Published as: *Neuron*. 2011 January 13; 69(1): 9–21.

Imaging voltage in neurons

Darcy S. Peterka[*], **Hiroto Takahashi**, and **Rafael Yuste**
HHMI, Dept. Biological Sciences, Columbia University, New York, New York 10027

Abstract

In the last decades, imaging membrane potential has become a fruitful approach to study neural circuits, especially in invertebrate preparations with large, resilient neurons. At the same time, particularly in mammalian preparations, voltage imaging methods suffer from poor signal to noise and secondary side effects, and they fall short of providing single-cell resolution when imaging of the activity of neuronal populations. As an introduction to these techniques, we briefly review different voltage imaging methods (including organic fluorophores, SHG chromophores, genetic indicators, hybrid, nanoparticles and intrinsic approaches), and illustrate some of their applications to neuronal biophysics and mammalian circuit analysis. We discuss their mechanisms of voltage sensitivity, from reorientation, electrochromic or electro-optical phenomena, to interaction among chromophores or membrane scattering, and highlight their advantages and shortcomings, commenting on the outlook for development of novel voltage imaging methods.

Keywords

Voltage imaging; membrane; neuron; Two-photon; SHG; nanoparticles; Qdots

Introduction

Because neurons communicate electrically, neuroscience has traditionally relied on measurements of the membrane potential using electrodes. But because electrodes are mechanically invasive there is scant data on how different parts of a neuron interact, or how assemblies of neurons communicate. As an example of this limitation, it is not possible to measure the electrical properties of dendritic spines, the primary sites for excitatory input in the brain, with electrodes because spines are simply too small for current electrodes. Electrical recordings also have significant limitations in studies of the thousands of cells that form neuronal microcircuits, where only highly invasive electrode arrays can be used to record the ensemble's electrical activity. Optical techniques, however, seem to be an ideal solution for measuring membrane potentials, for both spines and circuits, since they are relatively non-invasive and could work both at low and high magnification.

While voltage imaging in neuroscience has a long history and has provided many significant advances in neuronal biophysics and circuit function (reviewed in (Cohen, 1989; Cohen and Lesher, 1986)), our belief is that it is not yet achieved its full potential, particularly for the study of mammalian preparations. As Sherlock Holmes argued, to understand a situation one needs to evaluate not only was has happened but also what has *not* happened. In this case,

[*]To whom correspondence should be addressed at: dp2403@columbia.edu.

we find it useful to compare current voltage imaging methods with calcium imaging to understand what could be missing. For example, calcium indicators are very sensitive (Tsien, 1980) and have custom-tailored spectroscopic properties (Grynkiewicz et al., 1985). They can be non-invasively loaded into neurons (Tsien, 1981) and can be genetically encoded (Miyawaki et al., 1997). Calcium indicators are sufficiently bright and sensitive that they can be used in combination with two-photon microscopy, which enables measurements in highly scattering media, such as intact mammalian tissue, while maintaining good signal to noise without averaging (Yuste and Denk, 1995). Indeed, with calcium imaging it is possible to perform measurements of the spiking activity from hundreds to several thousand neurons in mammalian circuits, while still keeping track of the activity of each neuron, individually (Cossart et al., 2003; Yuste and Katz, 1991). In fact, individual action potentials can be measured optically, without averaging and with excellent signal to noise (Smetters et al., 1999). At the dendritic level, one can measure the calcium influx associated with quantal synaptic events at individual dendritic spines (Yuste and Denk, 1995).

However, calcium imaging is not without its shortcomings and cannot substitute for voltage imaging. First, the time scales related to membrane voltage changes can be significant faster than those captured by the calcium dynamics. Another major impediment is that calcium imaging is biased to supra-threshold signals. Small sub-threshold events are practically invisible in the cell body, making it very difficult to monitor the myriad of activity that actually drive the cell to threshold. In addition, when imaging action potentials with calcium indicators, it can be difficult to quantitatively assess the number of spikes and spike timing if there are high spike rates since sensitive, high affinity calcium indicators suffer from saturation effects. Finally, calcium dynamics are confounded by the biophysical constraints associated with the diffusion of calcium from its source (membrane entry points, normally), through the cytoplasmic shells, until it binds the free calcium indicator. Even worse, calcium dynamics are also shaped by the complicated interaction between different intrinsic or extrinsic calcium buffers, and the fact that the high-affinity indicators, normally used to report action-potential induced changes in calcium concentrations, also significantly buffer and alter those same calcium dynamics. These problems indicate that calcium imaging, while very useful, fails to faithfully measure changes in membrane voltage, and hence it cannot serve to report a complete description of the activity of neurons, or of their subcellular compartments.

Voltage imaging, on the other hand, could in principle capture the entire picture: reading out the electrical activity of each neuron in the circuit, including sub-threshold excitatory and inhibitory events, for all different cell types. Or mapping, with sub-millisecond precision and micron resolution, the electrical structure and dynamics of dendritic trees as they receive synaptic inputs and integrate their responses. High-resolution voltage imaging could be crucial to understand open questions such as the nature of dendritic integration, the electrical function of dendritic spines, how different forms of activity propagate through neural circuits, and whether or not there are functional modules in neural circuit. Given the structural complexities of mammalian dendrites and circuits, the spatial aspects associated with their function are likely going to be fundamental in understanding them. By directly documenting these spatial differences, voltage imaging could help answer these and other fundamental questions, and likely lead to novel insights in neuroscience.

Technical challenges for voltage imaging

Probably the reason that voltage imaging has lagged behind calcium imaging is the significant challenges associated with the biophysical constraints of the measurements themselves. The phenomenon to be measured is a change in the membrane potential of the neuron, caused by the rapid (sub-millisecond) redistribution of ionic charges across the plasma membrane along with the opening or closing of membrane ionic conductances. The

actual number of ions that enter or exit the membrane is small (less than 10^{-5} of the total ions in the cell) but these ions have a large effect on the electric field of the membrane, even briefly reversing its polarity. In fact, the membrane potential changes are sizable (100 mV), and given that they occur across a very narrow section of dielectric material, the plasma membrane (only a few nanometers wide), these changes are associated with an enormous electric field ($10^7 – 10^8$V/m!), which can be modulated at KHz frequencies by neurons.

While these electric fields are huge, and *prima facie*, an engineer may consider measuring these types of signals a technically easy problem, there are many difficulties that have to be addressed for successful voltage imaging in biological samples, making effective voltage imaging quite a formidable challenge. The first fundamental constraint arises from the fact that the plasma membrane is very thin, only a few nanometers, and is surrounded by charged and polarizable chemical species providing dielectric screening, so the electric field rapidly dissipates as one moves away from the membrane (Figure 1; (Offner, 1970)). The effective range over which the electric field is still significant (the Debye length) decreases exponentially with distance from the membrane, and is only on the order of ten angstroms from the surface of the membrane. This means that the sensor, for example, a voltage-sensitive chromophore, needs to be physically inside the membrane or directly contacting for it to actually “see” the field. Thus, whereas for calcium imaging or other cytoplasmic measurements the localization of the chromophore is not crucial because diffusion redistributes the chemical species to be measured, for voltage imaging, a displacement of the chromophore by a single nanometer could easily destroy the sensitivity of the measurement. This makes the delivery, targeting, and localization of voltage probes a fundamental issue, one with little room for error.

A second related biophysical constraint is that the plasma membrane is a thin, essentially two-dimensional surface. Thus, compared to the bulk cytosol, the maximum number of chromophores in a volume that can be used to report these electric field changes is physically limited. Even in ideal situations, the optical detection of the membrane potential can only be carried out with relatively few emitted photons. Because of this, for the signal to be distinct from the photon shot noise, one typically needs to use very efficient chromophores, very strong light sources, or extensive temporal or spatial averaging. Unfortunately, despite its great strength as an insulating layer and in maintaining cellular integrity, the plasma membrane is also a very delicate part of the cell, and does not tolerate intense illumination. The photodamage associated with excited state reactions, such as the generation of disruptive oxygen free radicals and other triplet state reactions, or simply by local heating, following photoabsorption by the chromophores used to measure the voltage signals, can easily compromise the integrity of the membrane and kill the cell. Indeed, some sort of photodamage is present in essentially all voltage imaging measurements and is normally the reason voltage imaging experiments are terminated. To make this situation worse, neurons, like most mammalian cells, have a significant complement of endogenous chromophores, such as flavins, cryptochromes, and phorphyrins, that absorb visible light and, in some cases, are even located near the membrane. So even illuminating *unstained* neurons can lead to the generation of oxygen free radicals, damaging the membrane and altering membrane conductances, and may even result in membrane perforations (Hirase et al., 2002). This endogenous photodamage is so prevalent that one sometimes wonders if neurons have light-sensing machinery, as unicellular organisms do, to monitor circadian light changes.

A third constraint arises from the fact that most of the membranes in cells are actually *internal* membranes. The plasma membrane, the only one across which the neuronal membrane potential exists, is only a small proportion of the total membrane surface in the neuron. Thus, any chromophore that binds indiscriminately to membranes will mostly bind

to internal membranes, which have no direct sensitivity to the plasma membrane voltage, and merely contribute background noise to the measurement. This is quite a significant problem, one that again does not exist for calcium imaging where the intracellular calcium eventually equilibrates by diffusion in the cytoplasm, in principle making every molecule of chromophore in the cytosol a possible contributor to measuring the signal. For voltage imaging, the desire to target only the plasma membrane and yet avoid internal membranes compounds the already strong localization requirements.

A fourth constraint is one that it normally not appreciated; the plasma membrane is an active component of the cell, so one cannot place arbitrary numbers of chromophores in the membrane without disrupting its properties. First, the electrical properties of the membrane can be altered by the physical addition of exogenous chromophores. In fact, most voltage dyes are either charged particles, or have significant dipoles, in order to be sensitive to changes in the electric field. But because they need to insert themselves in the plasma membrane for effective voltage measurements, they can significantly alter the electrical charge of the membrane and distort its normal behavior. In particular, the addition of fixed charges increases the membrane capacitance, to the point that staining with a voltage-sensitive dye can lead to major reductions in the action potential conduction velocity (Blunck et al., 2005).

The unwanted electrical effects of the voltage dyes in membranes are not their only side effect. In fact, many voltage indicators have substantial toxicity and a variety of pharmacological effects, probably related to their localization in a key cellular component such as the plasma membrane. Moreover, these effects are not easy to generalize and depend on the specific dye and the specific preparation used (A. Grinvald, pers. comm.). For example, a few voltage sensitive dyes have been shown to modulate the ionotropic GABA-A receptor with an effectiveness similar to that of drugs designed specifically for that purpose (Mennerick et al., 2010). Therefore, for each novel voltage chromophore a substantial amount of “homework” is required for each preparation.

Assuming that all the previously mentioned challenges have been met, there remains another substantial difficulty when using voltage indicators: calibrating their signals. Translating an optical signal into an electrical one requires a good understanding of the biophysical mechanisms of voltage sensitivity. While for some of the mechanisms and chromophores there can be linear relationship between voltage and optical signal, in many experiments this is not demonstrated. Understandably, neuroscientists are often interested in the overall biological results, and concentrate their efforts on getting the voltage measurements to work, rather than on understanding the precise details of how their measurements have actually worked. It is also likely that multiple mechanisms with differing time scales contribute to the overall voltage sensitivity of these molecules, confounding the calculated relation between photons measured and electrical signals. In simple situations one can carry out a combined optical and electrical measurement of the same signal, and thus have a direct calibration of the optical signal, but often such combined experiments are not practical, because the optical measurements are carried out precisely in locations or regimes where electrical measurements are impossible. Also, while it is typically assumed that the voltage sensing mechanism of a chromophore, and hence the calibration, is the same in different parts of a neuron, it is possible that differences in local membrane composition and environment, or chromophore concentration and localization, could generate different chromophore responses, rendering global calibrations from regional measurements such as somatic responses, incorrect for distal parts of the neuron. As one might imagine, this could be a serious challenge to calibrating voltage signals in small dendrites or dendritic spines, although, researchers can, and have used, the neuron’s own electrical signals, such as back-propagating action potentials, as internal standards for calibration (Nuriya et al., 2006).

Finally, the relatively high speed of the electrical responses of mammalian neurons also generates a serious challenge for voltage measurements. While infinite temporal resolution would be welcome, in practice most questions can be addressed with one millisecond resolution. As we will discuss in the next section, there are a variety of chromophores with different response times; but unfortunately the fastest ones normally provide the smallest signals, which has been a long standing problem in voltage imaging (Waggoner, 1979).

Voltage imaging techniques and their biophysical mechanisms

The reader can appreciate from the previous list of problems that for effective voltage imaging ones needs to solve some non-trivial challenges. At the same time, as mentioned, the electric field at the plasma membrane is very strong and can easily alter the physical, chemical, environmental, and spectral properties of any molecule located within it. This creates the potential to tap into a rich toolbox of different physico-chemical principles and harness them to measure changes in the electric field. As we will see, there are a great diversity of approaches that *have* achieved meaningful optical voltage measurements, a tribute to the determination and ingenuity of the scientists involved (Cohen, 1989; Cohen and Lesher, 1986). Most of the successful experiments with voltage imaging so far have been accomplished using single photon excitation with visible light, where the absorption cross-sections of the indicators are large. Also, some light sources (arc lamps, or now LEDs) can have very low noise, making it relatively easy to detect minute changes in signal, with ratiometric measurements at multiple absorption or emission wavelengths providing additional noise immunity and sensitivity (Yuste et al., 1997; Zhang et al., 1998). With typical light sources, wide field excitation is possible, and many photons can be collected from spatially extended areas, such as a section of dendrite, the entire soma, or many cells and their processes, increasing the integrated signal. But all the typical problems of single photon excitation apply - there is low penetration into scattering media like intact vertebrate brain tissue, and no native sectioning capability, requiring the use of confocal microscopes to afford cellular resolution. So while the signals from populations of neurons can be relatively large, it can be very difficult extract the signal of a single neuron, or neuronal compartment among a population of labeled neurons. Photobleaching and photodamage also are troublesome, although these effects are not unique to voltage imaging. These problems can be mitigated with the inherent sectioning and lower scattering of non-linear microscopy techniques, such as two-photon fluorescence and second harmonic generation (SHG), but unfortunately, new problems also arise. Two-photon absorption or SHG is much less efficient than single photon absorption, and the excitation volume small, so fewer chromophores are excited, leading to lower overall photon counts, smaller absolute signals, and currently, higher noise. Still, for optimal precision and imaging deep into intact brain tissue, non-linear imaging is a must, and the development of optimal two-photon or SHG active voltage sensors appears clearly necessary.

In the following section we discuss common methods of voltage imaging in neuroscience, focusing on mammalian preparations, which, to us, are where the limitations are most acute. We will not cover the history of this field or attempt to comprehensively review it. Instead, we will focus on providing examples of methods that tap into different biophysical mechanisms of voltage sensitivity. It should be stated that while some mechanisms and detection schemes theoretically allow for the absolute determination of the trans-membrane voltage, in nearly all experiments, what is actually measured is the *change* in membrane potential (Ehrenberg and Loew, 1993). As mentioned previously, it is important to note that voltage indicators can gain their overall sensitivity from a combination of mechanisms, each with different time scales, which complicates the calibration. However, in many cases, one particular mechanism appears to be dominant, and this dominant mechanism is typically used to describe the chromophore. We will describe these different dominant mechanisms

(Figure 2, Table 1), and illustrate them with data from mammalian preparations, chosen as examples of the best signal to noise measurements (Figures 3 and 4). We will only highlight only a few contributions from the literature, as representatives of a large body of work that will not be explicitly cited. We will also review some limitations of these current approaches, a critical exercise that seems to us necessary to move beyond the current state of these techniques. We finish with some thoughts on how to carry out these improvements.

Organic voltage indicators chromophores

Most efforts in voltage imaging involve the synthesis of organic chromophores that can bind to the plasma membrane. This line of work extends now for several decades, starting with invertebrate preparations, and has used chromophores for both absorption and emission (see reviews (Cohen, 1989; Cohen and Lesher, 1986; Gross and Loew, 1989; Waggoner and Grinvald, 1977)). These approaches rely on several different mechanisms of voltage sensing which are common to both absorption and fluorescence, so we will review them together.

Perhaps the simplest voltage sensing mechanism is *redistribution* (Figure 2A, Table 1A), whereby the change in the electric field causes the chromophore (for example, a charged molecule) to move in to or out of the cell, either completely or partly, changing the absolute concentration of the fluorophore in the cell, and hence the fluorescence from it (Ehrenberg et al., 1988). These types of chromophores are sometimes referred to as “Nernstian” dyes, because they redistribute according to Nernstian equilibrium, or alternatively “slow” dyes, because their insertion or detachment from the membrane is a relatively slow (lasting even seconds) equilibrium process, when compared with other mechanisms. The dyes do not have to completely leave the cell - it may be the case that the changing membrane voltage simply alters the portion of a fluorophore that is embedded in the membrane. The equilibrium partitioning of a fluorophore (or part of a fluorophore) between the water-rich cytosol and lipid-rich membrane is determined by the Gibbs free energy of the system, and depends on both the chemical interactions, and the presence and location of charges and electric fields. With changing membrane potential, the equilibrium shifts, altering the concentration and location of the fluorophore. The differences in chemical environment between membrane and cytoplasm (for example, differences in the electric field, in dielectric strength, and other intermolecular interactions) alter the relative stabilities and energies of the ground and excited states of the chromophore, changing its spectroscopic properties. The different environments can also lead to changes in the relaxation rates, altering the lifetime and quantum yield of fluorescence. This enables the optical readout of the redistribution and, indirectly, of the electric field change that caused it. But because of the significant change in chemical environment between the lipid-rich membrane and water-rich cytosol, the spectral changes are large, and thus they generate clear signals, although are only very useful for applications where high time resolution is not crucial.

A different mechanism is *reorientation* (Figure 2B, Table 1B), in which the chromophore lies in or on the membrane with a particular orientation, determined by the sum of the interaction forces on the chromophore. Changes in the electric field affect the chromophore by acting on the dipole moment, producing a torque that alters the orientation angle of the chromophore. The change in alignment then leads to changes in the interaction with the light field, usually by changing the effective extinction coefficient or the fluorescence spectra and quantum yield. The change in angle also changes the relative orientation of the transition dipole moment of the chromophore, so there will be changes in the anisotropy of absorption and emission of polarized light. Reorientation can be fast since it does not involve a significant movement of the chromophore.

A third mechanism, and the one that has received most interest, is *electrochromism*, i.e., the direct electrical modulation of the electronic structure, and thus the spectra, of a

chromophore (Figure 2C, Table 1C). Chromophores that exhibit strong electrochromism typically have large differences in the dipole moment of their ground state and their low lying electronically excited states, and are also highly polarizable, with large induced dipoles. The relative energies of these states thus depend strongly on strength and direction of the external electric field (Loew et al., 1985; Platt, 1956). These changes in the electronic structure lead to changes in both the excitation and emission spectra, which are then manifested as differences in absorption, emission, or lifetime, with respect to voltage. The typical electrochromic dyes are polar, lipophilic and are normally derivates of styryl or hemicyanine dyes, all of which undergo a large internal charge transfer when excited electronically (Fluhler et al., 1985; Fromherz and Lambacher, 1991; Fromherz and Schenk, 1994; Grinvald et al., 1982a). The electrochromic effect, also known as *Stark* effect, is fast since it only involves intramolecular charge redistribution, without chromophore movement. By generating spectral differences it offers a convenient method to monitor changes in membrane potential by measuring optical signals at selective wavelengths.

As an example of the work using organic chromophores with absorption measurements, it has been possible for many years to optically monitor action potentials with excellent temporal resolution, albeit only after extensive averaging (Grinvald et al., 1981; Ross et al., 1977; Salzberg et al., 1977). Similar experiments have been performed successfully using fluorescence, again in a variety of preparations, with the best signal to noise from invertebrate samples. For example, it is possible to measure action potentials in some invertebrate preparations with exquisite temporal resolution (Cohen et al., 1974), or monitor the activity of hundreds of neuron simultaneously during behavior (Wu et al., 1994). Considering these results, one could argue that at least for some invertebrate samples, voltage imaging is effectively a solved problem. Unfortunately, the same cannot be said for mammalian preparations, where similar experiments analyzing the voltage responses of many neurons in a circuit do not afford single cell resolution, when dyes are applied to the entire tissue. While the temporal resolution is high, measurements from bulk application of organic voltage-sensitive dyes on mammalian samples (Grinvald et al., 1982b; Orbach and Cohen, 1983), provide an optical signal that is more equivalent to an ensemble average of the postsynaptic responses of many neurons (Figure 3A and C; (Grinvald et al., 2003; Kuhn et al., 2008)). This “optical field potential” can provide deep insights into the dynamics of spontaneous and evoked neuronal activity (Figure 3A; (Arieli et al., 1996; Grinvald and Hildesheim, 2004), yet at the same time, does not permit the analysis of these responses with single cell resolution (Yuste et al., 1997), unless of course one injects the dye intracellularly (see below). In cultured mammalian preparations, however, voltage imaging of populations of neurons with single cell resolution is possible after bath application of the fluorophores (Grinvald and Farber, 1981).

In terms of the use of organic voltage sensitive dyes for probing subcellular compartments, one can microinject the fluorophores into isolated cells in brain slices, and after a relatively long wait for diffusion to occur, necessary for the fluorophore to distribute along the inner leaflet of the plasma membrane of the neuron, one can image dendritic voltage responses with enough signal to noise to visualize action potentials in dendrites and in spines with one and two-photon induced fluorescence (Figure 3B and D; (Antic and Zecevic, 1995; Holthoff et al., 2010)). The high lipophilicity of these fluorophores makes experiments difficult, because if any chromophore is released accidentally near the site of interest, it binds indiscriminately to all surrounding membranes, resulting in a strong fluorescent background, which contaminates the signal of interest. The lipophilic nature can be advantageous however, as once inside the membrane, the fluorophores migrate along the membrane and can be exploited for use as tracers for anatomical pathways and to enhance the staining (Wuskell et al., 1995). Finally, there has been an effort to synthesize newer families of red-shifted probes with good voltage sensitivity that are well suited for both one and two-photon

excitation (Kuhn et al., 2004), therefore enabling the high resolution voltage measurements from highly scattering media, with the optical sectioning capabilities afforded by non-linear excitation.

Genetic voltage indicators

Fluorescent proteins, most of them variants of the green fluorescent protein (GFP), have become widely used for *in vivo* cell labeling (Chalfie et al., 1994; Tsien, 1998). Combined with protein moieties that provide specific binding to a ligand, they can be engineered to report changes in intracellular free calcium and in other ions or small metabolites (Miyawaki et al., 1997; Tsien, 2009). Because they are genetically encoded, these probes enable the genetic labeling and specific targeting of the chromophore, properties that are ideal for their use *in vivo*.

There have been several different attempts to build voltage sensitive fluorescent proteins. Most use a voltage sensitive domain of an ion channel, or of another protein, as the voltage sensor that sits in the plasma membrane and experiences the electric field. This voltage sensor can directly fluoresce, or be attached to an additional component consisting of one or two fluorescent proteins that do not necessarily need to experience the electric field directly, and can therefore remain outside the membrane (Figure 2D–G). The mechanisms of voltage sensitivity of genetic voltage indicator differ among different constructs: in the simplest case, the voltage sensor or reporter molecule undergoes a significant conformational change that alters its spectra (Figure 2D; (Villalba-Galea et al., 2009)). In other cases, where more than one component is involved, one relies on allosteric interactions that reorientats or otherwise changes the environment of the fluorophore which changes their optical properties (Figure 2E). For example, Förster resonance energy transfer (FRET) or collisional quenching (Dexter energy transfer) can result from these molecular interactions and motions, leading to changes in fluorescence intensity that can be read out optically (Table 1D, E). Changes in lifetime can also be used to monitor these effects, and therefore, the membrane potential.

There are several examples of genetically-engineered fluorescent sensors for voltage. One early attempt was FlaSh5, a construct that uses a non-conducting mutant of a voltage gated potassium channel as the voltage sensor, and a fluorescent protein inserted into the C-terminus region of the channel protein as a reporter (Siegel and Isacoff, 1997). Another construct, SPARC, was generated by inserting a GFP molecule into a rat muscle sodium channel subunit (Ataka and Pieribone, 2002; Baker et al., 2007). A new popular design, termed voltage-sensitive protein (VSFP1, 2, etc) contains two consecutive fluorescent proteins (a FRET pair) attached to the voltage-sensing domain of a mammalian potassium channel or to the trans-membrane domain of a voltage-sensitive phosphatase (Akemann et al., 2010; Gautam et al., 2009; Lundby et al., 2008; Sakai et al., 2001; Villalba-Galea et al., 2009).

Genetic indicators have the added benefit of targeting. By linking expression of the protein to specific promoters, the activity of specific cell-type populations can be monitored without contamination from other classes of cells, so in this respect they could seem as an ideal method to pursue. At the same time, currently, it is still early to judge their usefulness, as most of the constructs have only been used in methodological tests and have not yet been used for extensive experimental programs. Development of genetic voltage sensors is ongoing, and they seem to be constantly improving. Nevertheless, though it is true that the existing proteins do exhibit voltage-induced changes in fluorescence (Figure 4A), in general, the observed changes in fluorescence are fairly small (<5% per 100 mV). More importantly, the responses can be slow (several ms), which results in significant filtering of fast signals such as individual action potentials. The slow response time is a reflection of the

mechanisms involved (redistribution or molecular movements of the protein), and this could be a fundamental limitation for voltage measurements with genetic indicators, one that does not exist for genetic calcium indicators, since their molecular transitions match the timescale of the slower calcium dynamics.

Hybrid voltage indicators

An interesting design for voltage-sensitive dyes is one with a mixture of organic and genetic components (Figure 2E). These hybrid strategies began with a FRET based system, composed of an oxonol derivative that functioned as the donor and a Texas Red labeled lectin as an acceptor (González and Tsien, 1995). Oxonols insert into the membrane and reside on one leaflet or the other depending on the membrane potential. The fluorescently labeled lectin is not membrane permeable, and sits only on the outside of the membrane, and through changes in the energy transfer efficiency between the two species, can be used to monitor the position of the oxonol, and thus, the membrane potential. Another strategy (Chanda et al., 2005) uses a hybrid voltage sensor (hVOS) that consists of a molecule of GFP fused to a farnesylated and palmitoylated motif that attaches it to the membrane. The second component is the synthetic compound dipicrylamine (DPA) that serves as a voltage-sensing acceptor and translocates across the membrane, depending on the electric field. Unfortunately, DPA increases the membrane capacitance, so care must be taken to ensure the concentrations used do not disrupt the native physiological responses. Recently, there have been some promising results from purely chemical hybrid systems, such as the DPA-diO hybrid (Figure 4B). This combination has high sensitivity to voltage, and uses low DPA concentrations, although more work needs to be done for consistent, calibrated voltage imaging in extended experiments (Bradley et al., 2009). Hybrid strategies appear more chemically flexible than pure genetic approaches, although at the same time, they are complicated by the application of exogenous species.

Second Harmonic Generation

It can be argued that fluorescence or absorption approaches are intrinsically flawed when optically probing interfaces, because of their lack of spatial specificity (Eisenthal, 1996). Unless a fluorophore or chromophore is selectively localized at the interface, the interface-specific signal will be greatly overwhelmed by the many other fluorophores/chromophores residing in the bulk solution and this argument can be extended to biological membranes. Second harmonic generation (SHG) solves this problem by only generating signal at the interface itself (Campagnola et al., 1999; Eisenthal, 1996; Moreaux and Mertz, 2001). SHG is a coherent hyper-scattering phenomenon by which the incoming light beam's electric field induces a second order non-linear polarization in the media, resulting in the emission of a photon of exactly twice the frequency (half the wavelength) of the incident photons (Figure 2F). In the asymmetric environment of interfaces, any molecule with non-symmetric chemical or electrical properties can spontaneously align themselves with respect to the interface, whereas in solution, or the bulk media, they will be isotropically distributed, and hence not oriented. Because SHG is strictly forbidden in isotropic environments it can only be generated by chromophores aligned in or on the plasma membrane, while the chromophores in the cytoplasm will remain "dark". As a coherent process, the SHG is strongly directed with respect to the incoming laser beam, and the signal scales as N^2, where N is the number of chromophores that are SHG active, so is strongly dependent on packing density. The strength of the overall SHG response depends on the effective $\chi^{(2)}$, the second-order susceptibility tensor, of the system, which in turn depends on the overall alignment and microscopic properties of the chromophores.

Besides its sensitivity to interfaces such as the plasma membrane, SHG appears particularly well suited for voltage imaging because, to a first order approximation, changes in the

membrane's electric field change the effective $\chi^{(2)}$ in a linear fashion (Table 1F), giving a direct readout of the voltage. In addition, SHG is a non-linear parametric process, and does not rely on the transfer of energy into the molecule, greatly diminishing the photodamage associated excited state processes, such as the generation of triplet states. Moreover, as in two-photon excitation (Denk et al., 1990), the nonlinearity of the process automatically produces optical sectioning in a laser scanning microscope, since SHG is only generated at the focal volume. This minimizes out-of focus excitation and photodamage. Because the *electro-optic* mechanism of SHG is essentially instantaneous, the signal originates only at the membrane, and the photons are emitted in a preferred direction with a well defined spectral signature, SHG seems to be the ideal method for optical recordings of membrane potential (Jiang et al., 2007).

Unfortunately, like with other voltage sensing modalities, current implementations of SHG have been limited. Though the pure electro-optic response is fast, other slower processes that depend on voltage can affect SHG by changing the chromophores spectra or alignment. Over time, the chromophores responsible for SHG can equilibrate across the membrane, reducing the asymmetry of the interface, and hence the overall response (Mertz, 2008). For the typical packing densities in the membrane, it is a relatively inefficient process and normally requires high peak photon fluxes (Campagnola et al., 2001; Eisenthal, 1996), from pulsed ultrafast lasers (Millard et al., 2003). Even so, typically few SHG photos are produced, generating an overall small signal and making photon counting measurements sometimes necessary (Jiang and Yuste, 2008). To increase the SHG signal, most experiments are done with photon energies close to an electronic resonance in the system, which enhances SHG but leads to direct photoabsorption. Also, most of the design strategies for molecules that increase their SHG activity and voltage sensitivity, such as having molecules with large dipole changes upon excitation, results in increases to their two-photon absorption cross sections as well, increasing the probability that the excitation beam will cause simultaneous transitions to real excited states, leading to photodamage, mitigating one of the theoretical advantages for SHG.

In spite of these challenges, in the last decade, the groups of Lewis, Loew, and others have pioneered the application of SHG to living cells and to measurements of membrane potential (Bouevitch et al., 1993; Campagnola et al., 2001; Lewis et al., 1999; Millard et al., 2003). The strategy pursued has been the application of organic dyes, based on styryl fluorophores with distinct electrochromic properties, originally synthesized for fluorescence voltage measurements (Bouevitch et al., 1993). SHG imaging of neurons has also been performed with the membrane-trafficking dye FM4-64, enabling high resolution measurements of voltage of somata, dendrites and dendritic spines (Figure 4C; (Dombeck et al., 2004; Dombeck et al., 2005; Nuriya et al., 2005). As an alternative strategy to the typical chromophores, one can use trans-retinal as a SHG chromophore to measure membrane potential (Nemet et al., 2004), since it exhibits a large change in dipole moment upon light excitation (Mathies and Stryer, 1976). Nevertheless, despite advances in the rational design of chromophores for non-linear imaging, relatively little work has gone into synthesizing chromophores specifically designed for SHG in biological samples that would maximize the SHG response while minimizing damaging alternative photoprocesses.

Intrinsic mechanisms

Finally, an alternative approach to measure membrane potential relies on intrinsic changes in the optical properties of the neurons, or axons. These approaches, which are among the earliest historically (Cohen and Keynes, 1971), are potentially very powerful because they do not need exogenous chromophores. At the same time, they can only be applied in optically very accessible preparations, such as neuronal cultures or some invertebrate

preparations. Also, they generate relatively small signals and extensive averaging is necessary.

These intrinsic approaches to measure voltage have exploited different type of optical measurements, mostly in invertebrate preparations. For example, changes in light scattering, changes in optical dichroism, and changes in birefringence have been explored (Ross et al., 1977). These changes are presumably related to alteration in the refractive index or small volume changes near the membrane, in response to the rapid osmotic changes associated with ion fluxes, and have been used to monitor action potentials (Cohen and Keynes, 1971; Ross et al., 1977; Stepnoski et al., 1991). Presumably these same intrinsic mechanisms allow for the detection of action potentials with optical coherence tomography, which uses interferometry to detect small changes in optical path length resulting from action potential activity in isolated neurons. However none of these methods have yet allowed for high-resolution voltage imaging of populations of neurons with single cell precision.

A final note relates to the use of a different type of intrinsic optical signals to monitor neuronal activity through its impact in blood flow or oxygenation (Grinvald et al., 1986). This work represents a large body of literature that has generated major advancements in systems neurosciences, and forms the basis of BOLD fMRI, a technique that has revolutionized brain imaging (Ogawa et al., 1990). Although blood-related intrinsic signals are important, the reliance on coupling to the circulatory system makes these techniques unlikely to generate single cell resolution data that is directly proportional to membrane voltage dynamics.

Future Outlook

Although currently used voltage imaging methods have some shortcomings, they *are* useful, and researchers *have* succeeded in measuring membrane potential in a variety of mammalian preparations. In addition, novel imaging modalities have been recently developed and, although they have not yet been implemented for voltage imaging, they could hold great promise for future work.

One example is the use of nanoparticles, such as nanocrystals or quantum dots (Hallock et al., 2005). These are small inorganic (metal or semiconductor) particles with well-defined electronic structure and precise quantum states. Composed of many atoms or molecules, the nanoparticles can have very strong interactions with the light field, leading to very large extinction coefficients and highly efficient emission (Figure 2I). The specialized structure of nanoparticles enables the generation of excitons, which can be sensitive the external electric field, resulting in strong modulations in the quantum yield, spectra, or lifetime with voltage changes. Most of these particles are coated with a passivation layer or specialized shell that limits direct interaction with the surrounding media, greatly minimizing bleaching, and in the cell, the generation of reactive oxygen species. Nanoparticles could be used alone, or combined with a conventional chromophore, as under certain conditions, they have been shown to greatly enhance optical signals, acting as an “antenna” for the light (Stiles et al., 2008; Tam et al., 2007). Thus when coupled to nearby chromophores, there could be large increases in fluorescence, Raman, or SHG. Already, membrane-bound, antibody-linked gold nanoparticles have been used to increase SHG from single dye molecules allowing site specific measurements of membrane potential (Peleg et al., 1999). On the negative side, nanoparticles can be large (>10 nm)) and difficult to properly deliver in biological samples, with coating procedures and functionalization seemingly more art than science. Nevertheless, if they could be properly targeted to the membrane, their optical properties and voltage sensitivity could make them ideal voltage sensors and some examples of their potential use have been published (Figure 4D; (Fan and Forsythe, 2008)).

As another potential strategy, one might be able to use other non-linear imaging modalities to optically interrogate intrinsic chromophores present in the membrane. For example, Raman imaging (Evans and Xie, 2008), sum-frequency or third-harmonic generation (SFG, THG; (Flörsheimer et al., 1999; Yelin and Silberberg, 1999)) or the recently developed stimulated radiation imaging methods (Freudiger et al., 2008; Geiger, 2009; Min et al., 2009), could potentially to be used to directly monitor the small spectral changes caused by the membrane potential in species intrinsic to the membrane environment, free from the constraints of exogenous labels. At the same time, these techniques would need to effectively solve the contrast problem raised above, and distinguish optical signals from the plasma membrane from those of other cellular membranes.

In terms of improving existing strategies, significant challenges need to be overcome. One major avenue for improvement is the rational design of novel probes, whether organic, inorganic or genetic. For example, it is known that the exact shape of trans-membrane proteins can strongly modify the local electric field, magnifying it, so that clever placement of a voltage sensing moiety in molecular pockets where the electric field would be more concentrated could lead to an improved voltage sensor. Also, for sensors based on energy transfer, conformational changes are not the only variable affected by voltage. The rates of energy transfer also depend critically on the spectral overlap of the donor's emission spectrum with the acceptor's absorption spectrum, and either of these can be altered directly or indirectly as a result of changing membrane potential. Because of the highly non-linear FRET dependence with spectral overlap of the donor-acceptor pair, it may be more sensitive than simply monitoring the spectral changes alone. As discussed previously, current SHG based measurements suffer because of concomitant absorption and subsequent photodamage, and non-traditional chromophores with large values of $\chi^{(2)}$, but with weak fluorescence could lead to new, useful voltage probes.

It seems particularly important for research groups with extensive experience in chemistry or the physical sciences to join these efforts, as it often occurs in science and particularly in biological imaging (as illustrated by the development of calcium indicators or of two-photon microscopy), it is from this interdisciplinary cross-fertilization that major advances are generated. In addition, more studies of the biophysical mechanisms of existing chromophores are necessary. This is not just an academic exercise, but it could be essential in the efforts to design better chromophores.

Also, it should be kept in mind that there may not be a universal voltage-sensitive dye, but it could be possible to use a combination of them, depending on the kinetics of the desired signals to be measured and constraints introduced by the specific preparations. This would be an situation analogous to the use of calcium indicators, with different affinity dyes being used to measure calcium signals with different amplitudes and kinetics (Neher, 1998; Tank et al., 1995).

A final note relates to the importance of identifying cell types in this type of optical experiments. Since most mammalian circuits are composed of different cellular elements, mixed together, and since it is likely that different subtypes of neurons serve different circuit functions, it appears essential, not only to monitor voltage responses with single cell resolution, but also to distinguish the specific cell type of each imaged neurons. In this respect, the use of genetically engineered animals where subsets of cells can be specifically labeled, or targeted, seems crucial. While ideally a genetic voltage indicator could be targeted specifically to a subset of neurons, one could also perform voltage measurements using a non-genetic method in animals where cell types are previously labeled with a genetic, or non-genetic, marker.

This is an exciting moment. Reliable, quantitative voltage imaging is arguably still the biggest current technical hurdle in mammalian neuroscience and we are now, as a research field, almost there. We ourselves remain agnostic as to which of the many different approaches discussed (organic fluorophores, SHG chromophores, genetic indicators, hybrid approaches, nanoparticles, intrinsic) is the most promising one but are hopeful for all of them. Our opinion is that, rather than a “winning horse”, it seems that at this point, the race has just started and none of these techniques has a significant advantage over the others, so parallel efforts should be undertaken to improve voltage imaging, rather than focusing on a single approach. A practical goal for voltage imaging would be to measure voltage signals at the soma, for example, with a S/N of 2 for individual action potentials, without averaging, allowing detailed monitoring of spontaneous and evoked activity in a population of neurons with single-cell specificity. Similarly, the voltage associated with quantal events in individual spines should be measured with the same S/N and without averaging. These are attainable goals, and ongoing improvements in voltage sensors could quickly break the logjam and enable what could be a new era for the study of neuronal integration and mammalian circuits. All hands aboard!

Acknowledgments

We thank members of our laboratory for comments, Janelia Farms for hosting a workshop on voltage imaging, and colleagues that attended the workshop for discussions. Supported by the Kavli Institute for Brain Science and the National Eye Institute.

References

Akemann W, Mutoh H, Perron A, Rossier J, Knopfel T. Imaging brain electric signals with genetically targeted voltage-sensitive fluorescent proteins. Nat Meth. 2010 advance online publication.

Antic S, Zecevic D. Optical signals from neurons with internally applied voltage-sensitive dyes. J Neurosci. 1995; 15:1392–1405. [PubMed: 7869106]

Arieli A, Sterkin A, Grinvald A, Aertsen A. Dynamics of ongoing activity: explanation of the large variability in evoked cortical responses. Science. 1996; 273:1868–1871. [PubMed: 8791593]

Ataka K, Pieribone VA. A genetically targetable fluorescent probe of channel gating with rapid kinetics. Biophysical journal. 2002; 82:509–516. [PubMed: 11751337]

Baker BJ, Lee H, Pieribone VA, Cohen LB, Isacoff EY, Knopfel T, Kosmidis EK. Three fluorescent protein voltage sensors exhibit low plasma membrane expression in mammalian cells. Journal of neuroscience methods. 2007; 161:32–38. [PubMed: 17126911]

Blunck R, Chanda B, Bezanilla F. Nano to micro -- fluorescence measurements of electric fields in molecules and genetically specified neurons. The Journal of membrane biology. 2005; 208:91–102. [PubMed: 16645739]

Bouevitch O, Lewis A, Pinevsky I, Wuskell JP, Loew LM. Probing membrane potential with nonlinear optics. Biophys J. 1993; 65:672–679. [PubMed: 8218895]

Bradley J, Luo R, Otis TS, DiGregorio DA. Submillisecond optical reporting of membrane potential in situ using a neuronal tracer dye. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2009; 29:9197–9209. [PubMed: 19625510]

Campagnola PJ, Clark HA, Mohler WA, Lewis A, Loew LM. Second-harmonic imaging microscopy of living cells. J Biomed Opt. 2001; 6:277–286. [PubMed: 11516317]

Campagnola PJ, Wei MD, Lewis A, Loew LM. High-resolution nonlinear optical imaging of live cells by second harmonic generation. Biophys J. 1999; 77:3341–3349. [PubMed: 10585956]

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science (New York, NY). 1994; 263:802–805.

Chanda B, Blunck R, Faria LC, Schweizer FE, Mody I, Bezanilla F. A hybrid approach to measuring electrical activity in genetically specified neurons. Nature neuroscience. 2005; 8:1619–1626.

Cohen, L. Optical approaches to neuronal function. In: Hoffman, JF.; De Weer, P., editors. Annual Review of Physiology. Palo Alto, CA: Annual Review Inc; 1989. p. 487-582.

Cohen LB, Keynes RD. Changes in light scattering associated with the action potential in crab nerves. J Physiol. 1971; 212:259–275. [PubMed: 5545182]

Cohen, LB.; Lesher, S. Optical Monitoring of Membrane Potential: Methods of Multisite Optical Measurement. In: De Weer, P.; Salzberg, BM., editors. Optical Methods in Cell Physiology. New York: Wiley-Interscience; 1986. p. 72-99.

Cohen LB, Salzberg BM, Davilla HV, Ross WN, Landowne D, Waggoner AS, Wang CH. Changes in axon fluorescence during activity: molecular probes of membrane potential. Journal of Membrane Biology. 1974; 19:1–36. [PubMed: 4431037]

Cossart R, Aronov D, Yuste R. Attractor dynamics of network UP states in neocortex. Nature. 2003; 423:283–289. [PubMed: 12748641]

Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. Science. 1990; 248:73–76. [PubMed: 2321027]

Dombeck D, Blanchard-Desce M, Webb W. Optical recording of action potentials with second-harmonic generation microscopy. J Neurosci. 2004; 24:999–1003. [PubMed: 14749445]

Dombeck DA, Sacconi L, Blanchard-Desce M, Webb WW. Optical recording of fast neuronal membrane potential transients in acute mammalian brain slices by second-harmonic generation microscopy. Journal of Neurophysiology. 2005; 94:3628–3636. [PubMed: 16093337]

Ehrenberg B, Loew LM. Absolute spectroscopic determination of cross-membrane potential. Journal of Fluorescence. 1993; 3:265–269.

Ehrenberg B, Montana V, Wei MD, Wuskell JP, Loew LM. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. Biophysical journal. 1988; 53:785–794. [PubMed: 3390520]

Eisenthal KB. Liquid Interfaces Probed by Second-Harmonic and Sum-Frequency Spectroscopy. Chem Rev. 1996; 96:1343–1360. [PubMed: 11848793]

Evans CL, Xie XS. Coherent Anti-Stokes Raman Scattering Microscopy: Chemical Imaging for Biology and Medicine. Annual Review of Analytical Chemistry. 2008; 1:883–909.

Fan, H.; Forsythe, C. Quantum Dots Enable Detection of Neuron Activation. In: Sandia, editor. Science Matters. Albuquerque: 2008.

Flörsheimer M, Brillert C, Fuchs H. Chemical Imaging of Interfaces by Sum Frequency Microscopy. Langmuir. 1999; 15:5437–5439.

Fluhler E, Burnham VG, Loew LM. Spectra, membrane binding and potentiometric responses of new charge shift probes. Biochemistry. 1985; 24:5749–5755. [PubMed: 4084490]

Freudiger CW, Min W, Saar BG, Lu S, Holtom GR, He C, Tsai JC, Kang JX, Xie XS. Label-free biomedical imaging with high sensitivity by stimulated Raman scattering microscopy. Science (New York, NY). 2008; 322:1857–1861.

Fromherz P, Lambacher A. Spectra of voltage-sensitive fluorescence of styryl-dye in neuron membrane. Biochimica et Biophysica Acta. 1991; 1068:149–156. [PubMed: 1911828]

Fromherz P, Schenk O. Voltage-sensitive fluorescence of amphiphilic hemicyanine dyes in a black lipid membrabe of glycerol monooleate. Bioch Biophys Acta. 1994; 1191:299–308.

Gautam SG, Perron A, Mutoh H, Knèopfel T. Exploration of fluorescent protein voltage probes based on circularly permuted fluorescent proteins. Frontiers in neuroengineering. 2009; 2:14. [PubMed: 19862342]

Geiger FM. Second harmonic generation, sum frequency generation, and chi(3): dissecting environmental interfaces with a nonlinear optical Swiss Army knife. Annual review of physical chemistry. 2009; 60:61–83.

González JE, Tsien RY. Voltage sensing by fluorescence resonance energy transfer in single cells. Biophys J. 1995; 69:1272–1280. [PubMed: 8534797]

Grinvald A, Arieli A, Tsodyks M, TK. Neuronal assemblies: Single cortical neurons are obedient members of a huge orchestra. Biopolymers. 2003; 68:422–436. [PubMed: 12601800]

Grinvald A, Farber IC. Optical recording of calcium action potentials from growth cones of cultured neurons with a laser microbeam. Science. 1981; 212:1164–1167. [PubMed: 7233210]

Grinvald A, Hildesheim R. VSDI: a new era in functional imaging of cortical dynamics. Nature reviews Neuroscience. 2004; 5:874–885.

Grinvald A, Hildesheim R, Farber IC, Anglister L. Improved fluorescent probes for the measurement of rapid changes in membrane potential. Biophysical journal. 1982a; 39:301–308. [PubMed: 7139029]

Grinvald A, Lieke E, Frostig RD, Gilbert CD, Wiesel TN. FUNCTIONAL ARCHITECTURE OF CORTEX REVEALED BY OPTICAL IMAGING OF INTRINSIC SIGNALS. Nature. 1986; 324:361–364. [PubMed: 3785405]

Grinvald A, Manker A, Segal M. Visualization of the spread of electrical activity in rat hippocampal slices by voltage-sensitive optical probes. J Physiol. 1982b; 333:269–291. [PubMed: 7182467]

Grinvald A, Ross WN, Farber I. Simultaneous optical measurements of electrical activity from mutiple sites on processes of cultured neurons. Proc Natl Acad Sci USA. 1981; 78:3145–3249.

Gross D, Loew LM. Fluorescent indicators of membrane potential: microspectrofluorometry and imaging. Methods Cell Biol. 1989; 30:193–218. [PubMed: 2648110]

Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem. 1985; 260:3440–3450. [PubMed: 3838314]

Hallock AJ, Redmond PL, Brus LE. Optical forces between metallic particles. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:1280–1284. [PubMed: 15647359]

Hirase H, Nikolenko V, Goldberg JH, Yuste R. Multiphoton stimulation of neurons. J Neurobiol. 2002; 51:237–247. [PubMed: 11984845]

Holthoff K, Zecevic D, Konnerth A. Rapid time course of action potentials in spines and remote dendrites of mouse visual cortex neurons. The Journal of physiology. 2010; 588:1085–1096. [PubMed: 20156851]

Jiang J, Eisenthal KB, Yuste R. Second harmonic generation in neurons: electro-optic mechanism of membrane potential sensitivity. Biophysical journal. 2007; 93:L26–28. [PubMed: 17604312]

Jiang J, Yuste R. Second-harmonic generation imaging of membrane potential with photon counting. Microscopy and microanalysis: the official journal of Microscopy Society of America, Microbeam Analysis Society, Microscopical Society of Canada. 2008; 14:526–531.

Kuhn B, Denk W, Bruno RM. In vivo two-photon voltage-sensitive dye imaging reveals top-down control of cortical layers 1 and 2 during wakefulness. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105:7588–7593. [PubMed: 18508976]

Kuhn B, Fromherz P, Denk W. High sensitivity of Stark-shift voltage-sensing dyes by one- or two-photon excitation near the red spectral edge. Biophysical journal. 2004; 87:631–639. [PubMed: 15240496]

Lewis A, Khatchatouriants A, Treinin M, Chen Z, Peleg G, Friedman N, Bouevitch O, Rothman Z, Loew L, Sheves M. Second Harmonic Generation of Biological Interfaces: Probing Membrane Proteins and Imaging Membrane Potential Around GFP Molecules at Specific Sites in Neuronal Cells of C. elegans. Chemical Physics. 1999; 245:133–144.

Loew LM, Cohen LB, Salzberg BM, Obaid AL, Bezanilla F. Charge shift probes of membrane potential. Characterization of aminostyrylpyridinium dyes on the squid giant axon. Biophys J. 1985; 47:71–77. [PubMed: 3978192]

Lundby A, Mutoh H, Dimitrov D, Akemann W, Knèopfel T. Engineering of a genetically encodable fluorescent voltage sensor exploiting fast Ci-VSP voltage-sensing movements. PloS one. 2008; 3:e2514. [PubMed: 18575613]

Mathies R, Stryer L. Retinal has a highly dipolar vertically excited singlet state: implications for vision. Proc Natl Acad Sci U S A. 1976; 73:2169–2173. [PubMed: 1065867]

Mennerick S, Chisari M, Shu HJ, Taylor A, Vasek M, Eisenman LN, Zorumski CF. Diverse Voltage-Sensitive Dyes Modulate GABA(A) Receptor Function. Journal of Neuroscience. 2010; 30:2871–2879. [PubMed: 20181584]

Mertz, J. Applications of Second-Harmonic Generation Microscopy. In: Masters, Barry R.; So, PTC., editors. Handbook of Biomedical Nonlinear Optical Microscopy. New York: Oxford University Press; 2008. p. 348-374.

Millard AC, Campagnola P, Mohler WA, Lewis A, Loew L. Second harmonic imaging microscopy. Methods Enzymol. 2003; 361:47–69. [PubMed: 12624906]

Min W, Lu S, Chong S, Roy R, Holtom GR, Xie XS. Imaging chromophores with undetectable fluorescence by stimulated emission microscopy. Nature. 2009; 461:1105–1109. [PubMed: 19847261]

Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY. Fluorescent indicators for Ca2+ based on green fluorescent proteins and calmodulin. Nature. 1997; 388:882–887. [PubMed: 9278050]

Moreaux L, Mertz J. Biological membrane imaging with multi-harmonic light microscopy. Biophysical Journal. 2001; 80:656.618. [PubMed: 11159434]

Neher E. Usefulness and limitations of linear approximations to the understanding of Ca++ signals. Cell Calcium. 1998; 24:345–375. [PubMed: 10091004]

Nemet B, Nikolenko V, Yuste R. Second harmonic retinal imaging of membrane potential (SHRIMP). J Biomed Optics. 2004; 9:873–881.

Nuriya M, Jiang J, Nemet B, Eisenthal KB, Yuste R. Imaging membrane potential in neurons with second harmonic generation. Cold Spring Harbor Synaptic Plasticity Meeting. 2005; 1:16.

Nuriya M, Jiang J, Nemet B, Eisenthal KB, Yuste R. Imaging membrane potential in dendritic spines. Proc Natl Acad Sci USA. 2006; 103:786–790. [PubMed: 16407122]

Offner FF. KINETICS OF EXCITABLE MEMBRANES - VOLTAGE AMPLIFICATION IN A DIFFUSION REGIME. Journal of General Physiology. 1970; 56:272. [PubMed: 5433470]

Ogawa S, Lee TM, Kay AR, Tank DW. BRAIN MAGNETIC-RESONANCE-IMAGING WITH CONTRAST DEPENDENT ON BLOOD OXYGENATION. Proceedings of the National Academy of Sciences of the United States of America. 1990; 87:9868–9872. [PubMed: 2124706]

Olivotto M, Arcangeli A, Carlà M, Wanke E. Electric fields at the plasma membrane level: A neglected element in the mechanisms of cell signalling. BioEssays. 1996; 18:495–504. [PubMed: 8787537]

Orbach HS, Cohen LB. Optical monitoring of activity from many areas of the in vitro and in vivo salamander olfactory bulb: a new method for studying functional organization in the vertebrate central nervous system. J Neurosci. 1983; 3:2251–2262. [PubMed: 6631479]

Peleg G, Lewis A, Linial M, Loew LM. Nonlinear optical measurement of membrane potential around single molecules at selected cellular sites. Proc Natl Acad Sci U S A. 1999; 96:6700–6704. [PubMed: 10359775]

Perron A, Mutoh H, Launey T, Knöpfel T. Red-Shifted Voltage-Sensitive Fluorescent Proteins. 2009; 16:1268–1277.

Platt JR. Electrochromism, a possible change of color producible in dyes by an electric field. J Chem Phys. 1956; 25:80–105.

Ross WN, Salzberg LB, Cohen LB, Grinvald A, Davila HV, Waggoner AS, Wang C-H. Changes in absorption, fluorescence, dichroism, and birefringence in stained giant axons: optical measurements of membrane potential. J Mem Biol. 1977; 33:141–183.

Sakai R, Repunte-Canonigo V, Raj CD, Knèopfel T. Design and characterization of a DNA-encoded, voltage-sensitive fluorescent protein. The European journal of neuroscience. 2001; 13:2314–2318. [PubMed: 11454036]

Salzberg BM, Grinvald A, Cohen LB, Davila HV, Ross WN. Optical recording of neuronal activity in an invertebrate central nervous system: simultaneous monitoring of several neurons. J Neurophys. 1977; 40:1281–1291.

Siegel MS, Isacoff EY. A genetically encoded optical probe of membrane voltage. Neuron. 1997; 19:735–741. [PubMed: 9354320]

Smetters DK, Majewska A, Yuste R. Detecting action potentials in neuronal populations with calcium imaging. Methods. 1999; 18(2):215–21. [PubMed: 10356353]

Stepnoski RA, LaPorta A, Raccuia-Behling F, Blonder GE, Slusher RE, Kleinfeld D. Noninvasive detection of changes in membrane potential in cultured neurons by light scattering. Proceedings of the National Academy of Sciences of the United States of America. 1991; 88:9382–9386. [PubMed: 1946349]

Stiles PL, Dieringer JA, Shah NC, Van Duyne RP. Surface-Enhanced Raman Spectroscopy. Annual Review of Analytical Chemistry. 2008; 1:601–626.

Tam F, Goodrich GP, Johnson BR, Halas NJ. Plasmonic Enhancement of Molecular Fluorescence. Nano Letters. 2007; 7:496–501. [PubMed: 17256995]

Tank DW, Regehr WG, Delaney KR. A quantitative analysis of presynaptic calcium dynamics that contribute to short-term enhancement. J Neurosci. 1995; 15:7940–7952. [PubMed: 8613732]

Tsien R. The green fluorescent protein. Annu Rev Biochem. 1998; 67:509–544. [PubMed: 9759496]

Tsien RY. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry. 1980; 19:2396–2404. [PubMed: 6770893]

Tsien RY. A non-disruptive technique for loading calcium buffers and indicators into cells. Nature. 1981; 290:527–528. [PubMed: 7219539]

Tsien RY. Indicators based on fluorescence resonance energy transfer (FRET). CSH protocols. 2009:pdb.top57. [PubMed: 20147227]

Tsodyks M, Kenet T, Grinvald A, Arieli A. Linking spontaneous activity of single cortical neurons and the underlying functional architecture. Science. 1999; 286:1943–1946. [PubMed: 10583955]

Villalba-Galea CA, Sandtner W, Dimitrov D, Mutoh H, Knèopfel T, Bezanilla F. Charge movement of a voltage-sensitive fluorescent protein. Biophysical journal. 2009; 96:L19–21. [PubMed: 19167283]

Vučinić D, Sejnowski TJ. A Compact Multiphoton 3D Imaging System for Recording Fast Neuronal Activity. PLoS ONE. 2007; 2:e699. [PubMed: 17684546]

Waggoner AS. DYE INDICATORS OF MEMBRANE-POTENTIAL. Annual Review of Biophysics and Bioengineering. 1979; 8:47–68.

Waggoner AS, Grinvald A. Mechanisms of rapid optical changes of potential sensitive dyes. Annals of New York Acadamy of Science. 1977; 303:217–241.

Wu JY, Cohen LB, Falk CX. Neuronal activity during different nehaviours in Aplysia: A distributed organization? Science. 1994; 263:820–823. [PubMed: 8303300]

Wuskell JP, Fink C, Wei M, Loew LM, Cohen LB, Tsau Y, Zecevic D. New potentiometric dyes for microinjection, neuronal tracing and intracellular labelling. Biophys J. 1995; 68:A291.

Yelin D, Silberberg Y. Laser scanning third-harmonic-generation microscopy in biology. Optics Express. 1999; 5:169–175. [PubMed: 19399061]

Yuste R, Denk W. Dendritic spines as basic functional units of neuronal integration. Nature. 1995; 375:682–684. [PubMed: 7791901]

Yuste R, Katz LC. Control of postsynaptic Ca2+ influx in developing neocortex by excitatory and inhibitory neurotransmitters. Neuron. 1991; 6:333–344. [PubMed: 1672071]

Yuste R, Tank DW, Kleinfeld D. Functional study of the rat cortical microcircuitry with voltage-sensitive dye imaging of neocortical slices. Cerebral Cortex. 1997; 6/7:546–558. [PubMed: 9276179]

Zhang J, Davidson RM, Wei MD, Loew LM. Membrane electric properties by combined patch clamp and fluorescence ratio imaging in single neurons. Biophysical Journal. 1998; 74:48–53. [PubMed: 9449308]

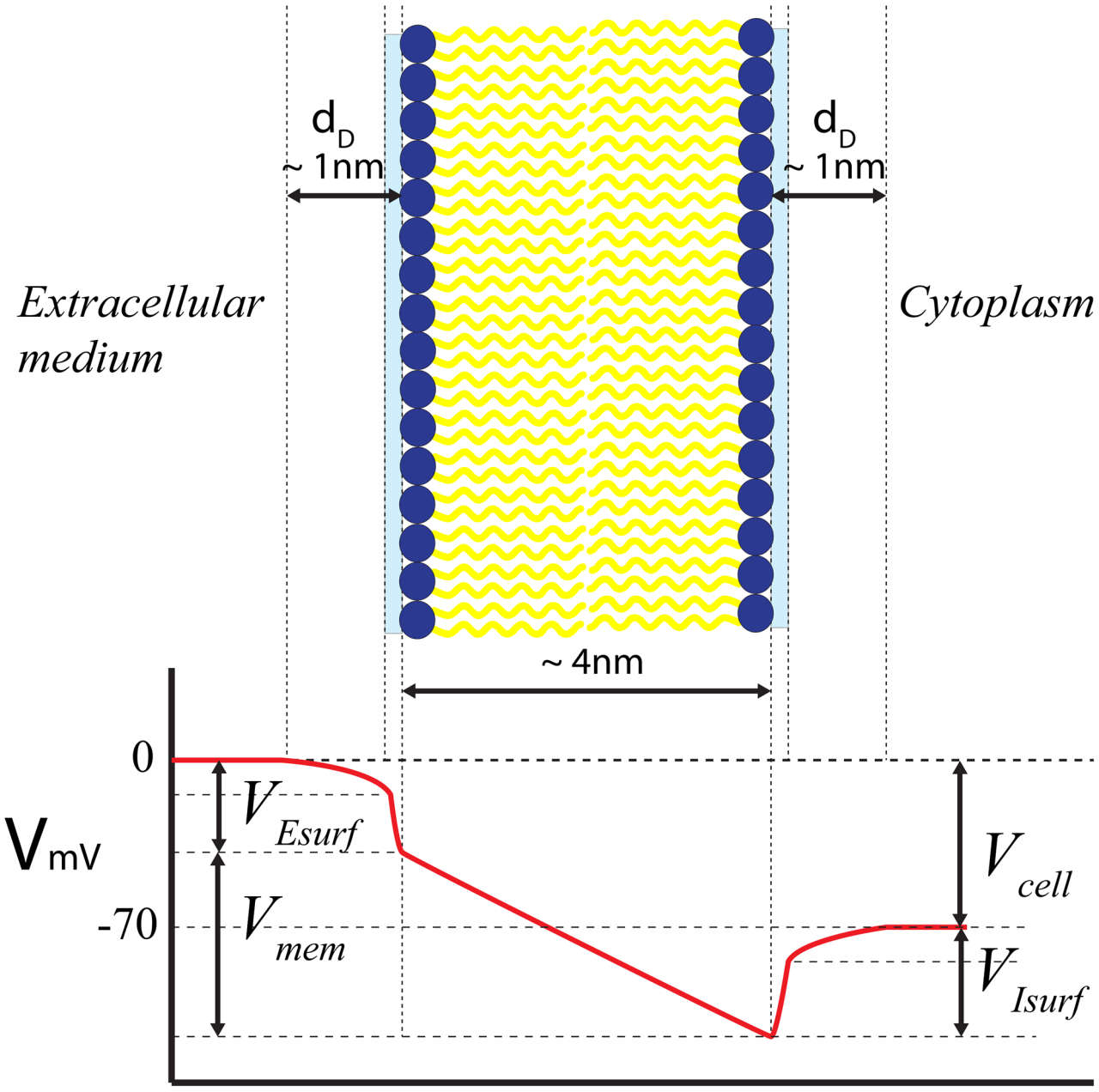

Figure 1. Biophysics of the plasma membrane

Illustration of the plasma membrane showing a simplified model (Gouy-Chapman-Stern) of the relevant structures, potentials, and distances involved in membrane voltage sensing (Olivotto et al., 1996). The cell's overall potential, $\mathbf{V_{cell}}$, is the difference in voltage between the bulk external media and the bulk internal media, as is governed by the concentration differences of ions in the two solutions. The local environment of the membrane has different potentials, however, reflecting electrical structures in the membrane. The negatively charged phosphate heads lead to strong polarization and alignment of water and ions immediately adjacent to the membrane. As one extends further into the bulk, the concentration of ions and water gradually transitions to that of the bulk and the field drops exponentially- the distance where the field drops to 1/e of the initial value is called the Debye length, and this region called the Debye Layer ($\mathbf{d_D}$). In the figure, $\mathbf{V_{Esurf}}$ and $\mathbf{V_{Isurf}}$

represent the external and internal surface potential surrounding the bilipid layer. The field inside the membrane, $\mathbf{V_{mem}}$ is illustrated as homogeneous, an assumption that is clearly not true locally, in the presence of transmembrane proteins and pores.

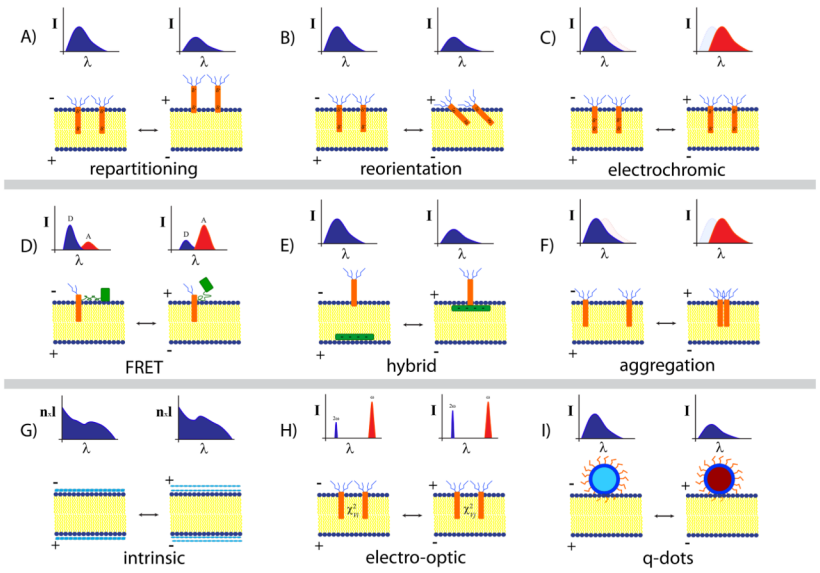

Figure 2. Mechanisms of voltage sensitivity

Schematic of the physical mechanisms leading to voltage sensitivity in plasma membrane measurements, along with typical spectral signatures. Starting from top left, going across the figure: **A**) **repartitioning**, where the dye molecules move in and out of the membrane with voltage changes (see Table 1A); **B**) **reorientation**, where the electric field acting on the chromophore's dipole produces a torque changing the relative alignment with respect to the membrane (see Table 1B); **C**) **electrochromism**, where the membrane potential changes the relative energy of the ground and excited states of the chromophore altering the excitation and emission wavelength (see Table 1C); **D**) **FRET**; where voltage induced conformational or spectral changes alter the efficiency of energy transfer (see Table 1D); **E**) **collisional quenching** is used in some hybrid schemes, where voltage induced motions lead to energy transfer, altering the fluorescence quantum yield and lifetime (see Table 1E); **F**) **voltage induced dimerization/aggregation,** where changing voltage induces aggregation of chromophores, altering the spectra; **G**) **intrinsic imaging** (complex refractive index changes due to action potential activity); **H**) **SHG (electro-optic)**, where changes in voltage alter the effective $\chi^{(2)}$, modulating the SHG signal (see Table 1F); **I**) **nanoparticles,** not a mechanism per se, but used as a novel chromophore, or as an sensitivity "amplifier" for existing nearby chromophores.

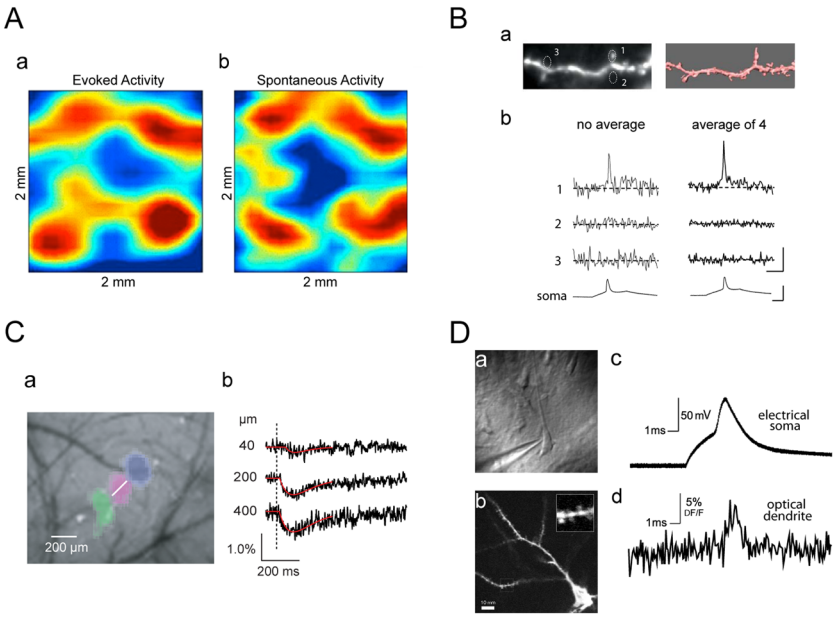

Figure 3. Voltage imaging in mammalian preparations with one photon and two photon microscopy

(A) One-photon voltage imaging from neuronal ensembles from cat neocortex *in vivo*. A photodiode array was used to monitor the responses over a ~2 mm by 2mm patch of cortical area stained with RH795. Pseudo color images represented as averages over all of the captured frames. **a**) Visual stimulation (eyes open) evoked a change in membrane potentials. **b**) Spontaneous activity (eyes closed) of the same cortical territory. Note how the optical recording in both cases revealed almost identical patterns. Reprinted from (Tsodyks et al., 1999) with permission. **(B)** One-photon voltage imaging of back propagating action potentials in individuals dendritic spines of rat neocortical neurons *in vitro*. **a**) raw confocal image is shown on left, and deconvolved reconstructed image is show in the right. **b**) left shows the individual signals recorded at positions 1–3 as indicated in panel a, along with the electrical signal measured at the soma, while b) right shows the averaged result from 4 measurements. Reprinted from (Holthoff et al., 2010) with permission. **(C)** Two-photon voltage imaging *in vivo*. **a**) Colored areas in the barrel cortex mark regions were intrinsic imaging showed reflectivity changes of >0.1% following whisker stimulation with white line marking area of two-photon voltage responses. **b**) Averaged (n=400) trials of the VSD response for three different focal depths (40, 200, 400 μm), with dashed line indicating onset of stimulation. Reprinted from (Kuhn et al., 2008), with permission. **(D)** Two photon voltage imaging *in vitro*. **a**) Transmitted light image of a neuron in acute rat brain slice. **b**) Fluorescence image of a cortical pyramidal cell filled with the fluorescent dye FM4–64. Inset: zoom onto the 10 X 10 μm area outlined in the image showing dendritic spines. **c, d**) Point-dwelling ability and photon counting permit optical recording of fast events with while maintaining significant signal-to-noise ratios. Electrical (c) and unfiltered optical (d) traces of an action potential in a rat cortical pyramidal neuron loaded with the potentiometric dye di-2-ANEPEQ. Traces were averages of four recordings. Reprinted from (Vučinić and Sejnowski, 2007) with permission.

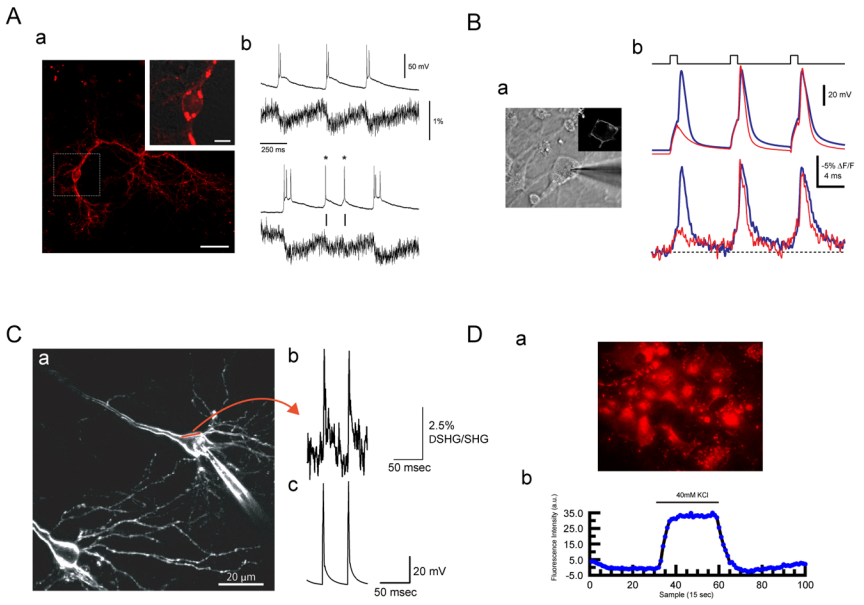

Figure 4. Novel modalities of voltage imaging

(A) Genetically-expressed voltage sensitive proteins can optically report membrane voltage of mammalian neurons. **a**) VSFP3.1_mOrange2 transfected into a cultured hippocampus neuron and expressed in the soma, axon, and dendrites (overview). High magnification image that demonstrated that VSFP3.1_mOrange2 is largely expressed in the plasma membrane with some fluorescence in perinuclear areas (insets). Scale bars are 40 μm in the overviews and 10 μm in the insets. **b**) Electrical (upper traces) and optical (bottom traces) recordings from a neuron expressing VSFP3.1_mOrange2. The voltage sensitive protein was able to sense evoked action potential bursts (upper right panel) and also spontaneous spikes (asterisks in the bottom panel) in a single sweep. Reprinted from (Perron et al., 2009), with permission. **(B)** The hybrid chemical sensor pair of DiO/DPA gives high fidelity for high-frequency bAPs. **a**) DIC and confocal (inset) image of cultured hippocampal neurons incubated with DiO and DPA. The region of interest is shown as a yellow cross in insets. **b**) Current injections at 100 Hz (top) evoked action potentials at the soma (middle), which induced voltage-dependent fluorescence changes (bottom). Optical traces were averaged: blue 6 trials, red 12 trials. The trial to trial fluctuations of the first episode during current injection led to successful (blue) and unsuccessful (red) firing of action potentials, which was accurately reported by the dye combination. Reprinted from (Bradley et al., 2009) with permission. (C) SHG signal captured membrane voltage transients in a hippocampal slice. a) Membrane restricted SHG signal was obtained by intracellularly loaded FM4-64 via a recording pipette. b) Line-scan recordings (red line in a) of SHG along the somatic plasma membrane revealed action potentials with high fidelity (S/N ~7–8) after averaging (n=55). c) The somatic membrane potential was monitored in current clamp mode. Super threshold depolarization elicited action potentials. Reprinted from (Dombeck et al., 2005), with permission. **(D)** Quantum dots (Q-dots) can sense electric field changes in mammalian cells. **a**) Q-dots successfully targeted the plasma membrane of cultured hippocampal neurons. **b**) Q-dots showed strong modulations in fluorescent intensity to membrane voltage changes induced by altering the potassium concentration surrounding the cells. Reprinted from (Fan and Forsythe, 2008), with permission

Table 1

Analytical functions underlying voltage sensing mechanisms. **(A)** Repartitioning is governed by Nernst Equation, which relates voltage differences to concentration gradients of a species. $\mathbf{E_{mem}}$ is the membrane voltage, **R**, the universal gas constant, **T**, the absolute temperature, **z**, the number of moles of charges transferred during the "reaction", and **F**, Faraday's constant. (B) Reorientation is a result of the electric field inducing a torque on the chromophore by acting on its dipole moment. τ, the torque, is equal to the cross product of the chromophores dipole, μ, with the electric field, $\mathbf{E_{mem}}$. **(E)** Electrochromism is a manifestation of the Stark effect. $\Delta\nu$ is the change in frequency of the electronic transition caused by differences in the permanent dipoles and induced dipoles, $\Delta\mu$ and $\Delta\alpha$, respectively, of the ground and excited states of the chromophore, with **h** being Planck's constant. **(D)** The rate of Förster resonant energy transfer, $\mathbf{k_{FRET}}$, is dependent on the inverse distance, as well as the strength of spectral overlap, between the two species, to the 6th power. Here $\tau_\mathbf{d}$ is the donor's lifetime, and $\mathbf{R_0}$ is the Förster distance. which depends on the orientation of the transition dipoles, κ, the donor's quantum yield of fluorescence, $\mathbf{Q_d}$, and the overlap of the donor's emission spectrum, $\mathbf{F_d}$, with the acceptor's absorption spectra, $\varepsilon_\mathbf{A}$. **N** is Avogadro's number, and **n** is the refractive index. Voltage changes can result in changes to **r**, and $\mathbf{R_0}$ **(E)** The rate of energy transfer in collisional quenching is exponentially dependent on distance, **r**, and is linearly proportional to the overlap of the donor's emission spectrum, $\mathbf{F_d}$, with the acceptor's absorption spectra, $\varepsilon_\mathbf{A}$. As with FRET, changing voltages can affect both **r** and the spectral overlap. **L** is the sum of the van der Waals radii of the donor and acceptor. **(F)** In SHG, the signal is proportional to $\mathbf{P^{(2)}}$, the second order polarization, and arises from the non-linear mixing of electric fields through $\chi^{(2)}$, the non-linear susceptibility of the system. To a first approximation, in the presence of the electric field across the membrane, $\mathbf{E_{mem}}$,$\chi^{(2)}_\mathbf{eff}$ is composed of two terms, the original $\chi^{(2)}$, and additional component, created by the mixing of the cell's electric field with the incoming light field, **E**, through $\chi^{(3)}$, the second order non-linear susceptibility.

A	Repartitioning	$E_{mem}=\frac{RT}{zF}\ln\left\{\frac{[dye\_outside\_membrane]}{[dye\_inside\_membrane]}\right\}$
B	Reorientation	$\tau=\boldsymbol{\mu}\times\mathbf{E}_{mem}$
C	Electrochromism	$\Delta\nu=\frac{1}{h}\Delta\mu E_{mem}-\frac{1}{2h}\Delta\alpha E_{mem}^2$
D	FRET	$k_{FRET}\propto\frac{1}{\tau_d}\bullet\left[\frac{\mathrm{R}_0}{r}\right]^6,\quad R_0^6=\frac{9000(\ln 10)\kappa^2 Q_d}{128\pi^5 N n^4}\int_0^\infty F_d(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$
E	Collisional Quenching	$k_{CQ}\propto J(\lambda)\exp\left[-2\cdot\frac{r}{L}\right],\quad J(\lambda)=\int_0^\infty F_d(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$
F	Second Harmonic Generation	$P^{(2)}(2\omega)\propto\chi^{(2)}:E\bullet E+\chi^{(3)}:E\bullet E\bullet E_{mem}$ $P^{(2)}(2\omega)\propto[\chi^{(2)}+\chi^{(3)}E_{mem}]E\bullet E$ $P^{(2)}(2\omega)\propto\chi^{(2)}_{eff}(\mathrm{E_{mem}})E\bullet E$