Chapter 1

Introduction

The right tool for the right problem

Neuroscience has many examples where technological development created a fertile ground for fundamental discoveries, and conversely, vexing biological questions that motivated technical development. Six years after the Nobel prize was established, in 1906 Camillo Golgi and Santiago Ramón y Cajal were awarded the Nobel prize in Physiology or Medicine for their work on the structure of the nervous system [1]. Key to their success was the development of reazione nera (black reaction) by Golgi and ultimately its meticulous application by Cajal, revealing the intricate morphology of various cell types in the nervous system (Figure 1.1).

Another turning point for neuroscience was the development of the patch-clamp technique and its variants by Bert Sakmann and Erwin Neher [2–5] for which they shared the Nobel prize in Physiology or Medicine in 1991. The method relied on pulling glass capillaries to obtain smooth micrometer sized tips and bringing the pipette tip in contact with the cellular membrane. Through gentle suction, the membrane stuck to the glass wall and a high-resistance seal of several gigaohms was obtained. If the membrane was left intact and with the help of pharmacological compounds, the kinetics of ion channels could be measured by stepping the pipette potential [6]. In this way they were able to demonstrate for the first time the existence of ion channels that gate the passage of ions across the membrane. By applying brief strong suction, the membrane would break and whole-cell access could be obtained while maintaining the gigaseal intact. In this configuration, the membrane potential of the cell could be stepped, current injected to elicit action potentials (APs) or synaptic activity be measured.

While initially whole-cell recordings were performed at the soma, the technique was gradually adapted to record from dendrites [7, 8]. This revealed that dendrites of most neurons are not simple passive cable-like structures. Instead, they contain active conductances that sustain the back-propagation of APs [9] (Figure 1.2), providing a Hebbian form of plasticity known as spike-timing-dependent plasticity (STDP) [10]. Moreover, active dendritic conductances such as sodium [11], calcium [12] and NMDA-
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Figure 1.1: Cajal’s drawing of various cell types in the retina using Golgi’s staining method.

Type glutamate receptors [13–15] have been shown to generate local dendritic spikes that precede somatic APs. In fact, as long as there is Ca$^{2+}$ influx through NMDA-type glutamate receptors or calcium channels, synaptic plasticity can occur even in the absence of somatic AP output [16], casting doubt on the usefulness of the STDP paradigm [17, 18].

The dendritic whole-cell recording technique, although successful, had several drawbacks. Like somatic recordings, the method was invasive with the potential of diluting the intracellular milieu and altering physiology. It required exceptional mechanical stability, limiting its use to the *in vitro* brain slice preparation. With great patience, initially only thicker dendrites could be studied while dendritic spines were inaccessible. Ultimately the bottleneck of this approach was the limited number of dendritic recording sites per cell, which stood in contrast with the distributed nature of electrical signaling within the dendritic tree. Another, less invasive, approach was needed.

In this work, several optical and technological advancements are described that complement and extend current electrophysiological techniques, with the ultimate aim of understanding how the brain functions. The approach taken here is on multiple levels. First, in terms of optical probes of neuronal function, chapter 2 details the development and application of membrane potential sensitive fluorescent dyes, which in combination with two-photon (2P) laser scanning microscopy (2PLSM), described
Figure 1.2: Active action potential backpropagation in the dendrites of rat layer 5 cortical pyramidal neurons (adapted from [9]). A brief 100 pA somatic current injection elicits an action potential, which was recorded both at the soma as well as at the dendrite, 443 µm from the soma. As the intracellular Na$^+$ channel blocker (10 mM) diffuses within the dendrite, after 100 s the amplitude of the dendritically recorded action potential diminishes, while the amplitude of the somatic action potential remains essentially unchanged.

below, have been successfully used to record back-propagating action potentials in dendrites. Second, in terms of optical instrumentation, chapter 3 describes the design, optimization and construction of optical components that are key to obtaining high-volume and high-quality images from neuronal tissue. This has been accomplished in a custom-built 2PLSM. Chapter 4 dispenses altogether with the need of using exogenous labels to visualize and manipulate the neuronal tissue by using another form of nonlinear optical imaging, third-harmonic generation. Chapter 5 addresses the problem of software flexibility and adaptation in an experimental setting by introducing the concept of hierarchical task control. The novel software architecture formed the basis of DAQLab, a custom written software to control a home-built 2PLSM. Finally, the intersection between the technical advancements shown here and neuroscience are demonstrated in a 2P glutamate uncaging experiment that reveals supralinear dendritic integration of synaptic activity.

Optical methods of measuring the membrane potential

As it became apparent that the passive cable model of dendrites was inadequate, progress was being made in developing Ca$^{2+}$ sensitive fluorescent probes. With the Ca$^{2+}$ fluorescent dye fluo-4 [19], a brighter and more photostable fluorinated counterpart of fluo-3 [20], Ca$^{2+}$ dynamics in dendrites and dendritic spines could be studied [21]. However, Ca$^{2+}$ imaging is only a poor proxy for the membrane potential as it relies on depolarization that leads to Ca$^{2+}$ influx from Ca$^{2+}$ ion-channels and NMDA-type glutamate receptors. This technique also did not allow the measurement of membrane potential hyperpolarization due to the inhibitory effect of interneurons [22, 23].

Early work on optical detection of electrical conduction through neuronal processes relied on intrinsic optical changes of invertebrate axons: axoplasm viscosity [24], UV light absorption [25], visible light absorption [26], fiber volume [27], visible light scattering [28, 29] and birefringence [29]. These initial experiments were soon followed by the application of UV excitable fluorescent dye 8-anilinonaphthalene-1-sulfonic
The majority of VSDs are constructed around a charge transfer chromophores where a $\pi$-electron donor and acceptor (commonly pyridinium) are linked through a conjugated polyene chain. Upon absorption of light and through the molecular Stark effect, generally the absorption and emission spectrum of the dye will shift in response to an external electric field:

$$h\Delta\nu = -q\vec{r} \cdot \vec{E} + \Delta\mu \cdot \vec{E} + \Delta\alpha |E|^2$$  \hspace{1cm} (1.1)$$

where $h$ is Planck’s constant, $\Delta\nu$ is the absorption or emission spectral shift induced by the electric field $\vec{E}$, $\Delta\mu$ and $\Delta\alpha$ are the change in dipole moment and polarizability upon excitation and $q$ is the amount of intramolecular charge transfer over the distance $\vec{r}$. However, because $\Delta\mu$ and $\Delta\alpha$ are negligible compared to the first term and because the orientation of VSDs within the membrane is parallel to the transmembrane electric field, the above equation simplifies to:

$$h\Delta\nu = -qrE = -qrV_m/d_m$$  \hspace{1cm} (1.2)$$

where $V_m$ is the membrane potential and $d_m$ is the membrane thickness. In this way, the spectral shift is linear with the change in membrane potential and if excitation is done at a wavelength $\lambda$ where $dA/d\lambda = \text{const.}$, then the emitted fluorescence intensity will be also a linear function of $V_m$. Though maximal fluorescence is obtained when exciting at the peak of the absorption spectrum, for a charge-shift based VSD it is also ineffective as $dA/d\lambda = 0$. Instead, to maximize the VSD response $\Delta A/A$, excitation must be done towards the red spectral edge [43], most effectively using laser sources to compensate for the low absorption. In the end, optically measured $V_m$ signal-to-noise ratio (SNR) depends on both the VSD response $\Delta A/A$ and fluorescence SNR, $\text{SNR}_{\text{fluo}}$:

$$\text{SNR}_{V_m} = \text{SNR}_{\text{fluo}} \cdot \Delta A/A$$  \hspace{1cm} (1.3)$$

Although the response $\Delta A/A$ can be increased asymptotically, to maintain the same $\text{SNR}_{\text{fluo}}$, the excitation power must be increased as well and eventually sample damage (thermal or phototoxicity) will set the upper limit on $\text{SNR}_{V_m}$. There are several ways to increase $\text{SNR}_{V_m}$ and reduce this problem. One approach is to spread the excitation over a larger portion of the sample as it is done in bulk loading the VSD and studying network level activity [49, 54, 61].

In chapter 2, however, the goal was to reduce phototoxicity by borrowing the same strategy of fluorination that was used to improve the photostability of Ca$^{2+}$ indicators while at the same time reaching a membrane potential sensitivity of $\approx 17%/100\text{mV}$ [76].

While the majority of VSDs have been designed to use the electrochromic effect, another notable development in the field have been two-component VSDs [72, 74]. In this approach, one side of the membrane is stained with the fluorescent neuronal tracer dye DiO while dipicrylamine (DPA), a Förster resonance energy transfer (FRET) molecule, is bath applied to the tissue. As the partitioning of DPA within the membrane is membrane potential dependent, DPA is able to modulate the fluorescence of DiO. Depending on the DPA concentration, at 2$\mu$M, a sensitivity of 26%/100 mV can be reached whereas at 5$\mu$M this can be as high as 56%/100 mV. The downside of using
a DPA concentration higher than 2 μM is that the added capacitance will distort the
AP waveform thus effectively limiting the sensitivity of this method to about 26%/100
mV. When compared to using electrochromic VSDs, an advantage of the DiO-DPA
FRET pair is that excitation can be done effectively at the peak of DiO absorption
so that a higher SNR can be achieved without thermal damage. On the other hand,
because it requires loading the tissue with DPA, this approach stands little chance of
being effectively applied in the intact in-vivo preparation.

A third class of recently developed VSDs is based on the principle of photo-electron
transfer across a molecular wire between an electron-rich quencher and a fluorescent
reporter [75]. At hyperpolarized membrane potentials, the transmembrane electric
field promotes electron transfer from the quencher to the excited-state fluorophore,
thereby quenching the fluorescence. At depolarized membrane potentials the electric
field hinders electron transfer, increasing the fluorescence. This process occurs on a ps–
ns time scale, which is practically instantaneous on a biological time scale. Importantly,
the change in fluorescence is linear within the range of neuronal membrane potential. In
this manner, a sensitivity of 20–27%/100 mV could be achieved. However, theoretical
considerations indicate that this type of VSD has the potential to greatly exceed the
sensitivity of electrochromic VSDs. In addition to this, because excitation can be
done efficiently at the absorption spectrum peak of the fluorescent reporter, a higher
SNR can be obtained. If fluorinated chromophores are used, phototoxicity could be
reduced as well.

Instead of applying fluorescent reporters exogenously, with the cloning of soluble
cytoplasmic green fluorescent protein (GFP) from the jellyfish Aequorea victoria [79]
and its fusion to the voltage-activated Shaker K+ channel, FlaSh, the first genetically-
encoded voltage indicator (GEVI) was created [80] (see review [81]). Its membrane
potential sensitivity of ≈5.1%/100 mV stems from the Shaker K+ channel gating
that couples to a GFP structural rearrangement, eventually a changing its fluores-
cence. Another early approach to GEVI design was in the form of VSFP1 construct,
which exploited the interaction between a FRET pair of fluorescent proteins to detect
voltage-dependent conformational changes in the Kv2.1 potassium channel, yielding
a sensitivity of 1.8%/100 mV [82]. Despite the early success in detecting a membrane
potential change, when expressed in mammalian cells, the first generation of GE-
VIs suffered from poor membrane targeting. This problem was solved by the second
generation of GEVIs, which were based on the simpler Ciona intestinalis voltage sensor-
containing phosphatase (Ci-VSP) [83]. This lead to the development of VSFP2.1 [84]
with a sensitivity of 6.5%/100 mV. Further optimization of the FRET pair-linking
amino-acid sequence [81] and FRET chromophores [85] (for other variants see also [86])
resulted in VSFP2.3 and VSFP2.4 with sensitivities of 9.5%/100 mV and 8.9%/100
mV respectively, somewhat faster bi-exponential kinetics compared to VSFP2.1, which
were used to measure membrane potential changes in the living mouse [87]. Subse-
quent work revealed that the faster ≈2 ms component was FRET independent and
was due to structural modulation of CFP alone [88], motivating the development of
VSFP3.1 where YFP component was left out. With one remaining fluorescent protein,
the next strategy employed was circularly permuting EGFP and mKate fluorescent
proteins [89], albeit the sensitivities were <1%/100 mV. In yet another approach
[90] the endogenous fluorescence of the microbial rhodopsin proton pump channel
Archaerhodopsin 3 (Arch) was found to be sensitive to the membrane potential with
a fast <0.5 ms linear response of 66%/100 mV. Upon illumination, the native Arch
proton pump also generates an outward photocurrent, which has been successfully used to silence neurons [91]. In a mutant Arch(D95N) variant, the photocurrent was eliminated while at the same time increasing the response to ≈100%/100 mV. Unfortunately this also slowed down its response time constant to 41 ms. While both Arch and Arch(D95N) have the highest sensitivity among GEVIs, their extremely low quantum yield of $9 \times 10^{-4}$ and $4 \times 10^{-4}$ respectively make them impractical. Still as Arch is one among hundreds of known microbial rhodopsins [92], it is likely that better candidates will be found.

Increasing the imaging depth

Equally important to the development of fluorescent reporters and with the ultimate goal of imaging deep within live tissue, optical instrumentation saw similar progress. In standard fluorescence microscopy, a bright mercury or xenon lamp source illuminates a portion of the sample through a filter cube containing a dichroic mirror as well as excitation and emission filters. For detection of fast membrane potential changes, early multi-site imaging experiments used photo-diode arrays to study AP propagation in snail ganglion neurons [36] and layer 5 neocortical pyramidal neurons [38]. Due to light scattering of brain tissue and degradation of image quality, wide-field fluorescence imaging is limited to sparsely labeled samples such as single neurons filled with VSDs. With the introduction of laser scanning confocal microscopy (for applications and historical perspective, see [93]), image resolution within scattering tissue could be retained by the use of a small pinhole conjugated to a raster scanned focal spot, which could reject out-of-focus scattered fluorescence. As imaging depth increases, so is the proportion of scattered vs. ballistic photons, and consequently the collection efficiency drastically drops, limiting the method to a depth of ≈<50 µm. Using this imaging method in conjunction with an earlier generation of VSDs (JPW-3028 i.e. di-1-ANEPEQ), the excitatory post-synaptic potential (EPSP) amplitude in dendritic spines of cortical neurons has been estimated to be between a few mV and up to ≈20 mV while back-propagating APs were found to fully invade spines without attenuation [71].

Whereas confocal laser scanning microscopy (LSM) obtains its sectioning capability by rejecting scattered fluorescence, two-photon laser scanning microscopy (2PLSM) [94, 95] obtains its sectioning power from the quadratic dependence of 2P excited fluorescence (Figure 1.3). Since the 2P absorption process already confines the excitation point spread function (PSF) to a transversal and longitudinal full width at half maximum (FWHM) of <0.5 µm and <1.5 µm respectively, there is no need to reject scattered fluorescence. Being a more efficient imaging method, 2PLSM imaging depths of >500 µm have been demonstrated in live brain tissue, with the achievable imaging depth $z_{\text{max}}$ [96] depending on the average power at the sample surface $\bar{P}$, scattering mean-free-path length $l_s$, pulse repetition frequency $f$, pulse width $\tau$:

$$z_{\text{max}} = l_s \ln \left[ \frac{\bar{P}}{\gamma} \sqrt{1/(f\tau)} \right]$$

and $\gamma = [\eta_2 \phi(z_{\text{max}})/\bar{P}(z_{\text{max}})]^{1/2}$, where $\eta_2$ is the fluorescence quantum efficiency under 2P excitation, $\phi(z_{\text{max}})$ is the collection efficiency and $\bar{P}(z_{\text{max}})$ the average excitation power at the focal plane.
A first approach to increasing the imaging depth is to increase the scattering mean-free-path length \( l_s \), which can be done by choosing an appropriate tissue preparation and by increasing the excitation wavelength. For \textit{in-vitro} brain slices from juvenile (postnatal day P14–18), young adult (P42–44) and mature (P90) rats, \( l_s \) at 800 nm has been measured to be 87±4, 68±3 and 47±5 \( \mu \text{m} \) respectively \cite{97}. In comparison, for the \textit{in-vivo} young adult preparation, \( l_s \) at 800 nm has been estimated to be \( \approx 200 \mu \text{m} \). Although there is a large difference in \( l_s \) between juvenile and mature \textit{in-vitro} brain slices, this may be due to the poor health of older slices and it remains unknown to what extent such differences can be seen in the healthy \textit{in-vivo} preparation of different ages. As for the wavelength dependence, \( l_s \) has been measured \textit{in-vitro} to increase 1.5 fold when using 900 nm instead of 750 nm. Using brain slices of young mice (P22–29) \cite{98} also found similar values for \( l_s \) at 800 nm and 950 nm of \( \approx 100 \mu \text{m} \) and 160 \( \mu \text{m} \) respectively, while increasing the wavelength from 750 nm to 900 nm, increased \( l_s \) approx. two-fold.

If instead either the repetition rate \( f \) or pulse duration \( \tau \) are changed, the maximum imaging depth would change by:

\[
\Delta z_{\text{max}} = l_s \ln \left( \frac{f_0 \tau_0}{f \tau} \right)
\]  

(1.5)

Assuming the pulse duration is unchanged, a reduction in repetition rate from the commonly used Ti:Sapphire lasers of 80 MHz to 200 KHz as shown by \cite{96}, can increase the imaging depth by 3\( l_s \), and with an estimated \( l_s \) of 250–300 \( \mu \text{m} \) at 1100 nm, the imaging depth can be further extended by 750–900 \( \mu \text{m} \) (in addition to \( z_{\text{max}} \approx 500 \mu \text{m} \)). Using this approach in combination with the genetically encoded calcium sensor GCaMP3 \cite{99} demonstrated functional imaging from the soma of cortical layer 5 pyramidal neurons. Despite the increased depth, the KHz repetition rate will severely limit the number of pixels in the image and ultimately the usable scan field size.

Given the widespread use of resonant galvanometer laser-scanning 2P microscopy for \textit{in-vivo} imaging, what would be optimal values for \( f \) and \( \tau \) and how much would the imaging depth limit be increased? With an 8 KHz resonant scanner, a frame rate of 31 Hz can be obtained using bi-directional scanning of an image with 512 lines. For a field of view of \( \approx 500 \times 500 \mu \text{m} \) and 512×512 pixels this typically offers sufficient resolution and scan speed to motion correct and segment neuronal soma \textit{in-vivo} and sample GCaMP6f \cite{100} fluorescence to estimate population firing rates. Assuming one laser pulse per pixel and 80% resonant scanner duty cycle (due to turn-around), \( \approx 512 \times 640 \) pulses are required per frame. Thus to obtain an image of 512×512 pixels at 30 Hz the optimal repetition rate is \( \approx 10 \) MHz. Considering pulse duration, the lower practical limit of 30–50 fs is set by the spectral overlap with the fluorophore and with the ability to compensate for pulse stretching through the optical components. For several years, Ti:Sapphire ultrashort pulsed lasers have been operating typically at 80 MHz and 80 fs. With development of new lasers \cite{101, 102}, if the repetition rate is reduced to 10 MHz and pulse duration to 50 fs, according to \textbf{Equation 1.5}, this will afford an increase of 1.3\( l_s \) or with \( l_s \) between 250–300 \( \mu \text{m} \) at 1100 nm, an increase in imaging depth of 319–382 \( \mu \text{m} \).

Certainly as \textbf{Equation 1.4} shows, the imaging depth can be further increased by increasing the average power \( \bar{P} \) at the sample surface, however, in practice thermal damage limits this value to \(< 170 \) mW \cite{103, 104}. Yet another approach short of increasing the 2P cross-section (which seems to have tapered to a few hundreds of
GM for best fluorescent probes) is to improve the fluorescence collection efficiency $\phi$ by using low-magnification high numerical aperture (NA) microscope objectives [97] in conjunction with efficient collection optics between the objective and detectors [105]. At the same time, high-NA, low-magnification objectives such as the Olympus 20× 1.0 NA or the Nikon 16× 0.8 NA can be used to sample the activity across a larger neuronal population, which may belong to separate cortical areas [106]. Though scanning with such objectives is appealing, to fully make use of their capability, the design of intermediate scan optics such as the scan and tube lenses over a wide wavelength range and large scan angles is not trivial.

In chapter 3 this issue is addressed through detailed optical modeling of the intermediary scan path and performance is verified over 750–1050 nm in a custom built 2PLSM.

As seen in practice and detailed by [107], another important factor in achieving deep tissue imaging is to reduce out-of-focus fluorescence by sparse sample labeling applied preferentially at the target imaging plane. Using three-photon excitation at 1700 nm with a custom built laser source, [108] showed that out-of-focus fluorescence can be dramatically reduced and demonstrated that imaging of red fluorescent protein expressing cells in the intact mouse brain is feasible at depths of up to 1.4 mm. This is especially interesting when considering newly developed red-shifted Ca$^{2+}$ genetically encoded fluorescent reporters such as jRGECO1a [109].

Other nonlinear optical processes

Besides multiphoton excitation (MPE), which is a non-parametric optical process by which the interacting light field changes the quantum state of the material (transition between ground and excited states), parametric processes such as second- or third-harmonic generation (SHG, THG) leave the quantum state of the material unchanged and can be similarly used in LSM. The appeal of using SHG and THG over MPE as basis for LSM is that addition of exogenous fluorescent labels is not required — provided that the material meets some conditions [40, 110]. For SHG, molecules must have a hyperpolarizability and a net orientation within the focal volume, in which case, the forward emitted signal frequency will be double the incoming excitation frequency. Such an arrangement can be obtained by staining a biological membrane with a dye (which need not be fluorescent) [40] or use naturally occurring molecules such as collagen [111]. For neurons stained intracellularly with VSDs, the molecular orientation requirement of SHG was exploited to isolate and calibrate the membrane potential sensitive signal from the otherwise intracellular background seen with 2P imaging [53, 55, 112, 113]. On other hand, to obtain THG, the material must have a sufficiently high third-order susceptibility $\chi^{(3)}$ and the excitation within the focal volume must satisfy phase matching conditions.

In chapter 4, lipid containing brain tissue with a high $\chi^{(3)}$ is shown to be a suitable material for THG and partial phase matching is achieved when the laser beam is scanned over small inhomogeneities. As a result, cell bodies are seen as shadows over a brighter signal-generating extracellular matrix, making this method suitable for optical biopsy without the need of exogenous labels.
Laser-scanning microscopy and general purpose data acquisition software

Software is an indispensable component in any modern experimental setting, and laser-scanning microscopy is a good example where various hardware components must function as a whole harmoniously. In a rush to get experiments going, it is often convenient to use commercially available software, but inevitably when the curious experimenter designs a new experiment, features are missing. Lack of source code access and little commercial incentive to implement features that are not justified by a sufficient market size can quickly put a stop to new ideas. Alternatively, one can use open source software where, at least in principle, there is a chance to make required modification, either within the research group, or in a collaboration. In neuroscience there are many great examples of open-source software, in particular, in laser-scanning microscopy several projects using different programming languages and tools are noteworthy: Helioscan (Labview) [114], ScanImage (Matlab) [115], ACQ4 (Python) [116], MPScope (Object Pascal) [117, 118].

As experimental design often changes, so must software, and considerable effort is needed for its adaptation. Key to modern software design is the concept of object orientation, where for example similar hardware from different manufacturers is exposed to the rest of the software through a common interface. This allows for ease of deployment in other laboratory settings where other hardware may be present. At the same time, inheriting most features from a parent class makes it easy for a child class to extend functionality.

As previous work showed, it is well possible to obtain a functional laser-scanning microscopy software based solely on a monolithic design [114, 115, 117, 118], where peripheral objects such as a data acquisition card or an XY-stage are coupled to a central laser scanning engine object. This hardly satisfies experimental variability. One source of variability is the need to redirect and distribute data flow between participating software components, for example when a certain waveform must be directed towards an analog output and displayed at the same time. This can be solved by providing some degree of flexibility at the point of loading a configuration file [116], but ideally, as it has been done here, this should be done through a graphical user interface (GUI). Often times data acquisition relies on hardware exchanging precise trigger signals, which establish a master-slave relationship between the participating devices. Similarly, these dependencies should be adjustable within the GUI. And finally, another source of variability, which has not been recognized previously, comes from the structure of the multidimensional dataset to be acquired. This can be thought of as a series of nested parameters that take the shape of a tree, with data, often in the shape of waveforms or images, being the leaves of this tree. Accessing a piece of data is easy, it can be done within a single execution thread and requires iteration over a set of indices. Data acquisition, on the other hand, is complicated by the fact that some processes at different nesting levels must run in parallel or use multiple threads. The problem to be solved is to map a desired data structure, which is easily understood by the experimenter, to the coordination required between various software and hardware components.

To this end, chapter 5, and the final chapter of this work introduces the concept of Hierarchical Task Control that extends software flexibility beyond what is afforded by an object-oriented approach alone. The novel framework was tested in a custom open-source software, DAQLab, totalling 61,200 lines of code and 72,218 comment
words that was used to steer a custom built 2PLSM and electrophysiology equipment. Beyond being a general purpose data acquisition software, DAQLab made possible cutting-edge experimentation to study supralinear dendritic integration by the method of 2P glutamate uncaging at dendritic spines [119].

Overview

As the pioneering work of not just great physiologists, but methods developers like Cajal, Sakmann and Neher shows, fertile ground for discovery is created with the availability of new tools. In a modest way, this thesis had the same motivation. In chapter 2, a new class of more responsive and photostable voltage sensitive dyes has been developed. Their improved optical properties afforded the measurement of back-propagating action potentials in the dendrites of neurons using 2PLSM. Chapter 3 improved upon the basic design of a 2PLSM by developing computer-optimized lenses and a high-quality laser-scanning engine. Chapter 4 takes nonlinear laser-scanning imaging one step further and demonstrates structural imaging without the need of exogenous labels. Finally, chapter 5 introduces a new software architecture and software, DAQLab, that achieves sufficient flexibility to perform cutting-edge imaging and physiological experiments as demonstrated by studying supralinear dendritic integration.
Figure 1.3: Principle of operation of a nonlinear laser scanning microscope. 

**a)** Schematic diagram. A pair of galvanometric mirrors deflects an incoming ultrashort pulsed laser beam while the combination of scan and tube lenses project the image of the deflected beam on the objective pupil, which will focus the beam at different location within the sample. 

**b)** Jablonski diagram comparing two-photon absorption (2PA), second-harmonic generation (SHG) and third-harmonic generation (THG). Note than whereas 2PA requires transition between electronic states with the possibility of fluorescence emission having a certain lifetime, SHG and THG are essentially instantaneous processes that do not require transition to the excited state. 

**c)** Comparison between one- and two-photon excitation of a cuvette filled with a fluorescein solution. The quadratic dependence of fluorescence on excitation intensity leads to confinement of excitation, making possible efficient detection of fluorescence in scattering tissue with non-descanned large area detectors (adapted from [95], photo credits: Steve Ruzin and Holly Aaron, UC Berkeley).
References

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