaussian).
<table>
<thead>
<tr>
<th></th>
<th>Visible AOD</th>
<th>UV AOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Tellurium Oxide, slow shear mode</td>
<td>Fused Silica</td>
</tr>
<tr>
<td>Acoustic velocity (m/s)</td>
<td>620</td>
<td>5900</td>
</tr>
<tr>
<td>Central Acoustic Frequency (MHz)</td>
<td>80</td>
<td>150</td>
</tr>
<tr>
<td>Acoustic Frequency Bandwidth (MHz)</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>RF Drive Power (maximal)</td>
<td>&lt;1W</td>
<td>3W</td>
</tr>
<tr>
<td>Diffraction Efficiency</td>
<td>&gt;80%</td>
<td>80%</td>
</tr>
<tr>
<td>Time-Bandwidth Product</td>
<td>64 / mm aperture</td>
<td>13 / mm aperture</td>
</tr>
<tr>
<td>Scan Angle Range (mrad)</td>
<td>~33</td>
<td>4.8</td>
</tr>
<tr>
<td>Beam/aperture size (mm)</td>
<td>~2</td>
<td>~1.5</td>
</tr>
<tr>
<td>Number of Resolvable Spots (nominal)</td>
<td>128</td>
<td>20</td>
</tr>
<tr>
<td>Access Time (μs)</td>
<td>3.2</td>
<td>26</td>
</tr>
<tr>
<td>Scan Angle Magnification</td>
<td>2.5X</td>
<td>12.5X</td>
</tr>
<tr>
<td>Magnified Scan Angle Range (mrad)</td>
<td>82</td>
<td>60</td>
</tr>
<tr>
<td>Estimated Spot Size (μm)</td>
<td>~2.5</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Table 4-1: Properties of visible and UV AODs in the Dual Scanner. Parameters in white reflect the manufacturer specifications. Shaded parameters are specific to the Dual Scanner system. Beam waist size given reflects the Gaussian beam waist parameter, rather than an apertured plane wave; thus the nominal number of resolvable spots (i.e. time-bandwidth product) given is artificially inflated.

For the visible scanner, TeO₂ deflectors (LS55V; Isomet) were elected owing both to their large figure of merit, as well as their ability to support a "slow shear" acoustic wave, which, as the name suggests, is an acoustic plane wave mode with slow acoustic velocity. Employing this mode, the nominal specification for the time-bandwidth product is 64/mm beam diameter, where "diameter" refers truly to only the axis along the acoustic wave. Given a 2mm beam diameter employed in the visible scanner (see Optical Layout section), this leads to a
nominally 128 spot resolution. Because of the slower acoustic velocity, meanwhile, it takes ~3.2μs for the acoustic wave to cross a 2mm beam. Given the scan rate of up to 100kHz discussed, the laser dwell time per site can be as short as 10μs; thus, increasing $D$ through the use of cylindrical optics is not a viable option here. In other words, the combination of spatial and temporal resolution achieved for the visible scanner is nearly optimal.

For the UV scanner, fused silica deflectors (SDM-1502B8; IntraAction) were chosen, because of its high UV transmittance and, thereby, its resistance to damage at the high Q-switched laser pulse energies. Compared to TeO$_2$, however, it is an inferior AO medium. Owing to its poorer figure of merit $M_2$, it is three times as long (~60mm) and is driven by three times the RF drive power to achieve comparable diffraction efficiency. Employing these measures, the specified diffraction efficiency is nearly comparable to that achieved by TeO$_2$\textsuperscript{8}.

Moreover, the acoustic velocity of fused silica is much greater, so the time-bandwidth product is only 13/mm beam diameter. Meanwhile, the temporal resolution is ~300ns for the 1.5mm beam diameter employed (see the Optical Layout section). Considering the UV laser repetition rate of 50kHz, this temporal resolution is excessive. In contrast to the visible AODs then, it would be propitious to improve the spatial resolution at the expense of temporal resolution by increasing $D$. For this reason, a new optical pathway based on cylindrical optics is currently under development.

\textsuperscript{8} In practice, the peak achieved efficiency with the fused silica deflectors is somewhat below specification, about 70% for the first deflector.
Polarization and Birefringent Bragg Diffraction

Materials for AO deflection range can be either isotropic or anisotropic. In the latter, more common case, the possibility of so-called birefringent Bragg diffraction arises. In contrast to an isotropic AO diffraction process, where the electronic polarization direction remains unchanged upon deflection to a higher order, in an anisotropic AO process, the polarization direction changes. If the medium is anisotropic, then the index of refraction changes, altering the determination of the momentum matching condition. The effects of this are well described in (Xu and Stroud, 1992), but are of little consequence to the practical user. However, of importance to this user is the fact that anisotropic materials form an important class of AO materials, in part because they are apt to support shear (transverse) acoustic waves, which may have a much slower acoustic velocity than the more conventional longitudinal waves, significantly increasing the time-bandwidth product and hence the scanner’s spatial resolution. This type of acoustic mode is employed in the TeO₂ deflectors used in the visible scanner.

In anisotropic AO media, the polarization of the incident optical wave affects the determination of AO parameters such as $M_2$ and $\theta_b$, as these are both based on the optical index of refraction. As a result, such devices are specified with an optimal polarization direction for the incident wave. As shown in Figure 4-4a, in order to satisfy the attain the optimal polarization for both the X- and Y-deflectors in a 2-D scanner, a half-wave plate must be used between the two deflectors, as shown in Figure 4-4a.
This scheme is employed for the fused silica UV AODs in the dual scanner, because, although fused silica is nominally isotropic, the UV deflectors used do show a preferred polarization, namely orthogonal to both the acoustic and optical plane vectors.

Some anisotropic devices, including the slow-shear TeO$_2$ AODs used in the visible scanner, exhibit optical activity; they rotate the polarization of the optical wave. In order to use such devices in an X-Y scanner, a quarter-wave plate can be used before both scanners to achieve a circular polarization state, which is an eigenmode of the deflectors. Alternatively, linear polarization can be employed, and no polarizing optics may be required; as shown in Figure 4-4b, if the AOD itself is designed so that it rotates the polarization $90^\circ$, ensuring the
optimal condition for both defectors. This latter condition applies for the TeO$_2$ AODs in the visible scanner, and no polarizing optics are employed there.$^9$

4.3 DUAL SCANNER OPTICAL PATHWAY: FOURIER OPTICS

Each AO scanner consists of a succession of relay telescopes: the beam expander, the AO scan optics, the scan magnification telescopes, and the final relay optics. Figure 4-5a illustrates an important concept: lenses effectively perform Fourier transforms between the input (back) focal plane and the output (front) focal plane. Thus, a relay telescope performs successive Fourier transformations, causing the original image to be reproduced at the output plane, save for a magnification factor $M$. We can apply this notion to get an intuitive understanding of the transformations performed at each relay telescope in the scanner pathway.

Figure 4-5b shows again a generalized linear AO scanner optical pathway. The shared focal planes within each relay telescope, where the beam comes to a focus at the current scan position are referred to as image planes; the shared focal planes between each relay telescope, where the beam is collimated (i.e. consists of rays parallel to the optical axis) and the optical paths from all scan angle positions converge at a single point are referred to as pivot points, or diffraction planes. This figure illustrates a key point that follows easily from viewing each lens as a Fourier transformer: the image and diffraction planes are Fourier conjugates, so that the size of the beam in the diffraction planes is

---

$^9$ An additional advantage, beyond the lack of required polarizing optics, is that, for these particular TeO$_2$ defectors, linear polarization produces a more uniform diffraction efficiency
Figure 4-5 Simple Fourier Optics View of Dual Scanner a) Relay telescope can be regarded as a double Fourier transformer b) Scan magnification telescope decreases the size of the pivot point, thereby increasing the size of the subsequent image plane. c) Each successive block of the Dual Scanner alters the beam size at the subsequent pivot point and image plane focus, with the two inversely related as a Fourier pair.

inversely proportional to the focal spot size in the image plane\(^{10}\). This is analogous to the inverse relation of \(\Delta t\) and \(\Delta f\) for a temporal Fourier transform pair.

The effects of these successive Fourier transformations at each stage of the AO scanner pathway are depicted in Figure 4-5c. For example, the beam expander increases the collimated beam diameter in the diffraction plane (i.e. across the scan angle range, albeit slightly lower than the circular polarization eigenmode case. \(^{10}\) The term “beam” is used in the diffraction plane, and “spot” in the image plane, because the laser beam is collimated in the former and focused in the latter.
The Fourier picture thus serves to explain, even for a single spot in the scan pattern, how the beam expander improves the system spatial resolution while the scan magnification telescope compromises it, by expanding and compressing the collimated laser beam, respectively. Of course, this picture is entirely consistent with the previously developed notions that expanding the beam before the AODs increases the time-bandwidth product $N$, and that expanding the angular scan range to fill the field-of-view with that limited time-bandwidth product reduces the spatial resolution in the specimen plane.

As will be shown (see Performance section below), the UV scanner focal spot size is <10μm, while the visible scanner focal spot size is ~2.5μm. This difference can be clearly deduced by examining the back focal plane of the objective. For the visible scanner, the laser beam diameter at the back focal plane is ~Ø1mm. For the UV scanner, the beam diameter at the back focal plane

---

11 This "scan pattern" can be seen as a stationary pattern if the laser is set to toggle very quickly between a few positions, as is done during alignment.
is comparatively very small (\(\sim 0.2 \text{mm}\)), such that the beam can no longer be considered fully collimated.

Considering the small size of the collimated beam at the final pivot point, it becomes clear that another way to understand the poorer spatial resolution performance of the UV scanner is simply that the scan angle magnification factor (and hence the beam "demagnification" factor) required to fill the field of view is too great compared to that of the visible scanner. In other words, the difference arises simply because the deflection angle \(2\theta_b\) of the visible AODs is much greater than that of the UV AODs, as seen in Table 4-1. Indeed, examining Equations (4.17) and (4.19), it is clear that, for a given beam diameter \(D\), a large time-bandwidth product \(N\) is tantamount to a large deflection angle \(2\theta_b\) (and hence a large scan angle range \(\theta_b\)).

One practical consequence of the small pivot point that is developed in the UV scanner optical path is that this leads to a very high energy density, given the small volume and the use of Q-switched pulses. This could give rise to laser damage, which must be guarded against (see below).

Another effect of the UV scanner's very small pivot point is the increased importance of exactly "imaging" this pivot point to the objective's back focal plane. To achieve the best possible focus at the objective, ideally the Gaussian beam waist coincides with the back focal aperture. Because the divergence of the UV beam after scan magnification is much greater, the effect of "defocus" of the pivot point is more pronounced, leading to a greatly increased focal spot size. As shown in Figure 4-6, defocus between the final pivot point and the objective's
back focal aperture has another effect, important for both the visible and UV scanners: the focusing pathway becomes off-axis. The “hourglass” shape of the focus will thus intersect other X-Y positions at planes slightly defocused from the specimen plane, somewhat diminishing the true spatial resolution.

Ultimately, then, it is important to correctly “relay” the pivot point to the back focal plane. To accomplish this, each of the relay telescopes in the laser scanner must be spaced by the sum of the two focal lengths of the lenses involved. This ensures true relay imaging—the output focal plane of each telescope coincides with the input plane of the subsequent telescope. This imposes a significant restriction, however, in that the position of every lens in the scanner pathway from the objective back to the AODs is constrained.

### 4.4 OPTICAL DESIGN OF THE ACOUSTO-OPTIC SCANNERS

The complete optical layout of the Dual Scanner, as currently configured, is provided in Figure 4-7. We refer to this figure in the following sections, which describe the choice of optics, the mechanical implementation, and finally the optical design of each section of the optical pathway.
Figure 4-7 Optical Layout of Dual Scanner
4.4.1 Dual Scanner Optical Layout

A brief description of the design and construction of each section of the layout is provided here.

Beam Expander

Immediately at the output of each laser is a “beam aligner”—a pair of adjustable mirrors with sufficient degrees of freedom to steer a laser beam. These are used to align the beam straight through both the subsequent beam expander and AO scan optics path.

The visible scanner employs an f40 (i.e. focal length=40mm) and f100 lens to form a 2.5X beam expander, which, given the ~1mm Gaussian beam waist of the Argon laser beam, serves to slightly overfill the 2mm short-axis of the AODs' aperture. This maximizes the spatial resolution to the greatest extent possible without employing cylindrical optics to fill the AODs' asymmetric aperture (7x2mm). The UV scanner, however, does not employ a beam expander in the current configuration. The laser's output beam is specified with a 1.5mm beam waist, so this somewhat underfills the short axis of the AODs' aperture (20x2mm). The beam expander was left out in order to maximize the power throughput of the UV scanner.

AO Scan Optics

Each scanner contains a pair of 1:1 relay telescopes, one following each of the two AODs. In the visible scanner, f40 lenses are used for each telescope. These are the shortest focal length lenses that mechanically allow the lenses to
be located a focal length away from the center of the AODs as well as to permit an adjustable slit assembly (20-532; AF Optical) to be located in each of the image planes. In the UV scanner, the deflectors are longer than standard AODs owing to the use of fused silica. Thus, longer focal length lenses are employed, f50 in the first telescope and f100 in the second telescope.

Some commercial AO scanners employ a simpler design—arranging the X and Y deflectors “back-to-back,” in contrast to the design employed here, with a telescope between the two deflectors. This telescope, however, provides the advantage that, by arranging the deflectors in equivalent optical planes, both the X and Y pivot points can be safely considered coplanar (insofar as the thick AO device can be said to give rise to a single pivot point). Appealing to the discussion above about the effects of defocusing the pivot point, the arrangement employed can be seen to ensure the spatial resolutions in both dimensions are both equal and optimized.

In the UV scanner, as discussed, a half-wave plate is located within the first telescope to rotate the polarization direction by 90°. It is located as far from the image plane (i.e. the slit plane) as possible, to avoid optical damage (see below).

Following the two AODs and telescopes, each scanner pathway contains a beam aligner that allows the angular scan pattern that is off-axis to be aligned straight through the subsequent scan magnification optics, as shown in Figure 4-5b.
Scan Magnification Optics

The scan magnification telescopes serve to boost the angular scan range of the scan pattern to better fill the objective’s field-of-view. In the visible path, a modest 2.5X magnification is provided, which produces a final angular scan range of 82mrad, as listed in Table 4-1. In the UV scanner, a much greater magnification is required owing to the small Bragg angle of the fused silica deflectors. A total of 12.5X magnification gives rise to a scan angle range of 60mrad.

The f100/f40 and f60/f60 telescopes provide the 2.5X magnification of the visible scanner. Meanwhile, the f100/f20 and f100/f40 telescopes provide the 12.5X magnification of the UV scanner in two steps. Although the magnifications achieved could be achieved with a single telescope, the two stage approach allows these magnification factors to be adjusted using readily available lenses, without any need to translate the preceding optics to maintain relay imaging. For example, the f60/f60 telescope could be replaced with an f80/f40 telescope to increase the field-of-view of the visible scanner by 2X without requiring any other optics to be moved.

Prior to the two telescopes that comprise the scan magnification, both scanners contain an additional f200/f200 telescope. This is inserted as a “buffer” between the AO scan optics and the scan magnification phase, which serves two purposes. First, this allows the AO scan optics to be located further from the microscope, which provides the mechanical space required for the lasers as well as the beam aligner used to center the scan pattern. Second, because of the
long focal lengths of these lenses, the effect of a small defocus (i.e. interlens spacing is not exactly 400mm) is not significant. This telescope, then, serves to relax the strict requirement for every optic from the objective back along the pathway to be in a particular position. This allows the AO scan optics and the scan magnification telescope to be adjusted more or less independently.

*Relay Optics*

The final telescope in both scanners' optical path is an f100/f100 telescope. The second lens is shared between the two scanners, and resides within the custom dichroic mirror slider in the inverted microscope. The two scanner pathways are combined with a dichroic mirror (*DCLP400*; Chroma Technology) that reflects the UV laser, but allows the visible laser to pass.

The importance of this final telescope is that, mechanically, the last lens can no be no less than ~100mm from the back focal plane. The final lenses of the scan magnification telescopes are, thus, too short to properly image the pivot point to the objective's back focal plane.

A final beam aligner is employed just before the relay telescope. These degrees of freedom are employed to align the scan pattern straight through the objective lens.

*Objective Lens*

Finally, the last, and very critical, optic in each scanner pathway is the objective lens. As mentioned, the Dual Scanner employs an oil-immersion, high NA (1.3), high-magnification (100X) objective lens (*Fluar*, Zeiss) with a field-of-view of ~200μm. An ideal laser-scanning microscope system would permit
diffraction-limited imaging, i.e. laser spot sizes as small as physics allows. Since
the lateral resolution limit is given by
\[
\Delta x_{\text{spot,min}} = \frac{\lambda}{2NA},
\]  
(4.20)
it is theoretically possible with the NA 1.3 objective to focus a 500nm laser beam
to a spot size of ~200nm. But, in order to achieve this spot size with an AO
scanner across the entire objective field-of-view, a very large time-bandwidth
product of \( N = (200\mu m / 200\text{nm}) = 1000 \) would be required. In practice, then, the
objective is not currently the limiting factor in the spatial resolution of the laser
scanners.

The high NA objective lens however is desirable for two reasons. First, the
excellent spatial resolution is utilized when performing either DIC or wide-field
fluorescence imaging. Second, the amount of fluorescence collected scales as
NA\(^2\); thus, the higher NA allows a stronger signal from the voltage-sensitive dye
to be collected during optical recording.

The dichroic mirror in the custom slider is chosen with a cutoff frequency
just above the visible laser wavelength generally employed, namely 488nm. A
495 DRXR dichroic mirror (Chroma Technology) is employed, which reflects both
the 355nm and 488nm laser wavelengths, and passes all light above the 495nm
cutoff, which corresponds to the fluorescence collected during optical recording.

4.4.2 Mechanical Implementation and Alignment

As has been discussed, the Dual Scanner optical paths were constructed
using an optical rod prototyping system (*Microbench*; Linos Photonics). This
provides several advantages including self-alignment of lenses, the ability to remove and reinsert sections of the optical path, and the availability of accessories for many common elements of the path. These include the adjustable slits (20-152; AF Optical) and the dichroic mirror holders (AF Optical).

Most of the optics are constrained to be centered on the optical axis, by virtue of the rod system. However, some are mounted on X-Y positioners of varying resolution. These can be categorized loosely as coarse, medium, and fine resolution x-y positioning (06-5040, 06-5070, and 06-5010, respectively; Linos Photonics). These allow degrees of freedom to be added at key points along the optical path.

Among the optics that are placed on positioners are the second lens of the telescope after the first deflector, as well as the second deflector. These adjustments allow the deflected first order from the first deflector to be maximally coupled into the second deflector. Another is the second lens of one of the scan magnification telescopes (the f20 on the UV scanner and the f40 on the visible scanner, see Figure 4-7). The effect of decentering this lens is to cause the whole scan pattern to be angularly shifted about the subsequent pivot point. This ultimately causes a direct spatial offset of the scan pattern in the specimen plane. This adjustment can be helpful along with the final beam steering mirror assembly to produce a centered, on-axis scan pattern. It also serves as a useful “quick and dirty” translation of the scan pattern (dirty because it also slightly repositions the pivot point, leading to off-axis focusing similar to the result of defocusing the pivot point).
Another important consideration in implementing the optical configurations is that no mirrors should be located too near to either a diffraction planes or an image plane. In the former case, the large beam energy densities can promote damage (in the case of the UV scanner), while in the latter case, small particles of dust or grime on the mirror will ultimately contaminate the scan pattern.

Finally, discussion of the alignment procedure for the AO scanner pathways is given in Appendix 1.

4.4.3 Optical Components

This section discusses the specific “optics”—i.e. lenses, mirrors, and other components employed in the Dual Scanner optical path.

Optical Aberrations

Reexamining Figure 4-4b, it is apparent that many off-axis rays and off-axis angles are incident on each of the Dual Scanner lenses; these make the optical system susceptible to spherical aberration and coma, respectively. These optical aberrations can limit the quality of focus achieved in each image plane and ultimately the specimen plane, undermining the system’s spatial resolution.

One means of reducing spherical aberration is to choose the appropriate “lens shape factor.” As discussed above, the laser beam outside each of the relay telescopes can be considered as collimated. Thus, every lens in the AO scanner pathway can, using the optical jargon, be said to image “from infinity” or “to infinity.” It is well known that the plano-convex lens shape (as opposed to biconvex or meniscus lens shapes) produces the minimum spherical aberration when performing such “infinite conjugates” imaging. Thus, the AO scanners
employ exclusively plano-convex lenses. The optimal performance occurs when
the curved surface of the lenses is oriented towards the side where the beam is
collimated, and the planar surface is oriented towards the side where the beam is
focused. Thus, as is seen in Figure 4-7, the curved surfaces of each lens in the
AO scanners "face" the diffraction planes and the planar surfaces face the image
planes.

Another means of reducing spherical aberration is to employ achromatic
lenses\textsuperscript{12}. These doublet (dual element) lenses are employed in the visible
scanner pathway. In the UV scanner, however, singlet (single element) lenses
are employed, because most commercially available achromats employ UV
curing cement to bond the two lens elements. This cement is susceptible to laser
damage at the high UV laser energy densities. Air-spaced achromatic lenses are
commercially available, but are prohibitively expensive.

No particular measures are employed to reduce coma. Fortunately,
however, the relay imaging configuration itself, used throughout the Dual
Scanner, is quite effective for reducing coma (Hecht, 1987,p. 225).

\textit{Materials for High Throughput}

In the visible scanner, the doublet lenses employed all are comprised of
common optical glasses for both lens elements. These glasses are very
transmissive at visible wavelengths. Meanwhile, standard aluminum mirrors can
be used for beam-steering in the visible pathway.

\textsuperscript{12} Achromats, as their name implies, also reduce chromatic aberration. This is of limited utility
here, of course, since each optical pathway is monochromatic.
Turning to the UV scanner: the standard optical glasses are all at least mildly absorptive at the 355nm UV wavelength. Because of the large number of lenses employed (12 lenses per scanner → 24 optical surfaces), it is important to maximize the transmission of each lens to achieve power sufficient throughput. Furthermore, all of the optics in the UV optical path require careful attention to damage thresholds. Coming from the laser at a beam diameter of ~1.5mm, the energy density of the 750mW, 50kHz UV laser is ~0.8mJ/cm². As discussed above, in the scanner optics the beam diameter is nominally demagnified by 12.5X, to 12mm. Consequently, the peak energy density in the path for a collimated beam is 130mJ/cm², already above the damage threshold for many standard optics. (The energy density is even greater in the image planes, where the focal spot size can be <100μm in the telescopes before the scan angle magnification. Care is taken to ensure that no optics are located near an image plane).

For these reasons, fused silica lenses are employed in the UV scanner; they provide >99% transmission and can safely tolerate high UV energy density. The mirrors employed in the UV scanner are high energy dielectric mirrors, which are specifically designed for high reflectivity at the 355nm wavelength (DLHS 355; Linos Photonics). The half-wave plate used to rotate the polarization between the two deflectors (as shown in Figure 4-4b) is an air-spaced (i.e. cement-free) quartz design, and is thus suited for high UV energies (05RP12-08; Newport).
Because of the large number of optical surfaces, it is important to employ anti-reflective (AR) coatings on all the lenses. The coatings employed are a conventional broadband visible AR coating for the visible scanner (ARB2 VIS; Linos Photonics), and a broadband UV AR coating for the UV scanner (ARB2 UV; Linos Photonics).

Finally, the UV transmission of the objective lens is a concern. At 355nm, the Fluar transmits greater than 90%. Nonetheless, inside the multi-element lens, the energy density can become quite high as the beam is focused, potentially causing damage to the cement. Such damage has been observed in testing the Dual Scanner.

One solution would be to use objective lenses designed for high UV transmission. However, to this author’s knowledge, there are no commercially available lenses that are 1) highly UV transmissive, 2) high NA, and 3) well corrected for aberration (i.e. suited for high resolution imaging)\textsuperscript{13}.

Fortunately, it appears that only prolonged exposure gives rise to damage mechanisms in the Fluar lens employed. As currently conceived, the Dual Scanner is designed only for short exposures of UV illumination (<1s); thus, damage is not expected during experiments. During alignment and testing, however, a “test pattern” is used (see Appendix 1) in which the illumination is prolonged. In order to protect the objective lens during this procedure, the beam is attenuated. For this purpose, a high damage threshold OD 2.0 dielectric attenuator is employed (UVDA-355-2.00-10; CVI Laser).
4.5 PERFORMANCE OF ACOUSTO-OPTIC LASER SCANNERS

Here we evaluate and compare the optical “performance” of the AO scanners, with attention to the spatial resolution, the uniformity of the scan pattern, and the power throughput.

4.5.1 Visible Scanner

To evaluate the visible scan pattern, a thin layer of fluorescent dye (fluorescein) was used as the specimen\textsuperscript{14}, the software was set to cycle through a grid pattern of scan positions rapidly, and the resultant fluorescent image captured by video or CCD camera. A video image of a 10x10 visible scan pattern is shown in Figure 4-8a. The programmed scan pattern covered the entire tuning range of the visible deflectors. It is apparent that the full scan range covers \(\sim 100\mu m\) in the objective’s field-of-view, which is approximately half the full field-of-view. Thus, an additional factor of 2X scan magnification could be employed to cover the entire field-of-view. From this image, it was estimated from the lowest row (where there are few saturated pixels) that there are 40 resolvable spots\textsuperscript{15} per dimension, implying a spatial resolution of \(\sim 2.5\mu m\), as given in Table 4-1. In Figure 4-8c, a line profile was taken of a portion of the lower line of the scan pattern in Figure 4-8a (Scion Image; Scion Image). It should be mentioned that, while the specimen was nominally “thin,” it is sufficiently thick that some out-of-

\textsuperscript{13} One high NA fused silica lens tested (03-0206; Partec) produced excellent focusing of the UV with minimal loss, but was poor at imaging, showing marked field curvature.
\textsuperscript{14} 10\textmu L of dye is “sandwiched” between two 25mm coverslips, which yields a 20\textmu m thick layer of dye if the dye spreads to fill the whole area.
\textsuperscript{15} A sharp visual resolution criterion was applied using Photoshop (Adobe Sytems) in which little overlap between spots was tolerated. This is a conservative estimate, therefore.
Figure 4-8 Visible Laser Scan Pattern a) Video image of scan pattern shows that ~100 μm of the field-of-view is covered. b) CCD image of scan pattern reveals the non-uniformity of scan pattern intensity. c) A line section of the lowest row of the image in (a) reveals the FWHM of one spot to be ~3.2 μm.

Focus fluorescence will be detected\textsuperscript{16}. As a result, the estimates of spatial resolution obtained are somewhat pessimistic.

In Figure 4-8b, a CCD image was taken (see description of CCD imaging pathway below), revealing the variation in intensity of the visible scan pattern to be somewhat stochastic: it's not strictly tapering towards the edges, as would be expected. Much of this variation may be attributable to the off-axis incidence of

\textsuperscript{16} Nominally, we would expect a spot size ~1 μm. The Rayleigh range of a Gaussian beam focused to a 1 μm waist is ~12.56 μm assuming 500 nm illumination. Assuming a Gaussian focus, the focusing "hourglass" would occupy an area of at least 1.8 μm in the 20 μm thick volume used.
the laser into the second (Y) deflector—adjusting the Bragg angle and position of this deflector can be used to alter the distribution of intensities. The variation in intensity, however, is not too problematic for the visible scanner, because the recording at each site-of-interest is normalized to the local intensity.

Examining Figure 4-8a closely reveals dim lines of fluorescence connecting the recording sites. This is visual evidence of the somewhat slower settling time of the TeO₂ deflectors, illustrating the importance of discarding the first several recording A/D samples following an update to the scan position (by the Vis Scan D/A converter).

4.5.2 UV Scanner

In Table 4-1, the estimated spatial resolution of the UV scanner is <10μm. This is verified in Figure 4-9a, where the laser spot size is directly compared to the size of a 10μm fluorescent bead (Fluospheres; Molecular Probes), again within a thin layer of fluorescent dye. As shown in Figure 4-9b, the number of clearly resolvable spots per dimension can be estimated as ~15, which coincides well with the nominally expected value in Table 1. In order to maximize the spatial resolution, the UV scan pattern fills less of the field-of-view than the visible scan pattern.

\[17\] The expected number of resolvable spots of 20 is based on the more lax Rayleigh criterion, so that 15 spots are clearly resolvable is quite good.
Qualitatively, compared to the visible scanner, the UV scan pattern has a more uniform intensity distribution. However, a strictly uniform, or at least calibrated, intensity distribution would be of significant benefit for the UV scanner. This would allow for straightforward mapping of receptor distributions, and the potential for quantitative analyses of post-synaptic properties.

A measurement at the back focal plane of a single central spot test pattern revealed a power throughput of \( \sim 25\% \)\(^{18}\). Since the Fluor objective is specified to transmit >90% at 355nm, we can expect a maximal power to the specimen as 169mW\(^{19}\), which corresponds to a maximal energy of 3.4\( \mu \)J/pulse. This is somewhat greater than the maximal energies used by other authors who either used flash lamps, arc lamps, or CW lasers, delivered over much longer periods (~1-10ms) (Schiller et al., 1998; Kandler et al., 1998; Rossi et al., 1997). This

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\(^{18}\) An optical power meter (LaserMate-1; Coherent) was positioned at the back focal plane of the microscope, with the objective removed. The scan pattern was set to one spot in the center of the field-of-view, so this should correspond to the maximal power throughput.

\(^{19}\) Based on 750mW nominal power specification; in fact, when operating well, a full 1W is obtained.
suggests that by simply varying the energy delivered in a single pulse, the entire range of reasonable activation strengths (i.e. to receptor saturation) can be covered. Thus the full temporal resolution potential of the 50kHz bandwidth should be utilizable.

Because the UV laser is pulsed, and the deflector access time is much shorter than the laser repetition period, there is no concern over illumination of undesired regions of the specimen as with the visible scanner. However, a diffuse background illumination is apparent in Figure 4-9b. This, it is found, appears attributable to the large amount of scattered light generated when blocking the zeroth order at the slit following each deflector. While it appears difficult to eliminate this source of background, its effect has been mitigated by programming the UV scan pattern buffer to turn off the laser, rather than just leaving all the power in the zero order, for prolonged “off” periods. See Appendix 3 for details.

4.6 DETECTION OPTICAL PATHWAY

The detection optical pathway of the Dual Scanner allows for video imaging, CCD imaging, and photometric detection with one or more photodiodes. In this section, each of these paths is discussed in more detail.

4.6.1 Shared Pathway

The inverted microscope is raised to provide access to the microscope’s bottom port. An image plane—i.e. a plane with an image of the specimen plane—appears just beyond that port, where an imaging detector would normally be placed.
In the Dual Scanner, a simple part was made to connect an assembly constructed of the optical rod prototyping system employed throughout the Dual Scanner (*Microbench*; Linos Photonics) to this bottom port. The approximately \(\varnothing 20\text{mm}\) image of the specimen plane here serves as the input image plane for the subsequent optics in each of the three separate detection arms. As is evident in Figures 4-10 and 4-11, each of these paths shares a single f200 lens, before the pathway trifurcates. This portion of the detection optical pathway is thus referred to as the “shared pathway.”

4.6.2 Video Pathway

The optical layout of the video imaging pathway is summarized in Figure 4-10a. The f200/f100 lens pair forms an imaging telescope, demagnifying the input \(\varnothing 20\text{mm}\) image to \(\varnothing 10\text{mm}\). This image plane serves as an input to the video zoom lens (*VZM300*; Edmund Scientific), which relays the image to the sensor on the video camera (*WV-1550*; Panasonic). This video output is made available to both a video monitor and to the frame grabber hardware in the system computer. At the lowest magnification setting, the full field-of-view can be seen\(^{21}\); by varying the zoom lens, up to 4X of “external” magnification can be added. This ability to add external magnification is of great importance while the experimenter is forming patch pipette seals at the cell body for

\(^{20}\) The image plane size of \(\varnothing 20\text{mm}\) is constant for all objective lenses. It is straightforward, therefore, to calculate the specimen plane field-of-view. For the 100X objective employed, the field-of-view is simply \(\varnothing 20\text{mm}/(100X)\), or \(\varnothing 200\mu\text{m}\).

\(^{21}\) The video camera employed has a standard 2/3" sensor size (8.8x6.6mm). The zoom lens operates from .75-4X magnification. At the lowest (.75X) magnification, the resulting image on the sensor surface is \(\varnothing 7.5\text{mm}\), so that most of the field-of-view is captured in the video image.
electrophysiological recording and/or dye loading. After a seal is formed, the external magnification can be removed, to allow sites-of-interest to be selected from throughout the (larger) dendritic tree.

As can be seen in Figure 4-10a, the zoom lens is parfocal, meaning that its front and back focal planes remain fixed even as the zoom factor is adjusted. Thus, the camera is attached and remains fixed on one end, while the input image plane is always located a specific working distance (60mm) from the (stationary) first surface of the zoom lens assembly.

The critical distances of the pathway are labeled in Figure 4-10a. The f200 lens in the shared pathway should be nominally 200mm away from the input

---

**Figure 4-10 Imaging Pathways**  
a) Video imaging pathway. The f200/f100 telescope creates a demagnified image at exactly the working distance of the parfocal zoom lens (VZM300; Edmund Scientific)  
b) CCD imaging pathway. The f200/f140 creates a demagnified image at the input plane of the telescope assembly (HR100NK; Diagnostic Instruments), which is at its first lens surface. Filling this lens leads to a final image well-matched to the CCD sensor size.
image plane, while the f100 lens should be 160mm from the first surface of the zoom lens assembly, to account for the 60mm working distance. The spacing between the lenses is not critical in this application: this is not relay imaging.

4.6.3 CCD Pathway

As has been discussed, the Dual Scanner was designed with the possibility of epi-fluorescence imaging in mind, in particular by switching the position of the dichroic mirror slider. Such imaging could be used to permit the visualization of various biochemical markers that could be correlated to the physiological results obtained using the visible and/or UV scanner. In order to achieve low light-level sensitivity, the Dual Scanner incorporates a CCD camera pathway for such epi-fluorescence imaging.

The CCD camera (CH250/A; Photometrics) employs a 386x576 pixel sensor (TH7883; Thompson) with a nearly standard 1" video sensor size (12.8x9.6mm). The CCD imaging pathway includes a fixed magnification (1X) telescope lens (HR100NK; Diagnostic Instruments), primarily because of the convenient mechanical fixtures it contains for mounting the camera.

The imaging pathway used is depicted in Figure 4-10b. An f200/f140 lens combination creates a Ø14mm image plane. This plane is made to coincide with the first lens surface of the telescope assembly (personal communication; Diagnostic Instruments), in order to produce a final image on the CCD sensor chip.
4.6.4 Photodiode Pathway(s)

Signal Photodiode(s)

The purpose of the photodiode pathway is to quantify the amount of fluorescence originating from the specimen plane, independent of the location of the fluorescence (although, using the visible laser scanner, at any instant of time, the fluorescence source is localized to a particular location in the specimen plane). Optically, this entails collecting all the light from a specimen image plane into a small spot that fills (or underfills) the active area on the \( \approx 1\text{mm}^2 \) photodiode employed (S1226-18; Hamamatsu).

It would be most straightforward to employ a larger photodiode area. This, however, gives rise to additional capacitance, which causes the detector response time to be too slow. Instead, it is found, empirically, that an eyepiece (10X in this case) can be used to effectively focus all the light to a small spot.

**Figure 4-11: Photodiode Detection Pathway** An 8X eyepiece is used to collect the fluorescence from throughout the field-of-view to a small “focus” at on the photodiode detector active area. A second photodiode is optionally employed for ratiometric detection, by separating the fluorescence into two spectral windows (required components are shown lightly).
The optical path is given in Figure 4-11. In sum, it consists of the shared f200 lens and a second f200 lens that because to gradually focus the bundle of rays back to an image, and serves to collect most of the fluorescence into the eyepiece. The eyepiece itself consists of a field lens and an eye lens. The first field lens further compresses the ray bundle, before the final eye lens brings the light to a final focus.

When testing the system with wide-field illumination, a small spot which appears to be ~1mm is formed beyond the eyepiece. Measurements of the photodiode response when using the visible laser scanner show that there is ample detection efficiency for all points in the scan pattern. The detection efficiency is apparently not uniform, however. One possible explanation is vignetting\textsuperscript{22}. Another is that the spot may somewhat overfill the detector area.

As shown in Figure 4-11, the photodiode pathway can consist of a dichroic mirror and a second photodiode detector. This is used for ratiometric imaging, which can be used to obtain calibrated membrane potential recordings (Bullen and Saggau, 1999). For the most commonly employed voltage-sensitive dye, di-8-ANEPPS, a DCLP570 (Chroma Technology) dichroic mirror is employed, along with a pair of 540/50 and 600/60 bandpass filters (Chroma Technology) to further prevent any spectral leak-through. The optical pathway is identical for both detectors.

\textsuperscript{22} The gradual fading out of an image towards the edges because the light collection cone angle of the system is reduced there. See (Hecht, 1987).
Reference Photodiode

When not employing ratiometric recording, a “Reference” photodiode is employed to measure the visible laser intensity fluctuations, so they may be cancelled (see Figure 3-7a). As seen in Figure 4-7, this photodiode pathway is located towards the end of the AO scanner pathway.

The dichroic mirror (DCLP400; Chroma Technology) that combines the UV and visible scanners reflects a small portion of the visible beam. The f40 lens in this reflected path forms a relay telescope with the preceding f100 lens in the visible scanner path. The reference photodiode is placed at the output focal plane, which is a small pivot point. Thus, the beam is stationary at this plane, always “crossing” the detector plane as the scan position varies. In order to prevent the small fraction of the UV beam that leaks through the dichroic from reaching the detector, a piece of GG455 Schott color glass is placed before the photodiode.

Improving the Signal Photodiode Optical Path

While the optical path for the signal photodiode described above is adequate, the author has recently devised an alternative, more straightforward and robust approach to achieve photometric detection of the entire field-of-view.

The f200 lens in the shared pathway forms a nearly 1:1 relay telescope with the tube lens that resides within the microscope. Thus an image of the back focal aperture (~5mm in size for a 100X objective) appears 200mm beyond this lens. This diffraction plane can be relay imaged to the back focal plane of another objective lens, e.g. a 40X objective. This would create a very small image on the
detector. Because 1:1 relay imaging is used from the back focal plane of one objective to another, the angular extent entering the second objective exactly matches that leaving the first objective. Thus, the resulting image size is just the expected field-of-view for that objective magnification. In other words, a 40X objective should produce a 20mm/40=500μm image at the detector, which is smaller than the detector size. This scheme will soon be tested in the hopes that it may provide better uniformity of detection efficiency.
As discussed in Chapter 3, the electronic signals of the Dual Scanner flow from/to DAQ boards in the scan computer to/from interface circuitry located in the 19" instrumentation rack used to/from the devices/instruments on or near the optical table. This general flow of signals is evidenced in Figure 5-1, which gives the "wiring diagram" for the Dual Scanner.

"Interface circuitry" refers to what the DAQ industry calls "signal conditioning"—the required transformation of electrical signals to match the voltage ranges and impedance characteristics of the DAQ hardware and/or the control/measurement device. As has been discussed, the Dual Scanner electronics is comprised of the following measurement/control systems:

- UV Scanner Control
- Visible Scanner Control
- Fast, High-Resolution Optical Recording
- Electrophysiology Recording and Control

In this chapter, the design and performance of the UV scanner control interface circuitry is presented and discussed here in depth.

The designs employed for the visible scanner control and the fast optical recording are also briefly discussed because they differ substantially from those employed in previously described work (Bullen et al., 1997; Bullen and Saggau, 1999). The changes are a result of employing a considerably faster A/D
converter\textsuperscript{1} in hopes of improving the quality of the optical recordings by allowing increased oversampling.

Both the UV scanner and visible scanner control and measurement have strict synchronization requirements that are discussed in the third section. Finally, the interface for the electrophysiology recording and control, as well as other electronics systems depicted in Figure 5-1 are briefly described in the last section.

\textsuperscript{1} The currently employed A/D converter (Chico/AIX; Innovative Integration) samples at 2.5MHz, compared to the 200kHz sampling used in the previous work.
Figure 5-1 Dual Scanner Electronics Wiring Diagram Footnotes are explained in Appendix 5.
5.1 UV AND VISIBLE SCANNER INTERFACE CIRCUITRY

The interface circuitry for the UV scanner and visible scanners are described here. A circuit diagram is provided for each of the three circuits involved: one each for the UV laser scan position and beam intensity control and one for the visible laser scan position control. In each diagram, the “backplane” section shows the assignment of signals to a DIN41612 connector, which becomes part of the “backplane” of the Eurocard card rack in which the circuits reside. The “front panel” section, meanwhile, shows the BNC connectors available on the card racks’ front panel.

5.1.1 UV Scanner Interface Circuits

The two D/A cards (AWFG; Keithley) that control the RF wave frequency and amplitude are referred to as the UV Scan and UV Mod cards, respectively. Both of these cards provide two analog output channels, which are used to provide tuning voltages to the deflector drivers of the “X” and “Y” AODs. The D/A cards also contain several digital outputs that are updated in synchrony with the D/A conversions. These digital channels on the UV Scan D/A board are used to control the UV laser Q-switch and provide timing signals associated with the scan pattern that is “played out.” These digital signals, as well as the digital interface circuitry required, are discussed.
*UV Interface Circuitry Design*

Both the UV Scan and UV Mod D/A boards (*AWFG*; Keithley) produce output analog voltages in the 0-10V range\(^2\), i.e. the 12-bits of resolution are assigned to this range, so the smallest voltage step is \(~2.5\text{mV}\). The tuning range of the deflector drivers (*DE1503-M*; IntraAction) is 0-1V for both the scan position and modulation inputs.

The interface circuits, then, have two purposes: first, they must attenuate the D/A voltage output by a factor of ten in order to preserve the nominal 12-bit resolution and, second, they must provide current amplification to drive the 50Ω input impedance of the deflector driver control inputs. The circuit must thus be able to source up to 20mA (1V/50Ω) of current.

The circuit designs for the UV Scan and UV Mod boards are shown in Figures 5-2 and 5-3. Each consists of two stages. First, the input voltage from the D/A converter is attenuated by 10x, using a 100kΩ potentiometer. Second, this attenuated signal is passed through a commercial unity-gain buffer amplifier, which can easily drive the 50Ω load.

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\(^2\) This was set by a DIP switch on the board. This unipolar option was elected so that only attenuation, i.e. no offset, is required.
Figure 5-2 UV Scan Interface Circuit
The required circuit performance characteristics are: 1) noise level sufficiently low to maintain as much of the 12-bit (at least 9 bits\(^3\)) resolution as possible, and 2) a "rise time" (defined throughout this chapter as the time to reach 99% of the final value, i.e. 4-5\(\tau\), where \(\tau\) is a first-order time constant) following a step input of <10\(\mu\)s. These requirements ensure, respectively, that 1) excellent positioning resolution is achieved for the UV scanner and that 2) the frequency and amplitude control signals "settle" within the 10\(\mu\)s period between updating the scan/modulation values and the next laser pulse (see Figure 3-7).

To meet these requirements, the BUF04 buffer amplifier was chosen for the UV Scan circuit and the BUF03 for the UV Mod circuit, both from Analog Devices. This allowed their performance to be compared, but the specifications of each (4nV/\(\sqrt{\text{Hz}}\) and 50nV/\(\sqrt{\text{Hz}}\), respectively; settling time <1\(\mu\)s) are more than adequate for the application. Comparing the two circuits, they are identical save for the choice of buffer amplifier\(^4\).

_UV Interface Circuitry Performance_

The output from the UV Scan interface circuit\(^5\) for a test pattern of 8 spots spanning across most of the scan range is shown in Figure 5-4a\(^6\). The steps

\(^3\) 9-bit resolution leads to 500 spot positioning resolution, which corresponds to ~400nm spatially, is chosen as a benchmark because it would enable single synapses to be targeted.

\(^4\) Actually, as shown in Figure 5-3, the UV Mod circuit contains an additional potentiometer per channel, connected between the BUF03 "NULL" pins, which serves to trim the offset voltage at the output. According to specifications, this output offset can reach 6mV when uncompensated. This potentiometer is adjusted so that a shorted input to the buffer driver produces a true zero volt output. The BUF04, meanwhile, has a much smaller offset specification; no offset control is required nor provided.

\(^5\) Specifically, the results from the "X" UV Scan interface circuit are shown. The "Y" circuit was also checked, to ensure similar results. The same is true for the UV Mod and Vis Scan circuits later.
occur at equally spaced intervals across the 0-1V tuning range. A similar trace is
given for the UV Mod circuit (Figure 5-5a), with a single spot test pattern
employed, where the acoustic amplitude is graded at four discrete levels in the 0-
1V tuning range.

In both traces, the tuning voltage level is updated every 20μs—the
repetition rate of the laser. The laser fires halfway between the voltage steps\(^7\),
during which time the tuning voltage should have reached a steady-state level.
That this is indeed the case is evidenced by Figures 5-4b and 5-5b, which show
the results of rise time measurements after transitions on the UV Scan and UV
Mod circuits. Based on a >99% settled standard, the rise times can be estimated
as 1μs and 400ns for the UV Scan and UV Mod circuits, respectively. Both of
these performance figures are more than adequate\(^8\).

Figures 5-4c and 5-5c show the measured RMS noise of the UV Scan and
UV Mod interface circuits, respectively, during the “steady state” when the scan
position and amplitude are constant. For the UV Scan circuit, a single
representative time interval revealed an RMS value of 5.1mV; for the UV Mod

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\(^6\) For all tests of D/A interface circuits, a digital storage oscilloscope (TDS3032; Tektronix) was
used. The circuit outputs were still connected to their destination devices (i.e. deflector drivers) in
order to preserve the loading encountered during actual use. The noise performance of this
oscilloscope was tested by measuring the output with a shorted input; it was found that the noise
level was ~1mV at the highest amplifications, which precludes an accurate observation of the
nominally <1mV noise expected in some cases (i.e. UV Scan and UV Mod). However, it does
allow a minimum performance level to be ascertained.

\(^7\) That the laser indeed fires halfway between the voltage steps is (unfortunately) evident in Figure
5-4a by the presence of a voltage transient in the exact center of each step. This transient is very
likely the result of the high-current digital circuitry that resides on the UV Scan interface circuit
(see next section). This spurious signal is of some concern since it could potentially modulate the
otherwise constant tuning voltage on the crystal just as the laser pulse arrives; however, it is
unlikely that such a short-lived transient could affect the scan position. Nonetheless, it would be
desirable to achieve better isolation between the digital and analog circuits.
Figure 5-4: UV Scan Interface Circuit
a) Pattern of UV Scan control signal used to create an eight-spot pattern ranging across most of the 0-1V tuning range. The laser position is changed every 20μs, coinciding with the laser repetition rate. b) Rise time during a step. The ~1μs rise time observed is very fast relative to the 10μs required. c) Noise measured during the “steady-state” period when tuning voltage is settled. RMS value calculated as 5.1mV.

circuit, a value of 6mV was seen. Given the 1V tuning range for both control signals, these values fall short of the ~500 spot resolution target for the UV Scan control. The noise waveform for the UV Scan circuit showed In both cases the noise exceeds the <1-2mV level that is sought. Based on their specifications, it is unlikely that this can be attributed solely to the buffer amplifiers. Thus, it is more likely that marked periodicity (~6MHz frequency) visible in the UV Scan noise waveform (Figure 5-2c) is a strong indication of an external source—such this “excess noise” may arise from pickup or parasitic capacitances in the layout of the circuit or the ICs themselves. These may be the result of employing discrete,

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It is surprising that the BUF03 outperforms the BUF04 in the rise time, however. The specifications suggest that the BUF04 should be considerably faster.
rather than surface-mount, components\textsuperscript{9}. Also, noise arising from EMI considerations in the card rack may also be a factor. Finally, the use of long, albeit RF shielded, cables between the interface circuits and the devices on the optical table may be a consideration.\textsuperscript{10}

The best possible solution would be to identify and eliminate the external source of noise. It may also be helpful to simply apply a filter at the input and/or output of the circuit, since the current settling time is much faster than required. And, although it is unlikely that the amplifiers used are directly to "blame," the use of a slower, lower noise buffer amplifier or op amp may yield better results. One

\textsuperscript{9} It is possible that some of the IC specifications only apply in the case of the surface mount component—a distinction is generally not made in data sheets.

\textsuperscript{10} However, a recent test showed no significant noise arising from an input via one (particular) of the long cables used—in other words, a short at the end of the long cable produced no more noise than a short right at the scope's input. The effect of the cables on the rise time, however,
viable choice would be the OP113 op amp from Analog Devices.

*Digital Signals*

Each of the D/A boards contains 8 digital lines that can be programmed and output simultaneously with the D/A samples. These lines on the UV Scan D/A card are employed for a variety of purposes, which are described in greater detail in Appendix 3. Briefly, four of these digital lines (DIO X0-X3) serve as signals of specific events during the stimulation pattern, such as the instant of the first pulse. These signals can be used to trigger other electronics and/or can be digitized\(^{11}\) to serve as a record of when particular events occurred during the experiment. Two of the other digital lines (DIO Y2-Y3) can be used as a digital control of the modulation inputs to the deflector driver, which allows binary (i.e. non-graded) control of the laser intensity.

The remaining two of these digital lines (DIO Y0-Y1) are used to control the UV laser Q-switch signals, “Q-Switch Clock” and “Q-Switch Gate.” These digital lines require a buffer amplifier because the digital inputs to the UV laser operate at TTL voltage levels and have a 50Ω input impedance\(^{12}\). This amplification circuitry is part of the UV Scan interface circuitry, and is visible in Figure 5-2. A commercially available TTL IC, the 74xx140, is employed. This is a 50Ω driver NAND buffer, which is wired to effect a simple inverting buffer. A

\(^{11}\) Commonly only one of the extra electrophysiology A/D channels (discussed below) is used to digitize the *PhotoStimActive* digital signal (see Appendix 3), which produces a digital pulse for every laser pulse directed at the specimen.
separate inverter element (74xx04) is used prior to the buffer to preserve the correct polarity.

Also visible in Figure 5-2 is circuitry to generate a "CLK Out" signal; this was part of one implementation (no longer used) used to synchronize the UV Scan and UV Mod D/A boards. See Appendix 4.

The TTL logic elements used require a +5V supply. A voltage regulator IC (LM2940CT-5.0; National Semiconductor) was used to derive +5V from the +18V supply available on the interface circuit. Note also in both circuits that the analog and digital grounds are maintained separately. While these grounds must meet within the D/A converter, they are separated elsewhere and the AWFG provides these grounds on separate pins. This separation is maintained throughout the interface circuit.

5.1.2 Visible Scanner Interface Circuitry

The interface circuit for the visible scanner is briefly described here—it is very similar to that employed in previous work (Bullen et al., 1997; Bullen and Saggau, 1999).

Vis Scan Interface Circuit: Design and Performance

The visible scanner requires only one D/A card, whose two channels are used to control the visible laser scan position. The deflector drivers employed (D322; Isomet) vary the RF output of each deflector across their 70-100MHz tuning range, in response to voltage inputs in the 3-13 voltage range.

\[12\] This is very unusual for a digital input and likely does not serve a particularly good purpose
The visible scanner interface circuit, shown in Figure 5-6, consists of two unity gain differential amplifiers. The +/-10V output range of the D/A card is added to a +10V input, generated by an adjustable positive voltage regulator LM317; National Semiconductor) from the available +18V supply, to produce a 0-20V output range. This covers the 3-13V tuning range. As the output range is twice the target tuning range, one of the 12 bits of AWFG D/A resolution is sacrificed. A well-matched set of 4 resistors was used for each amplifier.

Because of the requirement for a fast settling time in order to allow maximal time for oversampling (see Figure 3-7b), a low-noise, high-bandwidth op amp was sought. The HA3-2525-5 (Intersil) was elected. From its specifications, a 99% rise time of ~366ns\textsuperscript{13} and an output RMS noise ~1mV\textsuperscript{14} are expected. This 1mV noise over a 20V range would not significantly impact the achieved resolution.

Because the peak output voltage is +13V, a +/-18V voltage supply was employed rather than a more conventional +/-15V supply to power the op amps\textsuperscript{15}, in order to avoid any nonlinear effects that can arise when op amp outputs near the supply voltages.

The performance of the Vis Scan circuit is shown in Figure 5-7. The rise time performance shown in Figure 5-7b is ~1.5\textmu{s}, slower than expected. Such a

\textsuperscript{13} This is based on summing the time calculated for a 20V (full-range) transition at the manufacturer-specified slew rate (120V/\mu s) and settling time (~200ns) for any ripples to disappear.

\textsuperscript{14} This is based on an “average” 50nV/\sqrt{Hz} figure; this approximation is sufficient to estimate whether the expected output noise density should be of significant concern.
This +/-18V supply is employed throughout "Card Rack #2" which contains all the scanner control circuits. Thus, it was used in the UV scanner interface circuits described above.
settling time would "consume" much of the minimal 10μs pixel dwell period, and
limit the amount of oversampling. The slower than expected performance is
attributable to the two capacitances employed. First is the 1pF compensation
capacitor located at the COMP pin to prevent oscillations (i.e. promote unity gain
stability). The second capacitor is a 15pF feedback capacitance, which was
added to limit the strong ringing that was seen in the step response.

The noise performance evident in Figure 5-7c shows an RMS noise of
~3.5mV. While this is less than the device specification, this yields >2800 spot
positioning resolution given the 10V tuning range. In conclusion, then, the Vis
Scan interface circuit suffers from the opposite affliction as the UV Scan and UV

Figure 5-7: Visible Scanner Interface Circuit  a) Pattern of Visible Scan control
signal used to scan a ten spot pattern across the 3-13 V tuning range. b) Dynamics of a
scan position transition. The rise time of ~1.5μs is slower than desired, reducing some
of the time that can be spent oversampling. c) Noise measured during the "steady-state"
period when tuning voltage is settled. RMS value calculated as 3.5mV.
Mod circuits: it overperforms in terms of noise, and underperforms in terms of dynamics.

5.1.3 Summarizing Scanner Interface Circuit Performance

The actual performance of the interface circuits generally fell short of the desired specifications. However, in principle, the requirements for each of the scanner control circuits should be achievable. For the UV scanner interface circuits, the elimination of an identified source of interference/noise or the use of filtering (sacrificing the excess bandwidth) should improve the noise performance. Meanwhile, for the visible scanner interface circuitry, the use of a faster op amp\(^{16}\) should allow the rise-time performance to be improved.

5.2 OPTICAL RECORDING INTERFACE CIRCUITRY

The optical recording interface circuit serves to transform the range of voltages output from the photodetector circuit, 0 to \(-10\)\(^V\)\(^{17}\), to the +/-2\(^V\) input voltage range of the fast 16-bit A/D converter employed (\textit{AIIX/Chico}; Innovative Integration). In the other interface circuits discussed, the requirements were such that \textit{either} the settling time performance \textit{or} the noise performance needed to be, subjectively speaking, very good. For the optical recording interface circuit, \textit{both} must be very good. The settling time should be fast (ideally

\(^{16}\) Alternatively, the identification of layout or other external factors that gave rise to the strong ringing could be used to allow the bandwidth limiting feedback capacitance to be reduced or eliminated. Arguably, also, the ringing is not of great concern, since samples collected during this time can be discarded.

\(^{17}\) -10\(^V\) is chosen as the “maximum” voltage of the photodiode amplifier by mapping it to one of the extremes of the A/D converter range. This disallows measurements of light levels near to the photodiode amplifier voltage rail (-15\(^V\)), where the response function might be somewhat nonlinear.
<1\mu s), to allow maximal oversampling, while the noise performance must be excellent. To preserve the full 16-bit resolution over the +/-2V output range, the output noise must be <60\mu V.

The circuit design is given in Figure 5-8. A separate interface for each of the four A/D channels is provided. Each is a gain/offset circuit implemented by a classic summing inverting amplifier configuration, with all the inputs at the negative op amp input. To generate a bipolar signal from the unipolar input, a -5V reference voltage (the midpoint of the 0 to -10V range) is created using a negative voltage regulator IC (LM337; National Semiconductor) and summed with the input voltage. A gain factor of .8 is created using a 130k\Omega/163k\Omega resistor combination; this creates an output range of +/-4V. A 51\Omega resistor at the output of the amplifier forms a voltage divider with the A/D converter's 50\Omega input impedance, producing finally the desired +/-2V input range. Finally, a separate voltage regulator was employed for each channel amplifier. This ensures that each of the reference ICs is located near to the corresponding op amp input, ensuring the best possible regulation.

The OPA627 op-amp (Burr-Brown) was elected owing to its very low noise figure of 4.5nV/\sqrt{Hz}, its fast settling time figure of 550ns, and its stability for unity-gain (or, in this case, sub-unity gain) applications. A high-quality ceramic 10\mu F feedback capacitor was added with the intention of limiting the bandwidth passed by the amplifier to ~1/2 the sampling frequency (i.e. ~1MHz bandwidth), in accordance with the Nyquist theorem. This represents a common "bandwidth narrowing" approach to noise reduction.
To test the circuit, a square wave test function to simulate the photodiode was created with a function generator (3312A; Hewlett-Packard), and connected to the interface circuit input. The system control software was used to acquire the final digitized signal, as if an optical recording (at one site) were occurring. The measured trace, then, reflects the aggregate performance of both the optical recording interface circuit and the A/D converter board (AIX/Chico; Innovative Integration).

Figure 5-9b shows the result of propagating the signal through the A/D circuitry. The resulting rise time can be estimated as ~8μs. Figure 5-9a establishes that the measured rise time is indeed the result of the interface circuitry, and not the function generator itself. This rise time is much slower than intended, and can be wholly attributed to an error in designing the filter capacitance\(^{18}\), which can be corrected. Figure 5-9c shows the steady-state RMS noise level of each discrete level to be ~1mV. However, it was not possible to ensure that the test signal (i.e. the function generator output) was not producing this noise, so the result cannot be considered conclusive.

In conclusion, the temporal performance of the currently configured optical recording interface circuit can be made adequate (i.e. settling time <1μs), but the RMS noise level has not been accurately determined. Assuming further improvement is required, the use of surface mount components on a printed circuit board (PCB) is likely warranted for the performance required. Another
factor that might be of help would be the use of additional voltage regulators, closer to the op amps. Currently, shared, remote linear power supplies (Schroff) are employed to power each of the interface circuits. Although the regulation of these supplies is quite good (<1% ripple), it is possible that using an additional regulator to produce a very stable supply near each of the amplifier circuits would yield an improvement.

5.3 DUAL SCANNER TIMING/SYNCHRONIZATION

The Dual Scanner requires that several timing signals be generated to clock the D/A and A/D updates, and that certain sets of these signals be strictly synchronized. In total, three distinct classes of timing/synchronization "threads" can be labeled that run concurrently during an experiment:

1. **UV Scan/Mod Thread** The position and intensity D/A tuning voltages must be simultaneously updated throughout the

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18 The time constant of the RC filter in the feedback loop (10pF x 130kΩ = 1.3μs), corresponds to a (3dB) cutoff frequency f₀ of only ~120kHz, rather than the intended 1MHz, because
experiment. These D/A channels run at the same rate (100kHz) and must be *hard synchronized*, i.e. they must both be run from the same clock.

2. **Optical Recording Thread** The optical recording A/D card samples at a much faster rate than the Vis Scan D/A card in order to allow oversampling. The two must run off the same master clock (i.e. must be *hard synchronized*) so that the Vis Scan updates always coincide with the optical recording A/D samples. This allows the optical signal to later be “soft-demultiplexed” (i.e. time points in the record correctly assigned to particular scan locations).

3. **Asynchronous Threads** These refer to the various processes that are directly controlled by the control software during the experiments. This includes the interrupt-driven control of the electrophysiological A/D and D/A channels and the CPU timer driven control of the “asynchronous DIO lines.”

The hardware implementation used to provide the timing/synchronization of the UV Scan/Mod and Optical Recording threads is shown in figure 5-10. The software function calls starting each of these threads are made one after the other to start an experiment, in order that the “time=0” point is reasonably matched for each of these independent threads. The threads can thus be said to be *soft synchronized*. Further discussion of the implementation of both the hard and soft synchronization is given in Appendix 4.

\[ f_0 = \frac{1}{(2\pi r)} \]  
This author forgot the factor of \(2\pi\), and this should be corrected.
Figure 5-10 Timing/Synchronization of Dual Scanner Electronics

1. The LSB of the on-board decade counter was picked-off to effect a ÷2 counter.
2. The 82C54 chip contains 3 16-bit counter/timers. Both Timer0 and Timer1 see 5MHz as their input. Timer0 is set to +50, producing the 100kHz clock used to drive the UV Scan patterns. Timer1 is presently unused, but could be programmed (in software) any submultiple of 5MHz.
3. The AIX A/D module supports data acquisition rates up to 2.5MHz (per channel), and employs 8X oversampling. Generally, this maximal rate is always employed, so 8x2.5MHz = 20MHz is used.
4. 100kHz is a typical scan rate for the visible scanner (10 spots at 10kHz). Different scan rates are selected by the user for each experiment, but its period must always be a multiple of 50ns (=1/20MHz).
5.4 OTHER DUAL SCANNER ELECTRONICS

5.3.1 Electrophysiology Interface Circuit

The Dual Scanner was designed to allow conventional electrophysiological techniques to be used for control and for calibration purposes. A multi-channel DAQ board is employed (CIO-DAS08-AOL), which provides 8 A/D and 2 D/A channels at 12-bit resolution and up to 20kHz update rates. These channels are generally used in conjunction with the commercial patch clamp amplifier (BVC-700A; Dagan), but can be used for other purposes, such as digitizing the UV scan control digital traces (see above) or controlling other neurophysiological devices, such as puffer pipette or iontophoretic neurotransmitter application.

Minimal interface circuitry is required. The voltage range of the A/D converter (+/-10V) exactly matches the meaningful physiological range of the 100xV_m output from the amplifier that is employed, namely a +/-100mV range of membrane potential recordings.

Meanwhile, the D/A converter outputs, set to the board’s lowest voltage range (+/-2.5V)\textsuperscript{19}, still require attenuation to produce a range of input voltages to the Dagan amplifier that yield physiological current (+/-500nA) commands. Because the control inputs to the patch clamp amplifier (and other devices) are generally high-impedance, only a passive voltage divider (1.07K/10.7K) is used to provide nominal 10x attenuation.

\textsuperscript{19} This was selected by a DIP switch on the board
The voltage ranges described pertain to the current-clamp recording configuration, in which voltages are measured and current commands are applied. This is the most common recording configuration employed, but sometimes voltage-clamp recording would be preferable. In this case, the D/A interface currently employed is not adequate: the resulting output voltage range of +/-250mV from the attenuated D/A converter produces only a +/-5mV external command range. An improvement to the current arrangement would be to add a switch to allow the passive attenuation to be removed when in current clamp mode.

5.3.2 Other Dual Scanner Circuitry

Referring to Figure 5-1, there are several other electronics system in the Dual Scanner which merit mention. These include the CCD camera electronics, the custom photodiode amplifier circuit, and the custom shutter control circuit. These are each discussed in Appendix 6.
CHAPTER 6

PRELIMINARY RESULTS

The Dual Scanner extends the previously developed "visible scanner" for fast, random-access membrane potential measurements (Bullen et al., 1997; Bullen and Saggau, 1999) to include a new "UV scanner" designed for fast, localized, random-access, graded multi-site photolysis of caged compound for the optical stimulation of cultured hippocampal neurons. This chapter discusses the preliminary results obtained to date with the new UV scanner system.

6.1 MODULATION OF OPTICAL INTENSITY

An important feature of the UV scanner is gradability—the ability to modulate the amount of the energy delivered per pulse by varying the intensity of the acoustic wave in the AOD. This is accomplished in the Dual scanner by varying the amplitude of the RF wave applied to the X-deflector of the UV scanner.

This gradability was measured/calibrated in two ways. First, the laser was set to pulse continuously (at its 50kHz rate), so the resulting intensity could be measured using an optical power meter (LaserMate-1; Coherent), which was placed just above the microscope's objective thread. In the second approach, the laser was again pulsed continuously, and focused by an objective onto a dish of 5mM fluorescein. The resulting steady-state fluorescence was measured using

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1 During operation (i.e. experiments), the RF amplitude applied to the Y-deflector is always set to the maximal value. The only exception is when the desired modulation is zero acoustic intensity; then, the modulation levels of both deflectors are set to zero to best minimize the amount of light reaching the specimen.

2 The objective is removed because its NA is too large and hence the resulting divergence too great, making a power meter measurement subsequent to it difficult to impossible.
Figure 6-1 Optical Intensity as a Function of Applied Acoustic Amplitude. The Bragg theory of the AO interaction predicts that the diffraction efficiency varies according to $\sin(KA_{\text{acoustic}})$ where $A_{\text{acoustic}}$ is the acoustic wave amplitude. This amplitude was varied directly using the modulation input to the "X" driver (while fixing the input to the "Y" driver to the maximal value), and the resulting optical intensity (that which passed through the first-order selecting slit) was measured, in two ways: first, by an optical power meter located at the back focal plane of the microscope, and second by measuring the fluorescence of a 5mM sample of fluorescein using the signal photodiode detection pathway. Both approaches gave similar results and were well fit by the $I_0 \sin(KA_{\text{acoustic}})$ curve shown.

the photodiode detection pathway. The results are shown in Figure 6-1. Both approaches gave similar results.

By combining Eqns. (4.14) and (4.15), it is found that the diffraction efficiency for a Bragg type AOD is,

$$\eta = \sin^2 \left( \frac{\Delta \phi}{2} \right) = \sin^2 \left( K \Delta n_0 \right) = \sin^2 \left( K' \sqrt{I_s} \right)$$  \hspace{1cm} (6.1)

where $I_s$ is the intensity of the acoustic wave and $K'$ is a constant dependent on a variety of parameters. Since the deflector driver provides a control for varying the amplitude $A_{\text{RF}}$ of the RF wave supplied to piezoelectric transducer, which is
proportional to the $\sqrt{I_s}$ term in Eqn. (6.1), the optical intensity of the output beam (i.e. the first order) should follow $I_{opt} = \sin^2(KA_{RF})$.

As shown in Figure 6-1, the optical intensity data, normalized to unit intensity at 100% acoustic power, was very well described by such a function, using a least squares fit$^3$. An additional fit parameter $I_{max}$ was used such that $I_{opt} = I_{max} \sin^2(KA_{RF})$ to account for the possibility that the maximal acoustic wave amplitude the driver can supply does not correspond to the peak of the $\sin^2$ curve$^4$.

These fit parameters will be incorporated into the system's software, so the user can specify the relative optical intensity desired on a linear scale; the software will compute the nonlinear transform between a specified value and the required acoustic amplitude to effect this value.

6.2 PHOTOLYSIS OF CAGED FLUORESCEIN

As a prelude to the photolysis of caged glutamate, for which the measured response—currents/voltages elicited in a living neuron—cannot be relied upon as a calibrated detection, control experiments involving the uncaging of caged fluorescein (DMNB caging group/dextran conjugated; D-3310; Molecular Probes) were carried out. The change in fluorescence following controlled doses of UV

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$^3$ The *lsqfit* MATLAB routine (Optimization Toolbox; Mathworks) was used for this fit

$^4$ Ideally, the manufacturer adjusts the length of the AO interaction (i.e. the length of the device) such that the peak RF power exactly corresponds the maximal diffraction efficiency. Of course, Eqn. (6.1) does not exactly describe the actual diffraction efficiency, since 100% efficiency is not obtainable. Nonetheless, the $\sin^2$ functional form is an excellent approximation.
Figure 6-2 Photolysis of Caged Fluorescein. a) Three successive doses of UV laser energy yield additional uncaging at each step; the amount decreases at each step. Concentration 250μM. b) Change in fluorescence following photolysis varies linearly with the number of pulses delivered. Estimated energy: 800nJ/pulse. c) Change in fluorescence following photolysis varies with the acoustic intensity according to the previously measured sin² profile (shown in blue). Fluorescence changes normalized for unit change at 100% acoustic wave intensity.

Illumination could be measured using the Dual Scanner's photodiode detection pathway, serving as a reliable assay of uncaging efficacy.

An example of raw data collected is provided in Figure 6-2a. Three doses of UV energy were applied, nominally 3μJ each. The fluorescence was measured using the visible laser scanner and photodiode detection, with the visible laser excitation adjusted to be co-localized with the UV excitation. Each successive UV dose produced additional photolysis. The change in fluorescence decreases at each step, presumably reflecting the reduced pool of available caged fluorescein.
An interesting feature of this trace, observed in every instance (n=6), is the apparent time constant, on the order of tens of milliseconds, governing the change in fluorescence after uncaging. This time constant is much greater than the uncaging rate of the DMNB caging group used\(^6\), so the likely process occurring is diffusion. Because it is an *increasing* fluorescence that is observed, a likely explanation is that the uncaging pulse, in addition to producing new fluorescent molecules, bleached some of the pre-existing fluorescein molecules. Thus, the phenomenon observed is similar to that observed in the fluorescence recovery after photobleaching (FRAP) technique, used for measuring diffusion constants (Blonk et al., 1993).

6.2.1 Energy Dependence of Photolysis

Because little work has been done to date employing a pulsed laser for photolysis of caged compounds, it is important to establish the energy dependence of photolysis for the specific kinetics of the laser employed (33ns pulses, 20\(\mu\)s repetition period). While it is likely, it cannot be assume *a priori* that the amount of energy delivered exclusively determines the amount of uncaging, independent of the temporal profile of how that energy is delivered (i.e. pulsed vs. CW). Indeed, it is possible, because uncaging is a multi-step process, that concentrating the energy into 33ns bursts is more or less effective at driving the uncaging reaction than CW excitation and/or longer pulses. Another possible

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\(^6\) 2 pulses of UV illumination, with the beam attenuated to 10\%, were given. Given the nominal 20\(\mu\)J per pulse specification, this corresponds to 4\(\mu\)J from the laser. No scanner was used in this experiment, so the primary source of attenuation was the objective lens, which transmits \(~75\%\).
effect of the pulsed kinetics is that successive pulses could accumulate either sub- or supra-linearly, for instance, if the initial molecular excited state retains energy for greater than 20\(\mu\)s.

Figure 6-2b shows the dependence for the photolysis of 100\(\mu\)M DMNB-caged fluorescein on the number of pulses applied, where the pulses are applied in rapid succession at the full laser repetition rate. For each case, the change in fluorescence is quantified by the normalized difference in the fluorescence before and after each uncaging event,

\[
\frac{\Delta F}{F} = \frac{F_{\text{after}} - F_{\text{before}}}{F_{\text{before}}}.\tag{6.2}
\]

The relationship between \(\Delta F/F\) and the number of pulses delivered is clearly linear, indicating that no molecular mechanism of “memory” exists over the 20\(\mu\)s time-scale. Each pulse contributes equally and independently to the final amount of uncaging. This linearity also indicates that only a small fraction of the caged molecules were uncaged with each pulse, since any depletion would also produce a nonlinear response.

Figure 6-2c shows the effect of varying the amplitude of the acoustic wave at one of the UV scanner's AODs. The change in fluorescence observed followed exactly the acoustic amplitude dependence previously measured (Figure 6-1). This demonstrates the proportionality of the UV energy delivered and the amount of uncaging, which is as expected for a single pulse.

\footnote{It's possible the somewhat faster CMNB caging group was used in the experiment of Figure 6-2a, but the argument remains the same.}
Summarizing, it was shown that for small numbers of pulses (as few as one) separated by 20μs, the amount of uncaging is directly proportional to energy delivered. However, it has not been definitively determined whether the use of 33ns pulses either increased or decreased the effective quantum efficiency of the uncaging process. This would require a comparison of the absolute amounts of energy delivered for different types of excitation (i.e. pulsed vs. CW).

6.2.2 Tracking Fluid Flow using Caged Fluorescein

To demonstrate the viability of combining the multi-site photolysis capability of the UV scanner with wide-field fluorescence imaging (using epi-illumination), some results from an investigation of fluid flow in a narrow (100μm) capillary are shown here. These investigations were aimed at exploring the possibility that electroosmotic flow could play a role in cell motility, particularly in the extracisternal space of the outer hair cell (OHC) in the ear (Losavio et al., 2001). The narrow capillary serves as a scaled up model of this nanoscale channel in the OHC. Previous work describing electroosmotic flow and its imaging in capillaries can be found in (Paul et al., 1998).

As shown in Figure 6-3, the capillary was filled DMNB-caged fluorescein and the UV scanner was programmed to create a narrow line of uncaged fluorescein. Epi-fluorescence imaging was then used to track the flow of the recently uncaged molecules. In Figure 6-3a, the diffusion of the thin strip of fluorescence over a few seconds can be observed.

A pressure-driven gradient was created by raising one end of the capillary (outside the field of view). The resulting fluid flow shows a parabolic profile
Figure 6-3 Imaging of Electrokinetic and Pressure-driven Fluid Flow in a Glass Capillary: 100μM dextran-conjugated DMNB caged fluorescein was loaded into a glass capillary (100 μm ID). The UV AO scanner was programmed to produce pulses along a line. The resulting fluorescence was imaged using the CCD camera pathway at specified intervals after the uncaging event. A) Diffusive broadening of the fluorescein. Inter-frame interval: 600ms. B) Pressure-driven flow of the caged fluorescein, using an ~300Pa pressure gradient created by creating a height differential at the two ends of the capillary. The characteristic laminar flow parabolic profile is evident. Inter-frame interval: 890ms. C) Electrokinetic flow of the caged fluorescein, driven by a 30V/cm applied electric field. The characteristic "plug" flow profile is evident, superimposed upon the diffusive broadening profile. Inter-frame interval: 100ms.

(superimposed on the diffusive profile of Figure 6-3a) that is characteristic of laminar flow, as shown in Figure 6-3b. For comparison, a large electric field (30V/cm) was applied across the two ends of the capillary. This gave rise to a fast fluid flow, presumed to be electroosmosis\(^7\), which shows a characteristic "plug flow" profile, seen in Figure 6-3c.

This work merely serves as an illustration of the power of combining laser photolysis with sensitive fluorescence imaging. In addition to the fluid flow studies described here, this combination of techniques could be used for novel studies in

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\(^7\) The possibility exists however that the electrokinetic phenomenon in play was electrophoresis, rather than electroosmosis, because the fluorescein-dextran conjugate is not entirely uncharged.
Neuroscience, such as the imaging of biochemical processes (visualized by appropriate fluorescent markers) in response to multi-site synaptic stimulation.

6.3 PHOTOLYSIS OF CAGED GLUTAMATE

Prior to experiments directly addressing the issue of dendritic integration, it is vital to address first some basic questions regarding the viability of the technique:

- Is the laser energy sufficient to allow a full range of physiological stimuli to be provided with one or at most a few pulses?
- Does the optical stimulation cause any damage to the cells?
- Does the optical stimulation elicit any physiological response of itself?

In all experiments, CNB-caged glutamate, with the CNB caging moiety forming an ester bond at the γ-carboxyl of the glutamate molecule, is employed. This is the most commonly employed caged glutamate variety, and is readily available commercially (G-7055; Molecular Probes). It is characterized by a high quantum efficiency (~.14), rapid rate of uncaging (~20 μs), and high water solubility (Wieboldt et al., 1994). However, its absorption peak is at 260nm, and the 355nm laser pulses used excite only the tail of its excitation spectrum. Among the varieties of caged glutamate available, it also has a rather large rate of spontaneous uncaging; thus great care was taken to freeze the compound and shield it from light until immediately before use.
Figure 6-4 Uncaging Glutamate Controls
300 µM CNB-caged glutamate uncaged, at full system throughput. a) Large postsynaptic currents were evoked by photostimulation, but only when near a dendritic site. b) Elicited currents are highly repeatable, to within ±10%, which coincides with pulse-to-pulse variation in the laser energy. c) Elicited currents are clearly the result of activating glutamate receptors. Application of blockers for NMDA (APV) and AMPA (CNQX) receptors, abolishes the sustained and transient current components, respectively.

Patch-clamp recording techniques, namely voltage- and current-clamp whole cell recordings, were employed to measure the response of the cells tested at the soma. This experimental configuration, combination of multi-site optical stimulation using the UV scanner and single site, high quality electrical recording at the soma forms an appealing testbed for the study of dendritic integration.

6.3.1 Control Experiments
In Figure 6-4a, UV excitation was delivered both near a dendrite of a neuron held in voltage-clamp as well as far from the dendritic tree. When the
laser is focused near to the dendritic tree, a current pulse is evoked; this pulse is not observed when the laser is focused far from the dendrites. Thus, the observed current, it can be concluded, is a physiological excitatory post-synaptic current (EPSC) signal. The size of the elicited EPSC, it is found, is rather repeatable from trial to trial.

This repeatability of the response was quantified, by delivering identical stimuli at 2 minute intervals. Over ten trials, as shown in Figure 6-4b, a variability of ±4.2% (standard deviation) was observed. This is within the range specified for the pulse-to-pulse energy variation of the UV laser (specified at <10%). Thus, the variation observed may be due nearly exclusively to changes in the amount of energy delivered, and not to any variability in the post-synaptic response. This tentative result would agree with the observations of others who have also found little variability in the postsynaptic response of hippocampal neurons (Liu et al., 1999), prompting the conclusion that variability in synaptic transmission is largely of pre-synaptic origin.

An important conclusion to be drawn from the repeatability of the response is that neither the laser pulses themselves, nor the process of uncaging, gave rise to any damage mechanism affecting the cell's viability.

Figure 6-4c establishes that the responses observed are indeed entirely the result of activating glutamate receptors. The application of CNQX, an AMPA (and partial KA) receptor blocker eliminates the fast, transient current response associated with those receptors. Meanwhile, the application of APV, an NMDA

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It's expected to be less than this, however (Varela, O., DPSS Lasers, personal communication).
Comparison of Optical and Electrical Recording

Figure 6-5 Electrical and Optical Stimulation of a Cultured Hippocampal Neuron Compared A cultured neuron was stimulated first with a 400pA current injection, 50ms duration. Subsequently, 4000 pulses (80ms) from the attenuated UV laser were applied, photolyzing the 20µM caged glutamate in the bath. The gradual depolarization leading to eventual AP firing is likely the result of defocus followed by diffusive transport to the correct plane; this mechanism for delayed/suppressed action also would also explain the continued depolarization following the cessation of optical stimulation.

Specific blocker suppresses the slower, sustained current component.

Coapplication of these drugs abolishes the response altogether.

It can be seen, however, that both the AMPA and NMDA components do not decay back to zero-current, even after ~2 seconds. A possible explanation is that the perfusion employed in this experiment did not sufficiently clear the uncaged glutamate, and that a steady-state concentration of glutamate remained after the initial activation, giving rise to a steady-state current that can flow through either activated AMPA or NMDA receptors. It is significant to note also that the EPSCs in this experiment are very large (~10nA), suggesting that a very large amount of glutamate was released. Thus it is unsurprising that a significant amount of glutamate “hangs around” for a while. Lower pulse energy should have been used to presumably produce a more physiologically typical response.
In Figure 6-5, responses to electrical and optical stimulation of a single neuron are compared. The electrical stimulation here consists of a steady-state current injection (400pA) lasting 50ms, while the optical stimulation consisted of 4000 successive pulses, each attenuated to <1% of the peak laser energy. These stimuli are relatively prolonged and, as shown, in both cases the cell was depolarized to about a -50mV threshold, after which an action potential fired. In both cases, the threshold is identical, and the size and kinetics of the action potential are very similar. The ability to fire action potentials provides further confidence that the health and viability of a cell are unaffected by the localized UV illumination. That the depolarization during the optical stimulation was slow and prolonged is likely attributable to a defocus of the UV laser, such that the majority of newly created glutamate was required to diffuse towards the targeted dendritic region.

6.3.2 Graded Photolysis of Glutamate

Figure 6-6a shows the linear accumulation of evoked current in response to multiple pulses, on the very fast 20μs time scale of the laser repetition rate. This result is a natural extension of the linear accumulation of uncaging yield in response to repetitive laser pulses found above in the caged fluorescein experiments. That the evoked EPSC grew linearly, however, is a property of the population of glutamate receptors activated. Note, however, that the “system step response” is not wholly linear—the first pulse elicited a much larger response than the subsequent pulses. The linearity of the subsequent pulses and from the caged fluorescein experiments, however, provides confidence that this non-
linearity can truly be attributable to the system under study. In particular, it is likely that the initial large, saturating current can be attributed to a separate population of receptors from those mediating the subsequent linear response. Determining whether these populations are truly different receptor types or, perhaps, similar receptors in different biochemical states would require pharmacological dissection. For example, Figure 6-6b shows the AMPA receptor specific response over a large range of delivered energies.

It is important to note that this type of measurement, essentially a “dose-response” curve, has not previously been carried out. Other approaches,
including iontophoresis and CW laser uncaging, are unable to deliver a wide range of doses in such a short time, thus convolving the cell's dose response with its temporal response. The employment of a pulsed laser overcomes this limitation.

The best temporal resolution arises when all the required energy is delivered in a single pulse. Figure 6-6c illustrates the grading of an EPSC by varying the energy of a single pulse\(^9\). Significantly, it was found that a meaningful physiological response (peak \(\sim 200\) pA) could be elicited even at high attenuation. Given the configuration used in this experiment\(^{10}\), the energy delivered at the highest attenuation (nominally 99\%) could be no greater than 240\(n\)J. Responses of comparable magnitude physiologically have been attained by others, typically employing somewhat greater energies, ranging from \(\sim 1\,\mu\)J (Kandler et al., 1998) to \(\sim 10\,\mu\)J (Bullen and Saggau, 1999). These results are at least suggestive that the Q-switched laser photolysis employed here yields more efficient uncaging, perhaps implying that the short \(\sim 33\)ns pulses do indeed more effectively drive the photolytic chemical pathway, i.e. a greater effective quantum efficiency. This cannot be consider conclusive, however, as several factors, such as focal spot size and concentrations employed, would need to be controlled for to allow a meaningful comparison of different excitation types.

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\(^9\) Actually, a pair of pulses was used in this experiment.

\(^{10}\) This was conducted prior to the construction of the UV scanner, while evaluating the laser. Optical filter glass was used to provide the attenuation and the laser was simply gated on for as short a period as possible, which corresponded to two pulses. Because there was no scanner, the focal spot size was likely much smaller than is currently attained.
6.3.3 Preliminary Investigations of Dendritic Integration

Figure 6-7 shows the results of two experiments investigating one aspect of dendritic integration—spatial summation. In Figure 6-7a, the cumulative effect of stimulating at multiple sites was tested. As shown, the response was nearly linear, over a range of resulting EPSCs. The slight sub-linearity observed for the case of four and five stimulation sites likely reflects some saturation due to the large ~1nA EPSC that is evoked, rather than any intrinsic non-linearity of spatial summation. This result, although far from conclusive, at least does on first consideration agree with those of other authors who have found linear spatial summation to be the rule (Cash and Yuste, 1998), at least for well-spaced (>10μm) synaptic inputs, which was the case here.

Figure 6-7b shows a combinatoric spatial summation experiment, in which various combinations of three distinct “synaptic” (likely, multi-synaptic) inputs were investigated. In all cases here, the combined input yielded a smaller response than the arithmetic sum of the individual synaptic inputs. The individual inputs however, gave rise to large ~1nA currents, so the sublinearity may again reflect a saturation mechanism apart from those which may underlie a true sublinear spatial summation.

These results are highly preliminary, but nonetheless do demonstrate the UV scanner’s capability of multi-site stimulation of a living cell’s dendritic tree.
Figure 6-7 Preliminary Investigation of Dendritic Integration a) Spatial summation of multi-site photolytic stimulation. summation is slightly sublinear. b) Combinatoric spatial summation experiment again reveals sublinear spatial summation. Dotted lines indicate the arithmetic sum of the individual inputs. “Pre” and “Post” traces show that responses at each site are not altered during the experiment.

6.4 SUMMARY

The results given in this section indicate that optical stimulation of caged compounds can elicit physiologically meaningful responses from living hippocampal cells. The energy of the Q-switched laser employed is sufficient to allow a wide range of meaningful inputs to be reached using a single laser pulse. This implies the full “bandwidth” of the laser’s 50kHz repetition rate can be leveraged to create high temporal resolution multi-site stimulation patterns. For example, more sites can be quasi-simultaneously stimulated in a study of spatial summation.
The pulsed nature of the illumination may improve the uncaging efficiency and moreover appears to enable characterization of the glutamate dose-response characteristics of a living cell, on the time scale of actual synaptic transmission (i.e. <1ms). This may ultimately prove to be a powerful means for mapping receptor types present in various compartments of a cell that is akin to the current use of dendritic patch clamp techniques to map channel distributions (Stuart et al., 1993)\textsuperscript{11}.

Most importantly, no pattern of cell damage attributable to the optical stimulation has been observed to date. Taken together, then, these results indicate the suitability of the UV scanner for investigating dendritic integration. Preliminary investigations of spatial summation offer some verification of previous work showing that this summation is linear and position-independent.

\textsuperscript{11} Actually, receptor distributions can be studied with dendritic patch clamp technique (Spruston et al., 1995a), using the "outside-out" patch technique in which sections of membrane are excised from the dendrite. Acquiring a "map" using this approach is clearly more time-consuming than with the UV laser scanner.
CHAPTER 7 SUMMARY AND FUTURE DIRECTIONS

The results of the last chapter portend a positive future for the UV scanner as a tool to investigate dendritic integration in cultured hippocampal neurons. These preliminary observations showed that meaningful physiological responses could be elicited by a spatiotemporal pattern of stimulation, that the energy requirements for producing physiological results are well met by the apparatus, and that there is no evidence of photodamage in typical experimental scenarios.

These results immediately enable several of the possible investigations enumerated in Chapter 2. Here a trajectory of future work intended by the author is presented, in approximately increasing order of difficulty.

7.1 PHASE 1: OPTICAL STIMULATION ONLY

The first experiments intended are those involving only the UV scanner and which employ a single site of recording—an electrode (patch clamp pipette) located at the cell body. This experimental configuration avoids the technical difficulties of optical recording, such as dye loading, and allows direct observation of the summation (dendritic integration) of a complex spatio-temporal pattern at the soma.

The investigation of temporal summation is the most straightforward. The effect of multiple synaptic stimuli at varying temporal offsets can be easily investigated, at various locations and synaptic stimulation strengths. As discussed, this approach combines the strengths of the studies of Margulis and Tang (Margulis and Tang, 1998) and Magee (Magee, 1999).
Beyond temporal summation, the investigation of spatial summation—and ultimately spatio-temporal summation—represents the next level of difficulty. In particular, study, owing to its relative simplicity: there is no need for optical recording and the requirements for the spatial resolution are not strict.

Subsequent to the study of temporal summation, the next “level of difficulty” would be the investigation of spatial summation (and, subsequently, spatio-temporal summation). The principle challenge would be to improve the spatial resolution of the system. As presently configured, the spatial resolution of the UV scanner is limited to 5-10μm owing to the relatively poor figure of merit of fused silica as an acousto-optic medium. As mentioned, efforts are currently underway to improve this resolution. These include the current testing of a cylindrical optics based optical layout that allows the full aperture of the AOD to be filled, increasing the time-bandwidth product N. Another effort to assess the suitability of the superior (greater N) TeO₂ AO medium for use with the UV pulses is also underway. Improving the spatial resolution would allow the results of Cash and Yuste (Cash and Yuste, 1998; Cash and Yuste, 1999) to be tested for more nearby synaptic sites where nonlinear summation effects may be more pronounced (Koch and Segev, 2000).

7.2. PHASE 2: COMBINED OPTICAL RECORDING AND STIMULATION

As discussed in Chapter 2, the role of spiking activity in the dendritic tree has been implicated in many important processes. For example, dendritic spikes may act as local thresholding operations while back propagating action potentials may act as a global associative signal of importance in long-term plasticity. The
Dual Scanner can address these phenomena in the context of a spatio-temporal pattern of activation, by straightforwardly combining optical stimulation with optical recording. Measurements of spiking activity are well within the ~5mV resolution of optical recording previously documented (Bullen and Saggau, 1999).

Beyond the observation of spiking activity, a compelling use of the Dual Scanner system is the use of optical recording to directly observe the process of dendritic integration for subthreshold excitatory post-synaptic potentials (EPSPs) generated using optical stimulation. Observing the “process” implies the observation of the summation locally at the synapses and the subsequent propagation towards the soma. This could be either with or without the presence of active conductances—it merely implies that, if present, they are sufficiently inactive that dendritic spikes do not occur.

To make such measurements effectively, greater voltage resolution is required. The current Dual Scanner design employs a higher sampling rate A/D converter for optical recording than previously employed, to enable greater oversampling. The noise reduction afforded may provide the greater voltage resolution required.

7.3 PHASE 3: FUTURE DIRECTIONS

The measurements described, incorporating the Dual Scanner’s capability for fast, multi-site optical stimulation and recording, would represent significant advances in the technology for investigating neurophysiology. It is planned to employ cultured hippocampal neurons, although other neuronal cultures could
also be used. As discussed in Chapter 2, cultured hippocampal neurons are a suitable model for "in vivo" cells in a number of ways, but nonetheless, their use somewhat limits the conclusiveness of the results obtained.

However, cultured neurons remain an important model system in the field of Neurobiology, particularly in the dynamic visualization of biochemical processes in living cells. Recent examples include the visualization of the clustering of "delayed rectifier" K⁺ channels (Antonucci et al., 2001) and the insertion of AMPA receptors following pronounced ("tetanic") stimulation (Shi et al., 1999). Most of these approaches employ green fluorescent protein (GFP) as a means to tag a protein of interest (Tsien, 1998).

As currently configured, the Dual Scanner is designed to allow epi-illumination that is either focused (i.e. laser sources entering at the side) or wide-field (i.e. arc lamp coupled in at the rear port). These are currently separate "modes"—these cannot occur simultaneously. A powerful experimental paradigm, however, would be the ability to couple the well-defined spatio-temporal pattern of activation with wide-field imaging. This paradigm would provide a strong link between the physiological and biochemical approaches to Neuroscience. Currently, an alternative optical layout is under test that will allow the combination of the dual laser pathway with illumination from an arc lamp.

Finally, another approach that has begun in the author's laboratory that addresses the limitations of cultured neurons is to employ two-photon microscopy. This technique enables high-resolution optical recordings to be obtained within thick light scattering tissue (Denk et al., 1990). This technique
employs ultrafast (~100fs) IR pulses, generally from a titanium:sapphire (Ti:S) laser source, and is viable for both optical recording and, recently, optical stimulation (Tsien, 1998).

Thus, the development of an AO scanner for use with a Ti:S laser source would allow 1) a single laser source to be used for both stimulation and recording, and 2) imaging within light scattering tissue (e.g. acute brain slices). However, an AO scanner for an ultrafast Ti:S laser has not yet been developed, largely owing to concerns over the considerable dispersion (i.e. spreading due to wavelength-dependent properties)—both spatial and temporal—an AOD inflicts upon the ultrashort laser pulses. Recently, optical strategies to lessen these effects have been devised and tested (Iyer V et al., 2002). The possibility of investigating single neuron computation in living tissue with the capability for multi-site stimulation and recording therefore looms on the horizon.
REFERENCES


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This Appendix describes the procedure for "aligning" each of the AO scanners. The alignment can be broken into three steps:

1. Alignment of the AO scanning system itself
2. Alignment of the AO scan pattern through the objective
3. Alignment of the AO scan pattern with the live video image

Each of these is discussed in turn.

A1.1 AO SCANNER ALIGNMENT

Figure 4-5c showed the "image" which appears in each of the Dual Scanner planes at each of its produce a “test pattern” (see Appendix 5) where the beam is scanned rapidly across several points in some spatially meaningful pattern (e.g. a grid). The scanning is so rapid that the pattern appears as a static image that can be seen at each image plane.

When aligning the Dual Scanner, the user follows the propagation of the beam through the scanner step by step. The primary heuristic that the author follows is to ensure that the beam is collimated after each relay telescope by adjusting the lens spacing of the telescope. The spacing between the telescopes is less critical, and is simply measured and set to the nominal distance.

In order to observe the output of each relay telescope, depending on constraints such as the location on the table and the distance from the subsequent optics, one of two approaches is used. First, the subsequent optics can be removed and the telescope output projected to a distant wall. The optics can later be reinserted without realignment (see below). Otherwise, the telescope output can be
projected to the ceiling, using a mirror mount (the "Microbench mirror insert") that can be inserted anywhere in the optical rod prototyping system (providing there is space for it) to project the beam to the ceiling. Because propagation to "infinity" (i.e. the ceiling) effects a Fourier transform akin to that (made much closer) by a lens, the mirror insert allows planes in the optical pathway to be "picked off" and their Fourier transforms examined.

For the first telescope, the beam expander, the output collimation can be assessed by the invariance of the output diameter at several distances. After subsequent telescopes, the distant plane is a Fourier transform of the pivot point or diffraction plane intercepted, and is thus an image plane. The telescope lens spacing can simply be adjusted so that the scan pattern is in focus at this distant plane¹.

Meanwhile, the tilt angles of the AODs are also adjusted by looking at infinity either directly after the AOD or after the subsequent telescope. As the tilt is adjusted, multiple diffraction orders are seen; when the Bragg angle is reached, the intensity in the corresponding first order is maximized. In reality, sometimes a compromise is reached between maximal intensity and maximal uniformity of intensity across spots in the scan pattern. After the tilt is set, the slit can be translated until it resides exactly in an image plane, as assessed by the sharp focus of the blade edges. Then it can be closed down to block all but the first order.

After the two deflectors in each scanner, a two-mirror beam aligner is used to steer the scan pattern through the subsequent optics. As was shown in Figure 4-5b, this beam aligner centers the scan pattern through these optics. Although this is not

¹ In practice, slightly out-of-focus is best, since the distant plane is not truly at infinity.
strictly necessary, this reduces aberration effects from the lenses and makes subsequent alignment easier. This alignment is accomplished by first programming a test pattern containing only a single spot in the exact center of the scan range. This central spot can then be aligned using two well spaced pinholes along the output bench (with the lenses there removed).

**A1.2 ALIGNMENT OF SCAN PATTERN THROUGH OBJECTIVE LENS**

In order to ensure that the various spots in the scan pattern focus along the optical axis in the specimen plane (i.e. do not flare to the sides as they are defocused), it is important to ensure the scan pattern is centered through the objective lens. Practically, it is found that this alignment should be quite good before inserting the high-NA objective lens: if the incidence angle is only but slightly off-axis, the beams do not become focused in the specimen plane’s field-of-view.

The final beam aligner, one per scanner, is used to center the scan pattern through the objective lens. To achieve this, a single central spot test pattern is again programmed. The objective lens is removed, and an optical bench (i.e. a rod system) is threaded into the objective lens turret of the microscope, using the simple Microbench-to-objective turret adaptor designed. Along this vertically oriented bench, then, are placed two pinholes (or one pinhole and one centering target for the second point). By aligning the central scan pattern spot through these pinholes, the scan pattern is aligned.

Once this has been done, the objective can be inserted and the central spot (or a multi-spot pattern) will be easily found in the field-of-view, employing a thin fluorescent preparation as the specimen. The final beam aligner can be used to
center it, sometimes in a compensating manner—i.e. one mirror is used to move the pattern towards the center, while the other is used to adjust the angle of incidence for an apparently centered focus (i.e. no flaring to the side). It is found that adjusting the "x-y position" of the second lens in one of the scan magnification telescopes (one is placed on a positioner in each scanner) can be used to translate the scan pattern in the field-of-view at this point, with little effect on the angle of incidence.

A1.3 ALIGNMENT OF SCAN PATTERN WITH VIDEO IMAGE

Finally, once the scan pattern is centered in the objective's field-of-view, it is required to align the pattern with the video image. This alignment consists of adjusting the "gain" and "offset" of the transformation from AO position tuning voltages to the actual positions in the video image. As discussed in Appendix 5, the Test Pattern... and Show Guides... features of the Modify/Scanning Pattern... dialog box of the system control pattern are used to adjust the position of a "scan pattern bounding box" which bounds the area on the video image that correspond to the edges of the tuning voltage range. Assuming that there is no rotation (i.e. the X-deflector purely deflects in the X direction and likewise for the Y-deflector) and that the transformation is purely linear, then, once this bounding box is set, the correct tuning voltage to scan to any site selected within the bounding box can be directly calculated from a linear function of that relative location. In other words, the bounding box contains all the information required for scan pattern alignment.

In order to adjust the bounding box, a scan pattern of three well-spaced arbitrary (not on a line) spots is programmed, and the bounding box boundaries
and/or the scan pattern are translated until these desired spots coincide with spots seen on the video image of the fluorescent preparation.
APPENDIX 2  DUAL SCANNER CONTROL SOFTWARE

In order to be useful as a scientific instrument, the Dual Scanner requires an easy-to-use computer interface that allows experimenters to focus on the experiment, rather than the equipment. A program named “Neurometer,” which runs under the Windows™ (Microsoft Corp.) operating system, has been developed to coordinate the various data acquisition, control, and imaging tasks required¹. A brief introduction of the user interface and how the software handles each of the required tasks is provided here.

The names of particular dialog boxes that can be launched from the application’s main menu are referred to in these descriptions in **bold**, while particular buttons, radio buttons, and edit boxes within the dialog boxes are referred to in *italics*.

A2.1 NEUROMETER FUNCTIONS

This section discusses the various tasks that Neurometer carries out before, during, and after an experiment.

A2.1.1 Site-of-Interest Selection

Neurometer interfaces with the frame grabber to bring a live image of the specimen onto the computer display. Since the frame-grabber features “non-destructive overlay,” the live image can be displayed while the user selects sites-of-interest for UV laser stimulation and visible laser recording, which become highlighted on the screen. A screen-shot of the dialog box used for site-of-interest
selection *(Modify/Scanning Pattern...*) was shown as Figure 3-8. The set of sites of interest selected, along with other timing parameters in the case of the UV scanner, comprise what is termed the “scan pattern.”

*Specifying the Scan Pattern*

In this dialog box, the user can switch between specifying the sites for stimulation (blue) and those for recording (green). The squares define the “scan pattern bounding box,” which may differ for the two scanners. The edges of the bounding boxes correspond to the extremes of the scan pattern tuning voltage ranges (i.e. 3-13V for the visible scanner, 0-1V for the UV scanner, with the current hardware).

For optical recording, all that is required is to select the sites-of-interest within this bounding box. The scanner will simply cycle rapidly between the sites during an experiment. For optical stimulation, the user must specify more details about the timing of the pulses in order to generate a desired spatio-temporal pattern of stimulation. Neurometer provides a rather flexible interface that allows many practical timing configurations to be specified; it does not, however, permit an arbitrary pattern. More details of how the UV scan pattern is programmed are provided in Appendix 7. A brief description of the parameters available for specifying the timing of the stimulation pattern is given here.

The stimulation pattern begins a specified time after the start of the experiment, set with the *Pre-Stimulation Delay* edit box. It then cycles through the

\[1\] Not far from 99% of this software was developed by Saumil S. Patel, whose importance to the work described in this thesis cannot be understated.
selected sites of interest in a pattern that is termed a “visit.” The Visit Count (i.e. number of visits) and the Inter-Visit Interval can be set using the other edit boxes. The pattern of stimulation within each visit can be specified by right-clicking on each site of interest. Here the “scan order”
\(^2\) (i.e. which site is stimulated first, second, etc.), the number of laser pulses, and the intensity (i.e. the UV Mod value) for each site are specified. In addition, the user can specify an inter-pulse interval (IPI), which specifies the delay between pulses delivered to that site, and an inter-site interval (ISI) that determines the delay after the set of pulses at the particular site selected and the pulses to be delivered to the next site in the scan pattern.

The UV laser repetition period is 20μs; thus, the actual delays implemented can only be multiples of this period. The software chooses the nearest such multiple to the specified value.

**Aligning the Scan Pattern**

About once per day, the scan pattern needs to be “aligned” so that the spots selected give rise to AOD tuning voltages that result in illumination at exactly the locations specified atop the video image. This scan pattern alignment consists of adjusting the scan pattern bounding box.

To align the pattern, a “stationary” scan pattern is created using the Test Pattern… button. This causes either the UV or visible scan pattern (whichever is selected in the Scan Mode radio buttons) to be “played out” repetitively or a fixed number of times (Test Scans): the D/A channels cycle through the scan pattern—

\(^2\) Actually, as of the writing of this thesis, the scan order of each site cannot be directly controlled from the “right click” control menu—but this will be changed.
sequentially for the visible scanner, and according to the specified parameters for
the UV scanner. The scan position update rates are fast enough so the resulting
scan pattern appears stationary to the human eye.

With this stationary pattern visible on the live video image\(^3\) displayed in the
Scanning Pattern... dialog box, the scan pattern bounding box boundaries can be
adjusted after selecting the Show Guides... button. By using an arbitrary three-point
pattern (not arranged on a line) as the test pattern, the user adjusts the guides until
the sites selected match the location of the spots visible on the video image. This
completely adjusts for the scale and offset parameters mapping the ideally linear
relationship between the tuning voltage for one deflector and the spatial location in
that particular dimension.

Using the separate Modify/Scan Pattern... dialog box, the user can choose
to select a subset of the tuning voltage ranges, so that, for example, the edges of the
scan pattern bounding box correspond to .1 and .9 V for the UV scanner. This might
be done to prevent the use of more nonlinear portions of the tuning curve. In this
dialog box, the user also can select the “direction” by which the tuning voltages map
to the video image (i.e. increasing voltages to deflector channel 1 imply further to the
right), which is determined by trial and error.

A2.1.2 Scanner Control

Neurometer takes the recording sites and stimulation sites which the user has
specified, as well as the timing and intensity information about the latter, to fill

\(^3\) A thin fluorescent prep is usually used as the specimen with the Test Pattern, so that the scan
pattern is visible in the video image.
memory buffers physically located on the D/A cards (AWFG; Keithley) which control the two AO scanners. These buffers are “played out” during the experiment—repeatedly in the case of the visible scanner, once in the case of the UV scanner—without consuming any CPU resources. Neurometer merely sends a command to start the buffer playback at the beginning of the experiment. At the end of the experiment (set by the sweep time parameter in Modify/Timing...), it sends a command to end the buffer playback for the visible scanner (the UV scan buffer only runs once, and is constrained to always be shorter than the sweep time).

For the UV scanner, the scan pattern buffer is always played out at the same rate: 100kHz. This is discussed in Appendix 6. For the visible scanner, the user can select a desired sampling rate in the Modify/Timing... dialog box—this is the true bandwidth desired required for the recording at each site (e.g. 5kHz would allow adequate sampling to capture all features in an action potential or post-synaptic potential waveform). Based on this sampling rate and the number of recording sites specified, Neurometer calculates the amount of time available to “dwell” at each recording site. For example, for 10 sites and 5kHz sampling, the visible laser could dwell at each site for 20μs. The rate at which the visible scanner updates the laser position—i.e. the rate at which the buffer is played out—is thus set at 50kHz.

**A2.1.3 Optical Recording**

During an experiment (which typically lasts ~1s), the CPU’s resources are mostly dedicated to collecting optical recording data, typically from two channels, at high rates. Generally, the maximum sampling rate of the A/D card (AIX/Chico; Innovative Integration) is employed (2.5MHz). This permits oversampling, where
multiple samples are collected during the laser dwell time at a particular site. This excess of samples can be averaged to significantly improve the signal-to-noise ratio (SNR) of the optical recordings.

During the experiment, a dynamic link library (DLL) written by the author to interface between the A/D card's control library and Neurometer runs behind the scenes. The collected data is streamed over the PCI bus and stored in raw binary form into large files on a RAM disk allocated in the computer's memory, occupying much of the resources of both the PCI and the CPU. The program polls to see that enough data has been collected into the RAM disk, and ends the experiment when it has been.

After the experiment, the large data set per channel is soft-"demultiplexed" so that samples are assigned to the appropriate recording locations and time points. The detector/deflector settling time\(^4\) is set by the user (the detector settling time item in the Modify/Timing... dialog box), determining how many of the initial samples per scanning spot to throw away. The samples remaining are averaged, and then subsequent ratioing, correction, and filtering operations are carried out. The settings for ratioing can be set in Modify/Measurement..., for bleaching correction in Modify/..., and for digital filtering in Utility/Filtering.... These processes are depicted in Figure 3-7 of the text.

After these calculations, the resulting waveforms at each of the recording sites are displayed in small windows in the Neurometer main window, one per

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\(^4\) In reality, this parameter should reflect an aggregate (convoluted) settling time of the Vis Scan D/A circuitry, the deflector, the photodiode detector, and the A/D circuitry. In practice, the detector, and to a lesser extent, the deflector settling times are the "rate-limiting" steps.
recording site. Thus the user immediately has a handle on the signal of interest (e.g. membrane potential) at each of the recording sites, and can determine whether to continue or abort experiments with the particular cell under study.

**A2.1.4 Electrophysiology**

In principle, the Dual Scanner obviates the need for conventional electrophysiological approaches, such as patch clamping, iontophoresis, or field recordings. In practice, however, some or all of these techniques will be employed for calibration and control purposes. Neurometer incorporates control of a 12-bit D/A-A/D card *(CIO-DAS08H; Computer Boards)* that is dedicated to providing channels for both control and acquisition from electrophysiological instruments.

Before an experiment, the user can specify the number of recording (A/D) channels *(Modify/Channel Designation...)* employed. For the D/A channels, the user can specify control waveforms to play out on one of two supported control/stimulation channels using the *Modify/Stimulation 1...* and *Modify/Stimulation 2...* dialog boxes. These permit flexible specification of pulses and pulse trains (i.e. amplitude and delay parameters). Since the vast majority of control signals required for electrophysiology are simple square pulses or functions with multiple steps, this is not a significant limitation.

During the experiment, the D/A-A/D board requires a read and a write to RAM every sample; thus, these channels occupy CPU time and other resources. However, because the update rate for these measurements is much slower (15 kHz aggregate for each D/A and A/D), this does not cause any contention or other difficulty with the much faster optical recording that occurs simultaneously.
After the experiment, the collected A/D data is displayed in the main experiment window, after the application of any off-line digital filtering (i.e. FFT) or other processing that Neurometer currently supports. This waveform(s) is displayed alongside the optical recording site measurements in the main dialog box.

**A2.1.5 Digital Control Lines**

Neurometer provides the capability of driving eight digital (i.e. TTL) output lines, which can be used to control and/or synchronize a variety of instruments. Before the experiment, the experimenter uses the **Modify/Digital Lines...** dialog box to specify for each digital channel when during the experiment it should transition (or even before the experiment, which “starts” when the first recording samples are collected). These digital lines are used, among other things, to control electromechanical optical shutters (*Uniblitz* Series; Vincent Associates) on the Dual Scanner such as the one used to prevent laser illumination from reaching the specimen at all times except during the experiment.

**A2.2 FLOW OF OPERATION**

The data files associated with Neurometer consist of *protocols* and *experiments*. Protocols are collections of all the pre-experiment settings including the sampling rate, stimulation protocols, digital line timings, etc, as well as both the visible and UV scan patterns. Protocols can be saved and then later opened as the basis for a new experiment. Slight changes can be made (such as changing the locations of the sites of interest) without changing the underlying protocol. Thus, they are a good means to save a set of commonly unchanged settings.
Once a protocol is opened (although it is not necessary—there are default settings), a new experiment is created (Experiment/New Experiment...). The user can then modify the settings if desired using the various options in the Modify... menu. Finally, the experiment is started using Experiment/Start Experiment....

Depending on the settings in the Modify/Timing... dialog box, the data collection can occur in one of three modes: continuous, averaging, or sequencing. In continuous mode, the experiment is run repeatedly at intervals specified by the inter-stimulus interval. During this interval, the data is displayed (the interval must be longer than the time to process and display the data), and the user can then elect to end the experiment if he/she “likes” the data (or gives up on the experiment/specimen). This is useful in cases where one is looking for a somewhat rare event over a number of trials\(^5\). The data from this last trial remains stored temporarily by the program, and can be saved as a take of the experiment via Experiment/Save Take.... Comments can be added to the take via Experiment/Take Comments.... In the averaging mode, the user can specify that a number of trials be averaged together, to improve signal quality. As in continuous mode, the data is processed and displayed between trials; however, the data is averaged with the previously collected trials. Thus the user can watch as the signal quality (hopefully) improves. After the number of trials specified, the averaging process starts over. The user can stop the experiment at any point, and the last data displayed is available to be saved as a take.

\(^5\) A spontaneous EPSP would be an example.
In the sequencing mode, the user can again select to average a certain
number of trials and, furthermore, can set how many of these aggregate averaged
takes to collect. Each of these averaged trials is automatically saved as a take in the
experiment. This allows a number of trials to be collected without user intervention.

The takes, whether stored automatically in sequencing mode or manually in
continuous or averaging mode, are stored within Neurometer's "Data" directory as
binary files containing all the protocol information for that take (i.e. timing,
stimulation, scan pattern settings, etc.), the take comments, and all the collected
optical and electrical recording data. This directory contains folders for each date on
which any takes were saved, and within that date, contains folders for each
experiments. Each experiment, therefore, can be a set of takes that were all taken
with the same settings. However, it is not required that the takes all share the same
settings, since each take saves these. In actual use, each experiment consists of a
single cell, which may have a number of takes acquired using different settings (i.e.
different sites of interest, different stimulation strengths, different timings, etc.).

It is important to record the structure of the cell in relation to the selected sites
of interest. Using Experiment/Save Image..., a TIFF image file is saved in the
experiment directory. Thus, generally one image is collected per experiment.

A2.3 DATA RETRIEVAL

As described above, Neurometer catalogs all the experiments collected in its
"Data" directory. At a later time/date, an old experiment can be opened using
Experiment/Open Experiment..., and the user can select a particular take to open.
When a take is opened, the program switches to exactly the state it was in when that
take was saved. The settings in the Modify... dialog boxes are those used for that
experiment, including the scan patterns. The image (if any) that was saved is
displayed along with the data windows containing the various optical and electrical
recordings. The user can select Experiment/Convert To Text... to create text files
containing the data for each A/D channel employed (i.e. both electrical and optical
recording channels). These can be used for external viewing with commercial data
analysis software.

When a particular old take is loaded, because all the settings for the take are
active, the user can start a new experiment based on the same settings by calling
Experiment/New Experiment....

A2.4 SUMMARY

Here we summarize the actions associated with Neurometer before, during,
and after an experiment.

Before Experiment:

- Protocol can be opened to load a set of commonly employed settings
- Settings for the experiment (sweep time, sampling rate, scan pattern, stimulation
  pattern, etc.) can be altered/specified using the Modify... menu items
- Image of the specimen can be acquired to store with the experiment
- Optical alignment of the scanners can be done using the Test Pattern... feature of
  the Scanning Pattern... dialog box (usually done once a day—see Appendix 1)

During Experiment:

- Fast optical recording occurs under control of a separate DLL, which streams data
  collected onto large RAM disks pre-allocated in the operating system. Neurometer
itself repeatedly makes a call to this DLL to poll to see when enough data has been collected\(^6\): when it does, the experiment is ended.

- Electrophysiological D/A and A/D values are transferred from and into memory buffers pre-allocated by Neurometer. This is interrupt-driven: the hardware contains a timer and generates periodic interrupts prompting Neurometer to load/store a new value.
- Digital outputs are updated on the parallel port at various times relative to when the experiment is started as specified in the **Modify/Digital Lines...** dialog box. This timing is controlled by a set of operating system CPU timer calls, and is thus "soft."

**After Experiment:**

- Optical data collected is "soft-demultiplexed," processed per the experiment settings, and then displayed
- Electrical data collected for up to two A/D channels is displayed
- The user can save the take, and add **Take Comments...**, as desired

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\(^6\) This is done instead of polling a CPU timer to ensure the sweep lasts the duration specified as the sweep time. This approach is just as effective, but sometimes it causes
APPENDIX 3  UV SCAN PATTERN PROGRAMMING

The software/hardware implementation to control the two parameters of the UV scanner—position and amplitude—is designed to provide considerable flexibility in the stimulation pattern that can be produced by the experimenter, but must also work within several constraints imposed by the laser itself (Series 3505; DPSS Lasers) and the D/A boards used to control the scanner (AWFG, Keithley Instruments). This Appendix describes those constraints and then provides a general description of the software code used to program the D/A board’s on-board memory buffer which is “played out” during an experiment.

A3.1 UV LASER CONTROL CONSTRAINTS

The Q-switched laser from DPSS was modified by the manufacturer to allow the Q-switch to be externally controlled. This control is achieved using two digital signals: “Q-Switch Clock” and the “Q-Switch Enable” TTL inputs. The CLOCK actually causes the laser pulses, on the lo→hi transitions. The ENABLE input simply ANDs with the “Q-switch enable” switch on the front panel to control whether the unit is lasing at all.

There are several constraints that these signals must adhere to (O. Varela; DPSS Lasers):

1. The clock signal must run at 50 kHz to within say a 100 Hz. This is to ensure that the laser is pulsing at the frequency it was optimized for.
2. After the enable signal comes on, the first clock pulse (i.e. low→high transition) should come within 20μs, and not before a few μs. If this is not adhered to, as I understand, the FPS circuitry (discussed below) could cause damage.
3. The clock signal should run continuously for as long as the enable signal is active.

The other concern regarding the DPSS laser is the first pulse suppression (FPS) circuitry. This circuitry attenuates the first few pulses fired after the enable signal becomes active; were it not for this circuitry, the first pulse would have an enormous energy, since it's had more time to build up an inversion. The downside to FPS is that it takes a few pulses for the pulse power to ramp up to the steady-state level. Once steady-state is reached, the pulse power stability averages <10%.

A3.2 SCAN PATTERN BUFFER

As was discussed in Appendix 6, the user selects sites of interest and defines the timing of the pulses delivered to those sites in the Scanning Pattern... dialog box of the system control software. The software fills 32Ksample memory buffers on each of the two D/A boards (AWFG; Keithley) for the scanner, UV Scan and UV mod. The contents of those buffers are referred to as the scan pattern buffer. Each sample consists of 32 bits, 12 bits for each of the two analog output channels and 8 bits for the digital output channels. On the UV Scan board, all 8 digital outputs are programmed along with the analog outputs to create a variety of timing signals described below.

As currently implemented, the scan pattern buffer is not amenable to being repeated, because the first portion of the buffer is an initial delay that should not be repeated. Thus, the size of the memory buffer limits the length of the stimulation scan pattern that can be played out to 32kSample x10μs/sample = 320ms.

The scan pattern buffer consists of the following phases:
PreStimDelay

\[
\begin{align*}
\text{Num Pulses} \\
\text{Inter-Pulse Delay (IPD)} \\
\text{Visit(s)} \\
\text{Inter-Spot Delay (ISD)} \\
\end{align*}
\]

\text{Inter-Visit Interval(s)}

The PreStimDelay is the initial delay after the experiment started where no laser pulses are to be directed at the specimen. After the PreStimDelay, the buffer cycles through the selected sites of interest in what is termed a visit. In "visiting" each site, the buffer can "dwell" for Num Pulses samples (actually pairs of samples). The Inter-Pulse Delay is applied after each of these pulses for a particular site, save the last one. The Inter-Spot Delay for each spot is applied after all the pulses for that spot have fired. The Inter-Spot Delay is applied even if the spot is the last in the stimulation pattern.

\textbf{A3.3 SOFTWARE IMPLEMENTATION:}

The programming of the UV scan pattern buffers occurs in the software file "expcont.c," in the function \textit{SetBuffersForPhotoStim()}. It consists of repeatedly calling one of four helper functions:
AddHomeBufferEntry()
AddPosnBufferEntry()
AddStartupBufferEntries()
AddDelayBufferEntries()

First, the total length of the buffer is estimated, in milliseconds, and it's checked that the buffer doesn't exceed either the experiment sweep time or the maximum buffer length of 320msec.

Next, entries are added to the buffer one at a time, using one of two functions, AddHomeBufferEntry() or AddPosnBufferEntry(). Each function adds one entry to each of four buffers (two channels for each of the two boards) pointed to by the variables vars.ptr1, vars.ptr2, vars.ptr3, and vars.ptr4. These point to the buffers for XSCAN, YSCAN, XMOD, and YMOD, respectively. Each entry places a new short-length integer into each of these buffers. In addition, the 4 digital lines available on the XSCAN and YSCAN outputs are each updated with each entry. The values to be output with each entry are contained in the variables vars.x_mask and vars.y_mask, respectively.

The digital lines associated with each channel are:

X/Bit0: PhotoStimDone         Y/Bit0: Q-switch Clock
X/Bit1: PhotoBufStart         Y/Bit1: Q-switch Enable
X/Bit2: PhotStimStart         Y/Bit 2: Digital Modulation X
X/Bit3: PhotoStimActive       Y/Bit 3: Digital Modulation Y

These are described further below.
The AddHomeBufferEntry() function programs a buffer entry with the SCAN signals in the “home” position: the x-position is set to the far left of the scan range, and the y-position is set to a random location in the scan range, which is calculated anew every time the function is called. This randomization was done to avoid illuminating the same spot continuously during long periods of “zero” stimulation, even though it’s out of the field of view. Meanwhile the MOD signals are both set to produce a zero-intensity spot; both the x- and y- channels are modulated to 0, to achieve the maximum extinction.

The AddPosnBufferEntry() function programs a buffer entry with the XSCAN and YSCAN signals as specified for a particular spot in the scan pattern. The XMOD signal is set to the amplitude specified for the particular spot in the scan pattern. The YMOD signal is set to 100%.

During each call to AddHomeBufferEntry() or AddPosnBufferEntry(), the

![Pair of Home Buffer Entries](image)

**Pair of Home Buffer Entries**

- **Q-SWITCH ENABLE:** Can be either enabled or disabled.
- **Q-SWITCH CLOCK:**
- **SCAN/MOD SIGNALS:**
  - Deflector positions during this time
  - Laser fires here (if enabled)

![Pair of Position Buffer Entries](image)

**Pair of Position Buffer Entries**

- **Q-SWITCH ENABLE:**
- **Q-SWITCH CLOCK:**
- **SCAN/MOD SIGNALS:**
  - Deflector positions during this time
  - Laser fires here

**Figure A3-1 UV Scan Pattern Buffer Programming** The scan pattern buffer is filled up by successively adding pairs of either “home” or “position” entries.
variable vars.total_samples is incremented, so an accurate count of the number of entries in the buffer is created. During each call, the variable vars.clock_mask is toggled between 0 and 1. The UV buffer is played out at 100kHz, while the pulses only fire at 50kHz. This is done so that the position can be set a clock period (10\(\mu\)s) before the next pulse fires, giving the deflectors time to settle. To implement this, each of these functions is called in pairs—the first entry with the clock value at 0 and the second entry with the clock entry at 1. At the start of the second entry is when the laser fires. This “building-up” of the buffer by pairs of home or position buffer entries is depicted in Figure A7-1.

There are 2 other helper functions, AddStartupBufferEntries() and AddDelayBufferEntries(). Each of these in turn calls AddHomeBufferEntries() an even number of times, so all buffer entries still follow one of the pair patterns depicted above. These functions are used to implement delays in the pattern—either the pre-stim delay, inter-pulse delays, inter-spot delays, or inter-visit intervals.

Because modulating the beam to zero and positioning it beyond the field of view doesn’t completely eliminate the light reaching the preparation (which can be considerable, owing to light scattering at the slits), a rule is followed that the laser should be turned off during these delays and then turned back on. When turning the laser back on, 3 laser pulses are fired to the Home position, modulated to 0--to account for the FPS effect—before the first positioned pulse after the delay is fired. Thus, the laser is only turned off when the delay in question exceeds the number of clock pulses needed to startup again.
AddStartupBufferEntries() adds 3 laser pulses (6 clock cycles) of home buffer entries, to account for FPS. AddDelayBufferEntries() adds as many clock cycles (an even number) of home buffer entries to account for the delay. If the number of clock cycles is greater than 6, then the Q-Switch Enable signal is turned off for these entries, and the number of entries is reduced by 6, to account for a call to AddStartupBufferEntries() at the end of delay. If it’s less than 6, then the laser stays on during the delay.

A3.4 DIGITAL LINES DESCRIPTIONS

The description of each digital line follows:

PhotoStimDone: Goes lo→hi just as the photostim buffer finishes—could be used to signal that it’s OK to start recording.

PhotoBufStart: Goes lo→hi at the very start of the photostim buffer, and remains high throughout the buffer—can be used as a trigger to indicate time=0 with respect to the UV stimulation paradigm.

PhotoStimStart: Goes lo→hi after the pre-stim delay is complete, just as the first non-home position pulse of the protocol is fired—can be used as a trigger to indicate the true start of the UV stimulation paradigm.

PhotoStimActive: Is hi for every position buffer entry, and lo for every home buffer entry. Thus, it’s hi during all the pulses that “count”—i.e are directed at the prep. This signal can be digitized to give a “timing” diagram of the stimulation paradigm.

Q-Switch Clock: Digital signal to laser that determines when laser pulses are fired—runs at 50kHz.
**Q-Switch Enable:** Digital signal to laser that, when HI, "turns on" the q-switch—it is turned on and remains on for periods during which the laser continuously fires at 50kHz. It is driven LO, turning the laser off, during periods where several home pulses are required in a row, to eliminate background scattered illumination during these times.

**Digital Mod X/Y:** Digital signal that is HI during position entries and LO during home entries—designed as digital alternatives to XMOD/YMOD analog modulation signals, in the event these are unavailable. These turn off the illumination during delay periods.
As described in Chapter 5, there are three classes of timing/synchronization "threads"—the UV Scan/Mod thread, the Optical Recording thread, and the Asynchronous threads. The UV Scan/Mod and Optical Recording threads require hard synchronization of particular D/A and A/D channels. These threads as well as other less critical threads associated with the experiment—i.e. electrophysiological recording and stimulation and digital control signals—must in addition be soft synchronized so that the time stamps associated with each thread are all well-aligned.

A4.1 HARD SYNCHRONIZATION

The timing and hard synchronization of the UV Scan/Mod and Optical Recording threads was depicted in Figure 5-10. Each thread is controlled by a shared master timer.

A4.1.1 Vis Scan/Optical Recording Synchronization

The hard synchronization between the Visible Scan AWFG D/A card (Keithley Instruments) and the AIX/Chico optical recording A/D card (Innovative Integrations) is driven by an 80MHz crystal IC, bought and installed on the Chico baseboard, is used to derive two timers, Timer 0 and Timer 1, using the (software-) programmable on-board timer/counter ICs. Timer 0 drives the A/D conversions, which occur at 2.5MHz, but which require a clock 8X as fast, namely 20MHz¹. Timer 1 is also

¹ This 8X oversampling factor is a feature of the A/D converter itself, and is separate from the oversampling the Dual Scanner employs to reduce noise in the optical recording.
derived from the same 80MHz source, and is constrained to be a sub-multiple of the
20MHz Timer 0. The Timer1 output is then used to drive the Visible Scan D/A card
via its External Clock input.

The sub-multiple constraint ensures that Timer 0 and Timer1 are phase-
locked, so the fast oversamples driven by Timer 0 can be assigned to periods of
Timer 1, as was shown in Figure 3-7b. Figure 5-10 shows only one possible Timer 1
frequency, namely the maximal 100kHz. In the system control software, the user
enters the desired frequency of optical recording. This is multiplied by the number of
recording sites to compute the desired Timer 1 frequency. The software then
determines the actual optical recording frequency by choosing the sub-multiple of
the Timer 0 frequency that is closest to the desired frequency.

A4.1.2 UV Scan/UV Mod Synchronization

The hard synchronization of the UV Scan and UV Mod D/A boards is
achieved by driving the “External Clock” input of both the boards with an output from
a 100kHz timer derived from the 10MHz crystal on the CIO-DAS08-AOL board
(Computer Boards). A simple modification to the board was used to tie the output of
the on-board decade counter to one of the on-board 82C54 Timer/Counter ICs. The
resulting timer output is available on the board’s 38-pin connector interface, and is
routed directly to the front panel as part of the CIO-DAS08-AOL interface circuitry.

The more direct approach would be to generate the 100kHz timer from the
on-board oscillator on one of the UV D/A boards, and then use this timer to drive the

2 Note that a “sub-multiple” of the 20MHz source actually means that the resultant period must be a
multiple of the base period, namely 1/20MHz=50ns. This permits considerable frequency resolution.
other board. However, this approach proved troublesome due to the very short-lived Digital Data Valid pulses that are made available by the D/A board, which did not serve as a reliable “clock out” signal to trigger the second D/A board, in spite of the Schmitt-triggered monostable vibrator circuitry employed (see Figure 5-2) to prolong the pulse.

Another viable approach would be to derive the 100kHz clock from the 20MHz master clock used in the optical recording thread. Because only two timers are available on the Chico baseboard, however, and these are both employed in the optical recording thread, a separate, custom timer/counter circuit would need to be employed.

A4.2 SOFT SYNCHRONIZATION

The two hard-synchronized threads described as well as the two asynchronous threads—the asynchronous DIO lines and the electrophysiology A/D and D/A channels—must all be synchronized so that the “time=0” point is reasonably aligned for each\(^3\). This less stringent synchronization is termed here “soft synchronization” since it is effected by software.

The soft synchronization is effected through the designation of one of the asynchronous DIO lines as the “Take Sync” signal (seen on Figure 5-1). When starting an experiment, the software employs the Windows built-in timer functions determine when to make the digital lines transition (usually LO→HI) on the parallel port. Generally, these transitions occur before the experiment (i.e. to open a shutter

\(^3\) “Reasonably” here means that any offsets be physiologically insignificant, so <500\(\mu\)s would be a good target, for example.
a few milliseconds before recording starts). At the "time=0" point in this sequence of digital line transitions, the Take Sync bit switches HI. This signal is connected to the "gate" input of the master timer on the Chico baseboard; thus the Take Sync signal directly triggers the start of the optical recording thread.

The electrophysiology and UV Scan/Mod threads are driven by the same timer, on the CIO-DAS08-AOL board. This board does not provide a trigger input, and so this timer is simply started in software one line of code subsequent to the setting of the Take Sync bit.

In this way, all the threads are well-aligned to within only a few CPU clock cycles
APPENDIX 5  DUAL SCANNER "WIRING DIAGRAM" FOOTNOTES

This Appendix contains the footnotes to accompany Figure 5-1—the Dual Scanner “wiring diagram.” These footnotes and the Figure combined are intended to serve as a quick reference guide for the connections between the various circuitry of the Dual Scanner as currently configured.

1. AWFG (Keithley): 12-bit D/A converters for controlling AOD scanners
2. AIX module/Chico baseboard (Innovative Integrations): Fast 16-bit A/D converter for optical recordings
3. CIO-DAS08-AOL (Computer Boards): 12-bit A/D and D/A for electrophysiology recordings
4. IV-400 Frame Grabber w/ non-destructive overlay (μTech)
5. AWFG DAC cards contain 8 bits of digital I/O ports that are updated synchronously with DAC conversions. On UV Scan AWFG, these ports are used to generate digital signals that 1) control the laser pulsing: Q-Switch Clock and Q-Switch Enable (Y0-Y1, respectively), and 2) provide triggers associated with the scan pattern: PhotoStimDone, PhotoBufStart, PhotoStimStart, and PhotoStimActive(X0-X3, respectively). See Appendix 6.
6. “Shutter”: Typically Async DIO lines 0-3 are designated as “shutter” lines which are used to control the mechanical shutters placed before the visible light sources—the visible laser and an epi-illumination lamp. Also drive the digital (i.e. on/off) modulation inputs to the visible deflector drivers.
7. Q-switch Clock and Q-switch Gate signals are 50Ω digital inputs to the UV laser. The manufacturer added these inputs to allow the laser pulsing to be externally controlled, and thereby synchronized with the AO scanner. See Appendix 6.
8. AT200 Controller Card. Photometrics, Tucson, AZ
10. Schroff linear power supplies are interspersed in the two card racks. +28V: Visible deflector drivers; +/-18V: Visible and UV scanner gain/offset circuit supply; +/-15V: Optical recording gain/offset circuit supply and photodiode amplifier supply; +5V: Digital supply for shutter and UV scanner circuitry

11. **CCD Take Trigger:** Input signal allows “take” on CCD computer to be synchronized with “take” on Scan computer. This is usually connected to the PhotoBufStart signal to allow synchronization with UV scan. When a longer delay between the start of imaging and the start of stimulation is required, **CCD Take Trigger** is connected to the Take Sync signal. See Appendix ...

12. **Vis Scan Clk:** This signal clocks the visible scan pattern. It is generated from Timer1 on the Chico baseboard. It is derived from (i.e. is a submultiple of) Timer0 on the baseboard, which runs many times faster. Timer0 is used to clock the fast A/D channels for optical recording. In this way, the recordings are synchronized to the scan pattern. Many samples are taken at each scan position—this oversampling is used to greatly enhance the SNR. See Appendix ...

13. **UV Scan Clk:** This signal clocks the UV scan pattern. It is generated from an 82C54 counter/timer chip on the CIO-DAS08H board. **UV Scan Clk** is the CLK0 signal on this chip. The board was user modified so this signal is driven by the on-board 5MHz crystal. The signal is software programmed to divide by 50, generating the 100kHz clock required for the UV scanner. See Appendix ...

14. BVC-700A (Dagan Instruments). Commercial patch clamp amplifier suited for both voltage and current clamp recordings, but preferably the latter.

15. **Step Cmd Gate:** This square-wave signal is used to gate periodic step commands delivered to the patch clamp electrode, during seal and patch formation. The signal is generated on the Chico baseboard and controlled by a simple DLL application that allows the user to select the period and duty cycle of the square wave.

16. Silicon photodiodes (S1226-18BK; Hamamatsu) and custom photodiode circuits for optical recording and UV pulse energy measurements.
17. Model 3505 Pulsed UV (355nm) laser (DPSS Lasers). Modified by manufacturer for a 50kHz repetition rate and to allow external control of the Q-switch.

18. SDM-1502B8 (IntraAction). Fused silica deflectors driven at 100-200MHz.

19. LS55V (Isomet). TeO₂ slow-shear deflectors driven at 70-100MHz.

20. WV-1550 (Panasonic)

21. CH250A Camera Head and CE200-12/500 (12-bit, 500kHz) Electronics Unit. Photometrics, Tucson, AZ. Uses Thompson TH7883 CCD Chip (386x576 pixels).

22. Two DIO lines from Chico baseboard are made available on front panel. These can be programmed for special purposes, such as the Step Command Gate signal.

23. Take Sync: Digital signal generated by Scan Computer that marks the start of a take, indicating the start of optical recording. This is a “soft” trigger, meaning that it is synchronous to the start of recording to within a few computer instructions. See Appendix 8.

24. Visible Scan Interface Circuit: converts the +/-10V output range from the Vis Scan D/A (AWFG) to the 3-13V range required by the visible deflector drivers.

25. UV Modulation Interface Circuit: converts the 0-10V output range from the UV Mod D/A (AWFG) to the 0-1V range required by the UV deflector driver. It buffers the output to drive the 50Ω input impedance of the tuning voltage.

26. UV Scan Interface Circuit: converts the 0-10V output range from the UV Scan D/A (AWFG) to the 0-1V range required by the UV deflector driver. It buffers the output to drive the 50Ω input impedance of the tuning voltage.

27. Asynchronous Digital I/O (Async DIO): digital signals generated by software on the Scan Computer at its parallel port. These signals are simply routed straight through to the front panel.

28. Optical Recording Interface Circuit: converts the output of the photodiode amplifiers (0 ↔ -10 V) to the voltage range required (-2 ↔ 2 V) for the fast 16-bit A/D converters on the AIX module.
29. Electrophysiology Interface Circuit: 7 A/D channels and 2 D/A channels are available for patch clamp and other electrophysiological recordings and stimuli. The A/D channels are simply routed straight through; the D/A channels are passively attenuated (voltage divider) by a factor of 10 to match the meaningful range of $I_{CMD}$ inputs to the patch clamp amplifier. Finally, the board's CLK0 signal is also routed to the front panel.

30. Clk Out: The "Digital Data Valid" pin on the UV Scan D/A generates a short-lived pulse every time the D/A updates. The interface circuit uses a monostable vibrator IC to prolong this pulse. This output, however, has been found to be unreliable, and is thus not used in the synchronization.
This Appendix describes those electronics systems of the Dual Scanner not discussed in Chapter 5.

A6.1 CCD CAMERA ELECTRONICS

For wide-field fluorescence imaging, the Dual Scanner incorporates a cooled CCD camera (CH250/A; Photometrics). The image acquisition of this camera is controlled by a specific board (CE200A-12/500; Photometrics), which supports a 500kHz data rate and 12-bit imaging. This board is inserted into a separate CCD control computer, and a custom Windows program provides a GUI style interface with which the user can set the acquisition region, the desired frame rate, and the amount of binning. For small regions and/or with large binning factors, very fast (~100Hz) frame rates can be supported. Experiments can be specified in which a given number of frames can be collected, forming a “movie.”

In order to synchronize the movie acquisition by the CCD computer and the UV (or visible) scanning controlled by the Scan computer, a “sweep trigger” input line is made available on one of the parallel port pins of the CCD computer. The experiment on the CCD computer is started, and the acquisition begins when a sweep trigger arrives from a digital line generated from the Scan computer experiment. More detail about the synchronization of the two computers is provided in Appendix 6.

A6.2 PHOTODIODE AMPLIFIER

The four photodiode circuits used in the Dual Scanner are shown in Figure 5-1. These include the two signal photodiodes used in ratiometric recording, the
reference photodiode used for non-ratiometric recording, and finally a "UV reference photodiode" that could be used to account, off-line, post-experiment, for pulse-to-pulse variation in UV laser energy and/or variations in intensity across the UV scan pattern.

Except for the UV photodiode, each of these amplifiers is based on a two-stage op amp amplifier design. The first is a transimpedance amplifier that converts the generated photocurrent at the photodiode into a small but significant voltage by using a large feedback resistor, while a feedback capacitance is used to limit the bandwidth to only that required. The second amplifier is a noninverting voltage gain amplifier, used to boost the signal from the first if necessary. Both stages are based on the OPA606KP (Burr Brown), elected for its small current noise and, importantly, its low input bias current\(^1\).

### A6.3 SHUTTER CONTROL CIRCUIT

The Dual Scanner incorporates two “Uniblitz” mechanical shutters (Vincent Associates), one for the visible laser and another for an epi-illumination arc lamp for fluorescence imaging. As shown in Figure 5-1, a custom shutter controller is used to open and close these shutters. This shutter controller circuit\(^2\) receives TTL inputs from the Asycronous DIO lines which transition HI specified times before the experiments (~10ms, to allow time for them to open). This digital signal causes a pair of large (220\(\mu\)F) storage capacitors containing 60V to discharge across the two shutter inputs (\(\text{SH}^+\) and \(\text{SH}^-\)). This short-lived 60V pulse serves to overcome inertia

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\(^1\) The second op-amp amplifier should be one of low voltage noise instead; typically, an op amp has excellent voltage or current noise performance, but not both.
and open the shutter blade. This voltage decays and the shutter circuit holds the output at 5V, which is sufficient to hold open the blades. At the end of the experiment, the TTL input signal transitions back LO, and the circuit disconnects the 5V power from the SH⁺ and SH⁻ lines, causing the shutter blades to close. While the shutter is closed, the circuit uses AC power to charge up the large storage capacitors for the next shutter opening.

² The circuit was designed, constructed, and tested first in Munich by G. Wenzel and P. Saggau.