2 Design and Use of Organic Voltage Sensitive Dyes

Leslie M. Loew

2.1 HISTORY AND INTRODUCTION

The pioneering work of Lawrence Cohen in the mid-1970s led to the establishment of optical methods as a way to measure the electrical activity of large populations of cells either through a microscope or in bulk suspension where traditional microelectrode methods are not applicable. Cohen’s laboratory used an extensive and comprehensive assay of commercially available dyes on the voltage-clamped squid giant axon to screen for dyes with large optical responses to membrane potential changes (Cohen et al. 1974). This resulted in the identification of the merocyanine class of dyes as effective candidates for further refinement via alteration of both the chromophore and side chains (Gupta et al. 1981; Ross et al. 1977).

This laboratory joined the effort to develop potentiometric dyes by applying rational design methods based on molecular orbital calculations of the dye chromophores and characterization of their binding and orientations in membranes (Loew et al. 1978, 1979a). Several important general-purpose dyes have emerged from this effort including di-5-ASP (Loew et al. 1979b), di-4-ANEPPS (Flühler et al. 1985; Loew et al. 1992), di-8-ANEPPS (Bedlack et al. 1992; Loew 1994), and di-4-ANEPPDHQ (Fisher et al. 2008; Obaid et al. 2004). All of these dyes provide rapid absorbance and fluorescence responses to membrane potential and are therefore capable of recording action potentials. They have chromophores that are in the general structural category called hemicyanine or styryl dyes. The characteristics of this class of chromophores will be discussed in detail in the next section. In addition, we have developed TMRM and TMRE (Ehrenberg et al. 1988), which are slow responding dyes that are capable of measuring smaller changes in plasma membrane potential or mitochondrial potential via confocal imaging (Farkas et al. 1989; Loew 1993). But the primary focus of this chapter, in keeping with the theme of the book, is on fast dyes that are designed to image electrical activity in excitable cells. All of the above-mentioned dyes and several others from this lab are now available in the Molecular Probes/Invitrogen catalog, and some are also distributed by smaller companies.

The dyes have been of great utility to neuroscientists interested in mapping patterns of electrical activity in complex neuronal preparations with numerous examples spanning the past 20 years (Djurisic et al. 2003; Grinvald and Hildesheim 2004; Wu et al. 1998). Interestingly, our styryl dyes have been of increasing utility when microinjected in single cells (Antic et al. 2000; Antic 2003; Canepari et al. 2007, 2008; Djurisic and Zecevic 2005; Kampa and Stuart 2006; Milojkovic et al. 2005; Nishiyama et al. 2008; Palmer and Stuart 2009; Stuart and Palmer 2006; Zecevic 1996; Zhou et al. 2007, 2008) to follow patterns of electrical activity along very thin dendrites where direct electrical recording is precluded. Interestingly, the dyes developed in our laboratory, primarily di-4-ANEPPS, have also become the standard for optical mapping of electrical activity in studies of cardiac activity (Efimov et al. 2004; Loew 2001).

More recently, we have synthesized voltage-sensitive dyes (VSDs) with new hemicyanine chromophores that have absorbance and emission further toward the red end of the visible spectrum (Kee et al. 2008; Matiukas et al. 2006, 2007; Wuskell et al. 2006; Yan et al. 2008; Zhou et al. 2007). These dyes are often called “blue dyes” because by absorbing long-wavelength red light they appear blue. The longer wavelength absorbance and emission properties of these dyes make them useful for experiments where absorbance by endogenous chromophores, notably hemoglobin, needs to be avoided. Long wavelength also permit recording from deeper within tissue because light scattering, which limits the depth of optical penetration, is dependent on inverse fourth power of the wavelength.

There are several other labs that are currently actively working on synthesizing new potentiometric dyes. Rina Hildesheim has been working for about 25 years in the laboratory of Amiram Grinvald and has synthesized hundreds of dyes. The latest dyes to emerge from these efforts are RH1691 and RH1692, oxonols that absorb at around 630 nm (Derdikman et al. 2003; Shoham et al. 1999; Slovin et al. 2002). They have been successfully used for in vivo studies on awake animals. The relative response (ΔF/F) to electrical activity in mammalian brains for these dyes is ca. 10⁻³, but it is hard to compare this to other dyes as no controlled voltage clamp studies have been reported on isolated cells or membranes. The dyes have become available through Optical Imaging, Inc., a company founded by Dr. Grinvald. The laboratory of Peter Fromherz has long been interested in the photophysical properties of the hemicyanine chromophores (also called “styryl” dyes) that have been developed in this lab. Recently, they have developed a new chromophore series, the “ANINE-s” that are hemicyanines enclosed in a completely rigid annelated ring framework. ANINE-6 has been particularly promising (Kuhn and Fromherz 2003; Kuhn et al. 2004).
and a new version, ANINE-6P, has improved solubility so that it may be more readily used in neuroscience applications (Fromherz et al. 2008). They are both excited at shorter wavelength than d-4-ANEPPS. Dr. Fromherz was kind enough to provide us with a sample of both dyes; in some combined Second Harmonic Generation (SHG) and 2-photon-excited fluorescence (2PF) experiments on voltage-clamped cells, they show sensitivities similar to d-4-ANEPPS, but lower than the high responses we get from our new long-wavelength chromophores. However, a full characterization on our hemispherical bilayer apparatus was not possible with the quantities provided. It should also be mentioned that a recent paper (Salama et al. 2005) representing a collaboration between Alan Waggoner and Guy Salama in Pittsburgh reports on some cardiac experiments with probes that use the same longer wavelength chromophores that we have developed over the last several years.

An exciting new approach incorporates green fluorescent protein into engineered channel proteins (Ataka and Pierbone 2002; Guerrero et al. 2002; Sakai et al. 2001; Siegel and Isacoff 1997). This approach shows great promise because of the specificity with which the constructs can be targeted to specific cells or subcellular regions. However, these probes have been either too slow or too insensitive to be practical alternatives to the organic potentiometric dyes and they are generally not properly inserted into the plasma membrane (Baker et al. 2007). Most recently, however, a significant improvement has been reported for a construct that does insert into the plasma membrane of cultured cells (Dimitrov et al. 2007) and produces reasonable sensitivities of 2%/100 mV and a time constant for response to potential steps of 20 ms. More recently, a promising fluorescence resonance energy transfer (FRET)-based dual fluorescent protein sensor has been developed with a reported 40% sensitivity/100 mV (Tsutsui et al. 2008). Whether these proteins can be targeted to specific cells in transgenic mice and whether they can continue to be improved to become practical tools for mapping potential remains to be seen. This technology will not, however, replace dyes for either the single cell microinjection experiments or for global mapping of all neurons in a macro view experiment. It is also worth pointing out that although fluorescent proteins for calcium sensing have been available for many years and continue to be improved, they are still too insensitive to have replaced dyes in the overwhelming majority of neuroscience and cardiac experiments. Our plans to improve the photostability and delivery of the potentiometric dyes should enable an even greater assortment of experiments for mapping electrical activity that will surely complement the studies that may be enabled by fluorescent protein voltage sensors.

### 2.2 MECHANISMS OF DYE OPTICAL RESPONSES TO CHANGES IN MEMBRANE POTENTIAL

It is important to appreciate how VSDs work as voltage sensors in order to apply them intelligently to different experimental situations. The dyes all respond by some change in their spectral properties in response to a change in membrane potential, so a prerequisite to understanding the possible mechanisms by which they can respond, is an understanding of the physical chemistry underlying the absorption and emission of a photon.

The core of any dye is the chromophore – the portion of the molecule that actually interacts with the light. The absorption of a photon is possible when the difference in energy between the ground state of the chromophore and an excited state matches the energy of the photon, which is given by \( \frac{h}{\lambda} \), Planck’s constant divided by the wavelength of the light. The range of wavelengths that can excite the molecule is broad because of the existence of vibrational sublevels within the chromophore’s electronic states and, importantly, a range of possible interactions of the chromophore with its molecular environment. Once the molecule is promoted to an excited electronic state, it immediately relaxes to the lowest energy through vibrational relaxation processes. But it stays in this vibrationally relaxed, electronically excited, state for some time, usually a few nanoseconds, before losing its energy either by emitting a photon (fluorescence) or through heat to regenerate the ground state. The fact that the excited state has a chance to relax before it emits a photon, possibly adopting a conformation that is most stable for the state’s distribution of electrons and reorganizing the surrounding molecules to the most stable configuration, invariably produces a longer wavelength (lower energy) fluorescence emission spectrum compared to the band of wavelengths that is used to excite a given chromophore. It is also possible for the excited state to undergo a chemical change which would destroy the chromophore; this is generally known as bleaching. It is possible to engineer dyes so that an electric field can either interact with the chromophore’s electron distribution directly or cause the dye to change its environment. For such dyes, a change in membrane voltage can alter the absorbance or fluorescence spectrum. Good VSDs have chromophores that are bright (efficient excitation and emission), are highly sensitive to environment, and are photostable (i.e., resistant to photobleaching).

There are three established mechanisms by which dyes can respond to action potentials because of a change in their molecular environment: ON–OFF, reorientation, and FRET. The ON–OFF, reorientation, and FRET mechanisms all involve a change in the location of a charged dye as a result of the changing membrane voltage (Fig. 2.1). For the ON–OFF mechanism, the dye moves from the aqueous extracellular medium to the cell membrane; because of their environmental sensitivity, the dyes will typically display a substantial increase in fluorescence upon association with the membrane and this is how the voltage change becomes transduced to a fluorescence change. Dyes with cyanine and oxonol chromophores often utilize this mechanism (Waggoner et al. 1977). A problem with this mechanism is that although the sensitivity is large, the response time of the system is often too slow to record action potentials. In the reorientation mechanism, the changing electric field within the membrane causes a membrane-bound dye to flip from an orientation perpendicular to the cell surface to an orientation where its long axis is parallel to the surface. Again therefore, the molecular environment of the dye is changed and this produces a change in the spectral properties; moreover, there is also a change in the average orientation of the dye molecules with respect to the propagation direction of the exciting light and this can also produce a change in the efficiency of light absorption. Merocyanine dyes have been shown to often utilize this mechanism (Dragsten and Webb 1978). This mechanism can be very fast, but the sensitivity can be low and quite variable from preparation to preparation. In the FRET mechanism, a donor fluorophore is anchored to the outer surface of the cell membrane and transfers its energy to nearby acceptor chromophore which then emits fluorescence at longer wavelength. If the acceptor is a negatively charged membrane-permeant dye, it will redistribute to the inner surface of the bilayer when the membrane depolarizes, thus reducing FRET and reducing the long-wavelength emission. This idea was demonstrated for a coumarin-conjugated lipid donor and a permeant oxonol acceptor (Cacciatore et al. 1999; Gonzalez and Tsien 1997). The sensitivity can be high for this mechanism, but the requirement for the application of two dyes at relatively high concentration has impeded its widespread adoption. In all three of the cases...
illustrated in Fig. 2.1, the voltage change has to tip the equilibrium balance between two states of the dye molecule resulting in the movement of the dye to a new environment. Since the intrinsic properties of membranes are themselves quite variable and can therefore affect the equilibrium and/or the kinetics of dye relocation, a dye that is sensitive to potential in one preparation or set of experimental conditions may be completely ineffective in another.

It would be preferable to have a mechanism which produces dyes with sensitivities that are more robust and reliable from one experimental situation to another. In pursuit of this goal, we have concentrated on producing dyes with chromophores that interact directly with the membrane electric field by an electrochromic mechanism (often referred to as a molecular Stark effect). The idea is explained in Fig. 2.2, which uses the results of molecular orbital calculations and qualitative resonance structures to demonstrate how a dye which has an electron rich $\pi$-system on one end and an electron deficient $\pi$-system on the other can switch its electron distribution upon absorption of a photon to produce the excited state. di-4-ANEPPS, the most popular of the VSDs to emerge from our work, provides a specific example. The chromophore of this dye changes its electron configuration upon excitation such that the charge shifts from the pyridinium nitrogen in the ground state to the amino nitrogen in the excited state. This behavior is predicted from molecular orbital calculations (Loew et al. 1978) such as the results shown at the top of Fig. 2.2. A polar group is appended to one side of the chromophore in the form of a propylsulfonate moiety and two hydrocarbon chains are included at the opposite end. This, together with the intrinsic amphiphilicity of the chromophore, serves to anchor it in the membrane bilayer in an orientation that is approximately perpendicular to the surface. This orients the direction of the excitation-induced charge motion parallel to the electric field vector within the membrane, as depicted in the upper diagram of Fig. 2.3, where the dye is shown as if having been inserted from the outside of the cell. As a consequence, the ground and excited states are differentially stabilized by the intramembrane electric field, causing a shift in the spectrum when the membrane potential changes (bottom of Fig. 2.3). An equivalent way of thinking about this is to realize that the excitation-induced charge displacement moves along the direction of the electric field when the membrane is depolarized. Experiments from voltage-clamped bilayer membranes (Fluhler et al. 1985; Loew et al. 1979a; Loew and Simpson 1981) have provided evidence that di-4-ANEPPS and related hemicyanine dyes do respond to membrane potential via an

**FIGURE 2.1.** Mechanisms used by VSDs that can change their location in response to membrane depolarization.

**FIGURE 2.2.** Electrochromic mechanism of voltage sensitivity. The top shows how the electrons, and therefore the charge distribution, shifts upon excitation of a typical electrochromic dye. These images were generated from molecular orbital calculations where low electron density (i.e., regions of positive charge) are represented by blue shades and high electron density (i.e., negative charge) is represented by redder colors. The lower portion of the figure shows resonance structures for the ground and excited states of one of the most widely used VSDs, di-4-ANEPPS. In this chromophore, the donor moiety is an aminonaphthyl group, the linker is a simple double bond and the acceptor is a pyridinium moiety.
and nonlinear optical imaging of membrane potential. focusing on fluorescence, but then briefly discussing absorbance will describe how the properties of the VSDs should be considered, the speed and sensitivity of the instrumentation. In this section, we this book will describe the considerations required for optimizing ability of fluorescence and confocal microscopes. Other chapters in

The voltage-dependent shift in the excitation or emission spectrum to be expected from electrochromic dye is depicted in Fig. 2.3, where the dye is assumed to be bound to the extracellular surface of the membrane and undergo charge redistribution in its excited state in the direction shown in Fig. 2.2. Under these circumstances, the spectra shift to shorter wavelength upon membrane depolarization. The magnitude of the spectral shift will be linearly related to the change in potential. In most experiments using VSDs, a fixed band of wavelengths is continuously monitored, rather than attempting to scan the entire spectrum. Figure 2.3 shows that the choice of wavelength can have a drastic effect on the observed sensitivity of the dye response. Contrary to what might be an experimentalist’s first impulse, the wavelengths of maximal absorbance or emission are actually the worst choices for achieving optimal sensitivity. The best sensitivity is achieved at the wings of the spectra and will show changes in opposite directions at the high and low wavelength wings. Furthermore, measuring the change in the fluorescence relative to total fluorescence signal, \( \Delta F/F \), biases the optimal wavelengths even further to the extreme edges of the excitation and emission spectra because \( F \), the denominator, decreases steadily at the edges. An approximately linear relationship between the voltage change and the fluorescence or absorbance change is obtained at the wings because the local slope of the spectrum is approximately constant, at least until it starts flattening out toward the very high and low ends of the spectrum.

Another important consideration in choosing wavelengths, which mitigates against going too far out to the edges of the spectrum, is the signal-to-noise ratio (S/N). Essentially, the detection system must collect a sufficient number of photons per measurement so as to be able to determine the small modulation by the voltage with statistical reliability. For fluorescence measurements, the number of photons can be boosted by simply utilizing an intense excitation source; it should then be possible to pick wavelengths at the edge of the absorbance spectrum, where \( \Delta F/F \) is maximal, without sacrificing signal. This is because the low absorbance at the spectral edge can be compensated by a higher intensity light source. This will permit a sufficient number of excitation events to occur in a sufficiently short time to be able to record action potentials. But a key consideration that prohibits applying this argument with respect to the edge of the emission spectrum is the limitation imposed by photobleaching. By detecting only a narrow band at the emission spectrum edge, most of the emitted photons will be lost, effectively wasting most of the excitation events. It would then be more probable that the dye will bleach before collecting sufficient photons for a good S/N. Therefore, the best compromise between \( \Delta F/F \) and S/N is to use an intense narrow band excitation source at the edge of the VSD absorbance spectrum, while collecting the emitted light through a filter that will pass all wavelengths longer than the emission spectrum maximum.

We and others have also taken advantage of the opposite changes in the voltage-dependent optical signals at the low and high wavelength wings of the spectra (Fig. 2.3) to implement a dual wavelength ratio detection scheme for measuring membrane potential with VSDs (Beach et al. 1996; Bullen et al. 1997; Knisley et al. 2000; Montana et al. 1989). The idea is to collect light from both wings of the spectrum and calculate the ratio of their fluorescence intensities as a measure of the voltage change. The primary advantage is that the sensitivity of the ratio is approximately equal to the additive absolute sensitivities at each wavelength. A second advantage is that the dual wavelength ratio can, in principle, be used to report the absolute value of the intramembrane electric field. A single wavelength fluorescence measurement can only report a relative change in potential because the intensity depends

\[ \lambda_H - \lambda_D \propto E \]

FIGURE 2.3. The shift in the spectrum of an electrochromic dye upon depolarization. The upper panel shows how the electric field in the membrane, \( E \), can perturb the spectrum of an electrochromic chromophore, represented by the stippled rectangles embedded in the outer surface of a membrane. The spectra shown in the lower half of this figure can represent absorbance, excitation, or emission spectra. The relative change in fluorescence, \( \Delta F/F \), is shown as a percentage change for different choices of detection wavelengths.

2.3 MEASURING VOLTAGE CHANGES WITH VOLTAGE-SENSITIVE DYES

A number of modalities are available for measuring voltage-dependent optical changes from excitable cells and tissues. The most common is fluorescence. This is advantageous because of the high contrast and low background intrinsic to this technique and the wide availability of fluorescence and confocal microscopes. Other chapters in this book will describe the considerations required for optimizing the speed and sensitivity of the instrumentation. In this section, we will describe how the properties of the VSDs should be considered, focusing on fluorescence, but then briefly discussing absorbance and nonlinear optical imaging of membrane potential.
on the level of staining, which can be quite variable even along the membrane of a single neuron. The ratio effectively normalizes away any differential staining levels because the fluorescence intensity at both wavelengths will be proportional to dye density but oppositely responsive to intramembrane electric field. We introduced the idea of dual excitation wavelength imaging (Montana et al. 1989) and showed that it could be used to map the membrane potential along a neuron induced by an external electric field (Bedlack et al. 1992). Importantly, because the dye reports on the local electric field, any variations in dipole potential or surface potential may also produce variations in the ratio (Bedlack et al. 1994; Gross et al. 1994; Xu and Loew 2003); therefore, any spatial variations in ratio must be interpreted with caution and may not reflect a variation in transmembrane potential. On the other hand, fast temporal variations in the ratio are likely to reflect changes in transmembrane potential because the changes in lipid composition that would be required for changes in other sources of intramembrane electric field would be slow on the timescale of action potentials. A dual emission wavelength measurement is preferred for rapid spatial mapping of electrical activity because two fixed detection paths can be employed (Bullen et al. 1997; Bullen and Saggau 1999) rather than mechanically switching between two excitation wavelength filters. In general, dual wavelength ratiometric measurements require more complex instrumentation and analysis than simply monitoring $\Delta F / F$, so the adoption of this approach has been limited to experiments that require extracting a voltage-dependent signal from other confounding variables.

A commonly used alternative to fluorescence for optically monitoring electrical activity is to record or image the light transmitted through a specimen stained with a VSD (Glover et al. 2008; Sasaki et al. 2002). A change in the transmittance of a stained preparation simply reflects the change in the absorbance spectrum of the dye. Of course, the transmitted light corresponds to the light that actually does not get absorbed by the dye. The amount of light that is absorbed by a single dye-stained membrane depends on the size of the cell, the surface density of the dye molecules on the membrane, and the extinction coefficient of the dye at the chosen wavelength. But it can be estimated that this will never exceed 1 part in $10^3$ for even the largest cell. The modulation of the transmitted light signal by an action potential would therefore never be much greater than ~1 part in $10^3$. A comparable estimate for the modulation of fluorescence is 1 part in 10, because the entire light signal emanates from the stained membrane. So if $\Delta T / T$ is intrinsically so much lower than $\Delta F / F$, why would anyone prefer to use transmitted light for optical electrophysiological recording? Indeed, the technique is almost never used for recording from single cells. In experiments where the collective activity of a large population of cells in a tissue needs to be imaged, the sensitivity of the technique is increased because more of the incident light is absorbed. Furthermore, the $S/N$ in a transmitted light detection is not limited by photon statistics so simpler and less noisy photodetectors can be used.

Another approach that is just beginning to emerge for VSDs is 2PF (Fisher et al. 2008; Kuhn et al. 2008; Loew et al. 2002). There is really no difference in principle in the way that 1- and 2-PF measure the dye response, except, of course, that the 2-photon modality requires a femtosecond pulsed laser operating at twice the wavelength and a microscope configuration that is optimized for the purpose (Denk et al. 1990). The major advantage of 2PF is the ability to probe deep inside a specimen with high 3D resolution. The disadvantage, in addition to complex instrumentation, is that the laser scanning required to obtain a full image of the specimen is too slow for recording of action potential activity; therefore, line scans or special spatial sampling protocols are used to record optical signals from a small number of sites. Chapter 11 in this book is devoted to two-photon microscopy of VSDs.

Second harmonic generation is another nonlinear optical process (Campagnola and Loew 2003; Millard et al. 2003a) that can take place in a microscope coupled to an ultrashort laser. As in the case of two-photon excitation the probability of SHG is proportional to the square of the incident light intensity. While 2PF involves the near-simultaneous absorption of two photons to excite a fluorophore, followed by relaxation and noncoherent emission, SHG is a nearly instantaneous process in which two photons are converted into a single photon of twice the energy, emitted coherently. Furthermore, SHG is confined to loci lacking a center of symmetry; this constraint is readily satisfied at cellular membranes in which SHG-active constituents are unevenly distributed between the two leaflets of the lipid bilayer. The first examination of the nonlinear optical properties of VSDs was in a collaboration between this lab and Aaron Lewis’ lab, where we analyzed SHG from a monolayer of di-4-ANEPPS in a Langmuir–Blodgett trough (Huang et al. 1988). Subsequently (Ben-Oren et al. 1996; Bouevitch et al. 1993; Campagnola et al. 1999), we were able to show that the SHG signal from electrochromic dye stained membranes is sensitive to membrane potential. These experiments were continued (Clark et al. 2000; Millard et al. 2003b, 2004, 2005a, b; Teisseyre et al. 2007) in an attempt to characterize and optimize the SHG response of our dyes to membrane potential. They indicated that the mechanism could not be explained as a direct electrooptic effect because the kinetics of the response for some of the dyes was slow, although these were not always the conclusions reached by others (Jiang et al. 2007; Pons et al. 2003). Whatever the mechanism, the laboratories of Rafael Yuste, Kenneth Eisenthal, and Watt Webb (Araya et al. 2006, 2007; Dombek et al. 2004, 2005; Nuriya et al. 2006) were able to demonstrate the measurement of action potentials using second harmonic imaging microscopy. These studies used the hemicyanine dye FM-464 applied to the interior of the cell through a patch pipette. Because the SHG can only come from dye that is noncentrosymmetrically distributed on the spatial scale of the wavelength, the light emanates only from the internally stained plasma membrane and no signal appears from internally stained organelles with highly convoluted membranes such as the endoplasmic reticulum and mitochondria. Because of this elimination of background from these internal electrically inactive membranes, the voltage sensitivity of the SHG signal can approach 20%/action potential – much higher than is practically possible with fluorescence. However, these measurements had very low $S/N$ and required extensive signal averaging because of the low intensity of the SHG. Therefore, it is not clear how generally useful this modality will ultimately be until new dyes are developed with much stronger SHG efficiencies. Chapter 13 provides a thorough review of membrane potential measurements with SHG.

2.4 CHOOSING THE BEST DYE FOR AN EXPERIMENT

Many of the hemicyanine dyes synthesized in our and other labs are capable of sensing membrane potential changes with high sensitivity. But different experimental designs require different spectral properties, solubility properties, and membrane binding affinities. Furthermore, competing processes can sometimes diminish the sensitivity of a given dye in a particular experimental preparation. So there is a need for a large repertoire of dyes to meet these varying needs and this section will consider how to make these choices. We focus on dyes developed in this laboratory as exemplars, but the same principles can be applied to the dyes developed by others.
A prerequisite for choosing a dye is simply being able to appreciate the structural variety of VSDs and establishing some nomenclature that permits us to refer to them in a recognizable way. As discussed in Sect. 2.2, the hemicyanine chromophores are all characterized by one end that is electron rich in the ground state, also called an electron donor moiety, and another end that is electron deficient, an electron acceptor moiety (Fig. 2.2). When these groups are linked with a \( \pi \)-electron linker, it is possible for the electrons to readily shift in response to excitation. Chromophores with such electronic structures are commonly called “push-pull” chromophores because of the complementary tendencies of the electrons at the two ends. Figure 2.4 lists all of the donor, linker, and acceptor moieties that we have employed in our lab to develop push-pull chromophores. We have synthesized approximately 300 dyes utilizing over 50 of these push-pull hemicyanine chromophores. The R1 groups on the donor ends are usually hydrocarbon chains that anchor the dye to the hydrophobic interior of the membrane. The R2 groups on the acceptor ends are usually hydrophilic groups that protrude into the aqueous medium adjacent to the membrane and help to maintain the orientation of the chromophore perpendicular to the membrane surface (cf. Fig. 2.3). The laboratories of Amiram Grinvald and Alan Waggner have also developed large numbers of VSDs using some of these groups. Table 2.1 provides the spectral characteristics of most of the chromophores constructed from these parts in ethanol, water, and when bound to lipid vesicle membranes. An important attribute of almost all of these dyes is that they have strong fluorescence quantum efficiencies in the membrane bound forms, but typically at least two order of magnitude lower fluorescence in aqueous solution. This means that the background signal from unbound dye can usually be completely neglected even if the preparation is allowed to remain exposed to the aqueous staining solution. For the reasons discussed in Sect. 2.3, the optimal wavelengths for voltage sensitivity are typically ~50 nm above the maximum wavelengths reported for the lipid spectra in Table 2.1.

The systematic chemical names for these compounds are extremely cumbersome because of their complexity. Therefore, the field has adopted different styles of abbreviation. Most commonly, a dye is identified by the initials of the chemist who synthesized it followed by a number. For example JPW-3080 was synthesized by Joseph P. Wuskell and its synthesis was first recorded on page 080 of his third notebook. Similarly, RH-160 is dye number 160 prepared by Rena Hildesheim in Amiram Grinvald’s lab. Of course these abbreviations do not convey much information about the properties of the dyes and we have attempted to formulate more descriptive abbreviations for the more common hemicyanine VSDs. The general formulation we have adopted uses the following scheme:

\[
di-n-DLAH
\]

This provides a designation for their structural components as follows: alkyl chain lengths (\( n \)) – \( \pi \)-Donor – Linker(s) – \( \pi \)-Acceptor – hydrophilic Head group (Wuskell et al. 2006). A pair of alkyl chains, R1, is appended to the amino terminus of most of dyes (right side of each structure in Fig. 2.4). The number of carbons in these chains is indicated by \( n \), in the “Di-\( n \)” portion of the naming scheme. The \( \pi \)-donor moiety, D, can be aminophenyl (AP, -1- in Fig. 2.4), aminonaphthyl (AN, 2- in Fig. 2.4), etc. The linker, L, is ethene (E, -1- in Fig. 2.4) diene (D, -2-), ethene-furan-ethene (EFE, -7-), etc. The acceptors, A, can be pyridinium (P, -1), quinolinium (Q, -3) indolenium (In, -5), etc. Finally, we have dyes with various head groups, H, the most common of which are shown in Fig. 2.5. Thus, di-4-ANEPPS in Fig. 2.2 has a pair of butyl groups (\( n = 4 \)) attached to the amino group of an aminonaphthyl donor (D = AN), which is linked via an ethene linker (L = E) to a pyridinium (A = P) acceptor, which is appended with a propylsulfonate (H = PS) head group.

The chromophore determines the optical properties of the VSD (Table 2.1) and also its intrinsic voltage sensitivity. We screen every new dye using a voltage-clamped hemispherical bilayer apparatus (Fluhler et al. 1985; Loew and Simpson 1981; Wuskell et al. 2006). From these experiments, as well as experience in other preparations
including squid axon and lobster nerve (Loew et al. 1992; Wuskell et al. 2006), the chromophores with the best intrinsic voltage sensitivity are (DLA): APEP, ANEP, ANEQ, ANBDQ, APEThEQ, ANEThEQ, ANBDIn, APEThEIn, and ANEThEIn. These chromophores span a range of over 200 nm in optimal excitation and emission wavelengths. In particular, the more recently developed long-wavelength dyes also avoid those of light-activated proteins used to control neuronal activity, such as channelrhodopsin-2 and halorhodopsin (Zhang et al. 2007), making it possible to combine the use of VSDs with such proteins. But whether a given VSD is successful in a specific experimental application also depends critically on the side chains and head groups.

The choice of alkyl chains provides direct control of the solubility and membrane binding characteristics. Short alkyl chains impart good water solubility at the expense of strong membrane binding;
this would be appropriate for staining a thick tissue where the dye needs to penetrate many cell layers. However, VSDs with greater solubility can also be more readily washed out of a preparation. We have found that longer R1 alkyl chains increase the strength of binding of the dyes to the membrane and also slow the rate of internalization. Internalization is detrimental to the potentiometric response because as the dye equalizes on either side of the bilayer the voltage-dependent spectral shifts from the two leaflets will cancel each other (cf. Fig. 2.2). However, long side chains decrease the solubility of the dye, making staining slow and inefficient and impractical for thick tissue. This problem can be ameliorated through the use of vehicles such as Pluronic F127 (Lojewska and Loew 1987) or γ-cyclodextrin (Wuskell et al. 2006), which form complexes with the dyes to keep them disbursed in solution.

With respect to R2, the negatively charged sulfonate head groups (PS and BS in Fig. 2.5) are attractive because they provide internal counter ions for the positive charge of the chromophore, resulting in an overall neutral VSD. These are less likely to interact and possibly interfere with other cellular components. The positively charged head groups in Fig. 2.5 provide some additional water solubility and can therefore partially offset the solubility lowering effect of moderately sized R1 side chains. Indeed, di-3-ANEPPDHQ and di-4-ANEPPDHQ have proven to be excellent general purpose dyes for staining of multicellular preparations (Obaid et al. 2004). Similarly, several of the new long-wavelength dyes with positive head groups can be used for labeling for recording of large neuronal populations in brain slices (di-2-ANBDQPO in Fig. 2.6) (Kee et al. 2008). An important finding for the doubly positively charged VSDs with the positive head groups is that, given enough time, they can become internalized and will be retained in the cell interior because of the polarized resting potential. This has led to applications where long R1 side chain dyes with the PQ head group are applied in an ethanol solution to cause the dyes to precipitate out and remain localized in a confined anatomical region. They then can become internalized and be transported over a period of hours by retrograde flow or diffusion to regions of the neurons remote from where they were originally stained. This strategy was first employed with di-8-ANEPPQ and di-12-ANEPEQ where the dye was applied to chick spinal cord to ultimately label and record from remote neuronal cell bodies, dendrites, and axons (Tsau et al. 1996; Wenner et al. 1996). When the positively charged head groups are combined with R1 chains of just one or two carbons in length, the resulting VSDs are extremely water soluble, often at a solubility of several mg/ml. This is important for applications where the dyes must be applied internally to a single cell through a patch pipette. Such experiments are designed to allow imaging of membrane potential from different regions of a single neuron in a brain slice or in vivo (Antic et al. 1999; Antic and Zecevic 1995; Antic 2003; Canepari et al. 2007; Milojkovic et al. 2005; Palmer and Stuart 2009; Stuart and Palmer 2006; Zhou et al. 2007, 2008). For example, Palmer and Stuart (2009) have been able to image membrane potential from...
2.5 HOW CAN BETTER DYES EXPAND THE SCOPE OF POSSIBLE EXPERIMENTS?

One major area for improvement is dye photostability. Obviously, more photostable dyes will permit longer duration recording experiments; but as importantly, they will permit high excitation intensities with a resultant higher emitted photon flux. This is important because the \( S/N \) for recording fast electrical signals is limited by the stochastic shot noise of the number of photons that can be collected – the \( S/N \) is proportional to (photon count)\(^{1/2} \). Higher dye brightness (the product of extinction coefficient and fluorescence quantum efficiency) will also help in the same way.

Greater sensitivity of the optical signal to voltage changes would expand the range of applications for VSDs. We have produced dyes that can give tenfold or more fluorescence changes for a 60 mV change in potential (Ehrenberg et al. 1988; Loew et al. 1983), but these dyes depend on a voltage-dependent redistribution across the membrane that renders the response too slow to follow an action potential. Fast dyes necessarily have much smaller voltage sensitivities. The best of our fast dyes, when excited at the spectral edge, can produce relative fluorescence changes of \(-40\%/100\) mV. Frankly, we do not anticipate much improvement in this sensitivity, although improved instrumentation can further stretch the range of experiments accessible to the technology. On the other hand, SHG represents a relatively unexplored optical modality that could produce much greater sensitivity than fluorescence and this is the rationale for our continued interest in developing this technique despite the \( S/N \) limitation. Our reasoning is that by resolving the mystery of how SHG responds to potential, we should be able to use this understanding to significantly improve the already high sensitivity by optimizing the design of the dyes and the optics.

Dyes, light sources, and optical modalities are required to permit deeper penetration into thick tissue or in vivo imaging. Longer wavelength dyes will allow for deeper penetration because their spectra are removed from interference by endogenous chromophores and because light scattering diminishes as the inverse of the fourth power of wavelength. Nonlinear optical modalities like 2PF and SHG will allow deeper optical sectioning if they can be implemented with longer wavelength ultrafast pulsed lasers.

One major area for future chemistry is to devise methods for more specific delivery of the dyes to individual, preferably multiple, cells in a complex preparation. Indeed, this is one of the main motivations for the intense activity in trying to devise a genetically encodable fluorescent protein voltage sensor. But by combining engineered organic dyes, photochemistry, and new optical technologies, it should be possible for chemists to devise general solutions to this challenge as well.

ACKNOWLEDGMENT

I am indebted to the many talented chemists, microscopists, and neuroscientists who have collaborated with me and who have carried out much of the research summarized in this chapter. Most notably, I wish to acknowledge my long-term collaborators Larry Cohen, Aaron Lewis, Mei-de Wei, and Joe Wuskell. The more recent work in my lab has benefited from collaborations with Ping Yan and Srđjan Antic. This work was supported by NIH EB001963.

REFERENCES


Membrane Potential Imaging in the Nervous System
Methods and Applications
Canepari, M.; Zecevic, D. (Eds.)
2011, X, 168 p. With online files/update., Hardcover
ISBN: 978-1-4419-6557-8