Chapter 6

Discussion

The goal of this work was the development of technical means to probe the structure and function of the nervous system, focusing on methods to study dendritic and synaptic physiology.

Membrane potential imaging

Chapter 2 presented a new class of voltage sensitive dyes (VSDs) that demonstrate a fluorescence response of $\Delta F/F$ of 17%/100 mV and improved photostability thanks to chromophore fluorination. This allowed for the measurement of back-propagating action potentials in dendritic spines (in the following simply referred to as spines) using two-photon (2P) laser scanning microscopy. Though somatically measured excitatory post-synaptic potentials (EPSPs) are fairly small <1 mV, it has been a matter of debate how large EPSPs are in the spine head itself. At the same time, spines come in a variety of shapes and sizes, ranging from thin filopodia-like to short and stubby structures [1]. To model accurately electrical signaling within the spine head, it is important to determine the spine neck resistance accurately. Recently, using one of the fluorinated VSDs described in this work, di-2-AN(F)EPPTEA, in combination with 2P glutamate uncaging, [2] estimated that spine head EPSP in basal dendrites of layer 5 cortical pyramidal neurons to be in the range 6.5–30.8 mV, with an average of 13.0 mV. As for the spine neck resistance, the same work found this to be 179±25 M$\Omega$ (mean±SE) ranging from as low as 23 M$\Omega$ and up to 420 M$\Omega$, in agreement with fluorescence recovery after photobleaching (FRAP) experiments conducted within the same study. Earlier work [3] using an older generation of electrochromic VSDs, di-1-ANEPEQ (referred to as JPW-3028 in the study), found comparable values for spine head EPSP, ranging from a few mV and up to ≈20 mV, while placing an upper limit of ≈500 M$\Omega$ for spine neck resistance. The reason for not having a narrower estimate was a rather low signal-to-noise ratio of the measurement, as well as, evoking the EPSP by electrical stimulation, which inevitably activated several spines and biased results toward a higher spine neck resistance. However, using the same dye but improved optical recording conditions and single spine glutamate uncaging, in a following study [4] found the spine neck resistance in basal dendrites of layer 5 cortical pyramidal neurons to be 27±6 M$\Omega$, while the input impedance of the parent dendrite (at 70 Hz) was measured to be 275±27 M$\Omega$. Though there is a several fold difference in
spine neck resistance between the studies of Acker et. al. and Popovic et. al., both studies support the conclusion that, from an electrical standpoint, synapses on basal dendrites of layer 5 cortical pyramidal neurons are effectively coupled to the parent dendrite. Further studies will be needed to establish if this conclusion also holds for spines located on other parts of the dendritic tree.

The VSDs studied here are based on the electrochromic effect, where a change in the membrane potential results in a shift of the emission and absorption spectra. To this end, to detect optimally such a change, excitation must be done at the red edge of their absorption spectrum, which necessitates a significant amount of laser power. Typically for in-vitro experiments in the brain slice preparation this has not been a major obstacle and optical recordings from dendrites and dendritic spines can be done routinely. However, one potential concern may be the upper limit of safe average excitation power at the brain surface <170 mW [5, 6] before thermal damage occurs.

As mentioned in chapter 1, while keeping the average excitation power at the brain surface constant, functional imaging using 2P excitation in brain tissue can be made more efficient in several ways: a) shorter laser pulses, b) larger 2P cross section, c) longer excitation wavelength, d) lower pulse repetition rate and consequently higher pulse peak energy, e) increasing reporter fluorescence change, f) lowering phototoxicity and g) increasing fluorescence collection.

In terms of lasers, the Ti:Sapphire and fiber-based sources have found widespread use: 1) Coherent Chameleon Ultra II with 140 fs at 800 nm, 80 MHz pulse rate, 3.3 W average power, 2) Newport Spectra Physics Mai Tai Deep See eHP with <70 fs over 690–1040 nm, 80 MHz pulse rate, 2.4 W average power at 800 nm and 3) Coherent Fidelity-2 with <55 fs at 1070 nm, 70 MHz pulse rate and >2 W average power. As it can be seen, these sources all have more than enough average power that exceeds the safe thermal limit, even if losses of coupling the laser beam to the microscope setup are considered. As for pulse duration, only lasers 2 and 3 fall in the ideal 50–70 fs range. However, all lasers have ≈8-fold higher repetition rates compared to what can be ideally used in a resonant laser scanning setup (see chapter 1). Thus, addressing points a), c) and d), development of new sources is greatly needed and significant progress is to be expected in the following years [7, 8].

While it is always desirable to have larger 2P cross sections, and peak 2P molecular cross-sections hardly exceed a few hundreds of GM, the only possibility to satisfy point b) is to excite the chromophore at the peak of its absorption rather than at the red spectral edge. To this end, and compared to electrochromic dyes, there are promising developments using molecular wire-based sensors, which can be optimally excited at the peak of their absorption. In a recent study, using 2P excitation, a rhodol-based molecular-wire sensor achieved a membrane potential sensitivity of 28% ΔF/F/100 mV [9]. Addressing points c) and f), further improvements in photostability may be possible if fluorinated chromophores would be used, as well as higher sensitivities can be expected based on previous thermodynamic considerations [10].

Voltage-sensitive fluorescent proteins (VSFPs) have also seen considerable development. While initially their sensitivity was a few percent for a 100 mV change in the membrane potential, and their temporal response was on the order of several tens of ms [11], in one of the latest developments, using 2P microscopy, a sensitivity of 17.5%/100 mV and an on/off kinetics of ≈2 ms has been achieved [12].

Compared to VSDs, VSFPs have the convenient advantage that they may be delivered using adeno-associated viral vectors. Alternatively, they may be altogether
expressed using genetically engineered mice. In all single cell imaging studies so far, VSDs have been delivered using whole-cell patch clamping. This approach is certainly feasible for the in-vitro brain slice preparation, however, due to motion artifacts, this is more challenging under in-vivo conditions. Another method of VSD delivery with higher success rate, which I have tested together with my colleague Rajeevan Narayanan from the research group of Christiaan de Kock, uses the same approach of single-cell juxtasomal labeling with the morphological tracer biocytin [13]. After achieving a loose seal, with a seal resistance of <50 MΩ, a series of positive current pulses of several nA are applied to the membrane to create nanoscopic pores and thereby allowing the VSD to diffuse across the membrane. In a similar manner VSFP plasmids can be electroporated in a few cells and expression of VSFPs occurs typically within 24–48 h.

Ideally, a membrane potential-sensitive optical sensor should measure the absolute value of the membrane potential. Unfortunately, VSDs stain intracellular and extracellular membranes indiscriminately which preclude their calibration. For proximal dendrites and spines, the optical signal may be calibrated by voltage clamping the soma to a hyperpolarized membrane potential, however calibration of distal dendrites and spines is less reliable due to voltage attenuation. To remedy this problem, instead of using 2P excitation, it has been shown that second-harmonic generation (SHG) is able to suppress the signal originating from randomly oriented chromophores staining the intracellular milieu, and favor the signal originating from the highly ordered chromophores staining the extracellular membrane [14]. Compared to 2P excitation, SHG imaging of membrane potential is even more challenging due to low SHG efficiency, which prevented its widespread adoption. To some extent VSFPs suffer from the same problem, however, targeting expression to various cellular compartments is better understood. In fact in [12], the fluorescent reporter is efficiently targeted to the extracellular side of the membrane, which could in principle be used to measure the absolute membrane potential, assuming the resting membrane potential is the same throughout the cell.

**Optical design and instrumentation**

In addressing point g), to improve functional imaging, previous work [15] showed that fluorescence collection using a 20× objective compared to 63× at an imaging depth of 500 μm is ≈ 10× greater, provided that the larger acceptance angle of the objective is matched by the relay optics directing the light towards the photomultipliers [16]. On the other hand, at shallower imaging depths, fluorescence collection is dominated by ballistic photons, in which case a high numerical aperture (NA) is desirable. Therefore, to obtain flexible imaging, both at shallow and great depths, use of high-NA, low-magnification objectives is desirable, such as the Nikon 16× 0.8 NA or the Olympus 25× 1.05 NA, which also comes with a spherical aberration correction ring that can be used to correct for depth-dependent refractive index mismatch between water and tissue. At the same time, high-NA, low-magnification objectives are ideally suited for recording neuronal activity from a large population of neurons, provided that the intermediary scan optics is designed appropriately.

In chapter 3, a thorough analysis of optical aberrations was presented in the context of designing the intermediary scan optics of a 2P laser scanning microscope using high-NA, low-magnification objectives. For their scan lens, a great majority of home-built
laser-scanning microscopes use a simple off-the-shelf achromatic doublet (achromat). While this may be acceptable for small scan angles, high-NA, low-magnification objectives require a 4–5× expansion between the scan and tube lenses, which results in large >10° scan angles that introduce various aberrations. As shown, at large scan angles, one severe limitation of using off-the-shelf achromats is the curvature of their scan field which results in loss of telecentricity and beam wander in the objective pupil. In turn this leads to loss of power and worsening of the point-spread function, which can be seen as a dimming of the image with increasing distance from the field center. The problem is that off-the-shelf achromats are optimized for on axis performance. To remedy this, chapter 3 offers a two-pronged approach. First, it shows that the convenience of using off-the-shelf achromats can be retained if two-such lenses are combined in a Plössl type eyepiece configuration. This solves the problem of telecentricity and the laser beam remains centered on the objective pupil during scanning. It also solves the problem of f-theta calibration, where the position of the laser beam within the sample is proportional to the scan angle. Second, for more demanding optical performance, a scan lens that operates over 420–1600 nm was obtained using a computer-optimized design process. With such a wide wavelength range, it should be well possible to design a laser scanning microscope that can be used for both confocal single-photon, as well as two- or even three-photon [17] imaging.

Label free imaging

Chapter 4 demonstrated label free structural imaging and manipulation of brain tissue using third-harmonic generation (THG). With this method it was possible to visualize cell bodies, which appeared as dark regions in an otherwise bright background generated by the extracellular matrix, as well as axonal fiber bundles, blood vessels and red blood cells. This contrast method allowed for guidance of a transparent glass microelectrode to perform in-vitro patch-clamping similar to previously described 2P shadow-patching method [18], however, without the need of expelling a morphological dye from the pipette, which limits tissue contrast after internalization. A useful observation that can be made here is that if functional imaging is not desired, the 2P shadow-patching approach can be improved considerably if instead of filling the pipette with a morphological dye, a low intracellular baseline fluorescence Ca²⁺ indicator is used such as the fluo-4 pentapotassium salt. In a related approach [19], patch pipettes coated with quantum dots could be visualized deeper within the tissue under 2P illumination and patch-clamping could be performed. However, targeted cells need to express a fluorescent protein and the quantum dot coating procedure is rather time consuming. Here instead, THG allows for both the pipette and the cell body to be visualized without additional labels. It is also encouraging that THG imaging was performed using 200 fs pulses at 80 MHz from an optical parametric oscillator and with new laser sources [7, 8] there is still room for improving this imaging modality.

THG, in combination with SHG, has been used in a wide variety of biological preparations [20] with applications ranging from the study of zebrafish embryo development [21] to detection of brain tumors [22]. For example, during mitosis, THG provides information on the cell’s morphology while SHG aids in visualizing chromatin dynamics. THG and SHG proved to be as effective as established histopathological methods, allowing tumor progression to be studied from the initial diffuse astrocytoma to the final glioblastoma stage.
Rethinking software design

Finally, chapter 5 presented a new software framework, termed hierarchical task control (HTC), that formed the basis for DAQLab, a custom written software in the C programming language built on the National Instruments LabWindows/CVI platform. Compared to a purely object oriented design, the HTC framework adds multiple levels of coordination between various software and hardware components that facilitate rapid prototyping of experiments. A first level of coordination was achieved by allowing the execution of each component to be controlled by a generic task controller (TC) which was modeled as an extended state machine with a clear set of states and events. Multiple TCs could be assembled in a hierarchy which reflected the multidimensional and hierarchical structure of the experimental design. Second, the HTC framework allows for a flexible exchange and distribution of data between various components, as well as, specifying hardware master-slave trigger relationships to ensure correct starting order between parallel processes. The ability of the HTC framework and its implementation in DAQLab was demonstrated by performing a complex two-photon laser-scanning and glutamate uncaging experiment to elicit dendritic spikes that are a hallmark of supralinear dendritic integration of synaptic activity [23]. However, due to its flexible design, DAQLab goes beyond being just a laser-scanning microscopy software [24–28] as its data acquisition and instrument coordination capabilities can be incorporated in other experimental designs as well. At the same time, the design principles presented here can be implemented in other preferred programming languages and environments such as C++ and the quickly growing QT-framework.

Future directions

Complex systems such as the nervous system have proven difficult to study using classical reductionist methods that have been so far at the heart of scientific progress. Therefore, in any attempt to study the nervous system, such as when preparing in-vitro brain slices or cell cultures, it is essential that the phenomenon under study is not perturbed. After more than 60 years from Cajal’s first description of dendrites, dendritic physiology is moving increasingly in the direction of using intact brains in awake animals performing a behaviorally meaningful task [29]. Under these conditions, it is essentially inconceivable how the standard dendritic patch-clamping method used successfully in brain slices could withstand extensive mechanical vibrations. Therefore, the progress of this field depends largely on improving optical methodology to probe neuronal activity in such small structures.

First, laser instrumentation still has ample room for improvement and efforts should be concentrated on developing sources with a repetition rate of about 10 MHz, pulse duration of 40–50 fs and wavelengths >980 nm with an average output power of at least 1–2W. Second, although the development of GCaMP6f [30] proved to be very successful in large scale network imaging, deep tissue imaging would benefit from red-shifted probes excitable at wavelength 1040 nm and beyond. Regarding this, the development of jRGECO1a and Cal590 [5, 31] is a significant step in this direction.

In terms of membrane potential imaging, both synthetic, as well as, genetically encoded sensors will be used increasingly. Out of all synthetic dyes, molecular-wire based probes [9] have the greatest potential for improved sensitivity and photostability
if fluorination is applied to the chromophore. Though initially genetically encoded probes were slower and less sensitive compared to synthetic dyes, their performance now is comparable [12]. On the long run, and with large scale genetic engineering and screening, even higher sensitivities can be expected. Similar to Ca$^{2+}$ sensitive probes, effort should be made to develop red-shifted membrane potential sensitive probes.

Where application of a fluorescent label is not possible, second- and third-harmonic nonlinear imaging provide a viable alternative. These imaging modalities are expected to accelerate the field of developmental biology [21] and non-invasive diagnostics based on tissue morphology [22].

With increasingly complex experiments, robust coordination between multiple devices and software is indispensable. The hierarchical task control framework presented here demonstrated complex coordination between various modules within the DAQLab application. Although it is conceptually attractive to have a single do-all software, in practice, this is rarely the case. Therefore, better coordination between disparate software should be sought that goes beyond merely exchanging a ‘start’ trigger. Fortunately, the hierarchical task control framework can be adapted and task controllers can be in charge of an entire software instance and exchange of events and data can be done through standard network protocols.

Neuroscience is one of the fastest growing and most diverse disciplines as it draws on knowledge and techniques from across the scientific spectrum. As it has been shown here, technical innovation using chemistry, physics and computer science gives us better means to study the nervous system, which still stands as one of the great mysteries of our time.

References


REFERENCES
