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Potentiometric dyes: Imaging electrical activity of cell membranes

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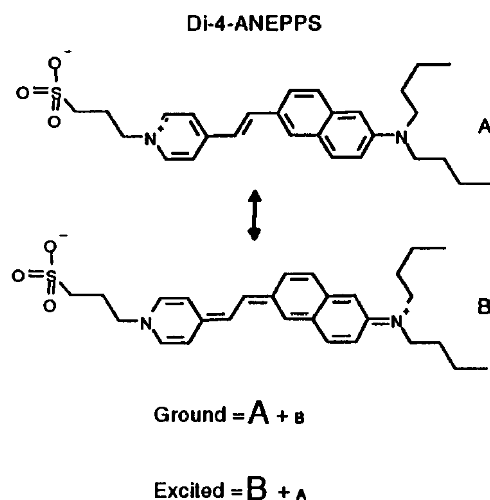
Abstract. Voltage sensitive dyes offer the opportunity to monitor neuronal electrical activity where microelectrode measurements are unsuitable or inadequate. This technology is especially powerful for the study of patterns of activity in complex multicellular preparations. It also makes possible the measurement of spatial and temporal variations in membrane potential along the surface of single cells. The fast dyes which have been developed in this laboratory are in the structural class called styryl or naphthylstyryl. These are amphiphilic membrane staining dyes which usually have a pair of hydrocarbon chains acting as membrane anchors and a hydrophilic group which aligns the chromophore perpendicular to the membrane/aqueous interface. The chromophore is believed to undergo a large electronic charge shift as a result of excitation from the ground to the excited state and this underlies the putative electrochromic mechanism for the sensitivity of these dyes to membrane potential. We have used these dyes to map the membrane potential along the surface of single cells with high resolution digital imaging microscopy techniques. These compounds also display remarkable efficiencies for second harmonic generation. Further, the second harmonic signal can be generated from a dye-stained membrane and is modulatable by changing the membrane voltage.

The development of potentiometric dyes has been motivated by the needs of neuroscientists to monitor neuronal electrical activity where microelectrode measurements are unsuitable or inadequate. This technology is especially powerful for the study of patterns of activity in complex multicellular preparations. It also makes possible the measurement of spatial and temporal variations in membrane potential along the surface of single cells. The dye technology may be suitable for suitable for other applications including photonics and laser technology and it is hoped that this paper will bring the availability of these compounds to the attention of a broader scientific audience.

Many of the fast dyes which have been developed in this laboratory are in the structural class called styryl or naphthylstyryl. These are amphiphilic membrane staining dyes which usually have a pair of hydrocarbon chains acting as membrane anchors and a hydrophilic group which aligns the chromophore perpendicular to the membrane/aqueous interface. The chromophore is believed to undergo a large electronic charge shift as a result of excitation from the ground to the excited state and this underlies the putative electrochromic mechanism for the sensitivity of these dyes to membrane potential (1,2).

The concept of electrochromism and how it can lead to a potentiometric dye is illustrated in Figure 1. According to our molecular orbital calculations (3,4) the naphthylstyryl pyridinium chromophore has most of its positive charge localized near the pyridinium nitrogen in the ground state and near the arylamino nitrogen in the excited state. When a hydrophilic moiety is attached to one end and a pair of hydrocarbon chains to the other, as illustrated for the dye di-4-ANEPPS, the molecule intercalates readily among the amphiphilic lipid molecules in a biological membrane and the chromophore is oriented perpendicular to the membrane surface. This orientation assures that the excitation induced charge redistribution will occur parallel to the electric field within the membrane. A change in the voltage across the membrane will therefore cause a spectral shift resulting from a direct interaction

between the field and the ground and excited state dipole moments. The electric fields in a membrane amount to ca. 10^5 V/cm and the difference between ground and excited state dipoles is about 15 Debye. The result is a shift in the absorbance or fluorescence spectra of just a few nm. But this has been sufficient for many applications of the dyes to biological problems.



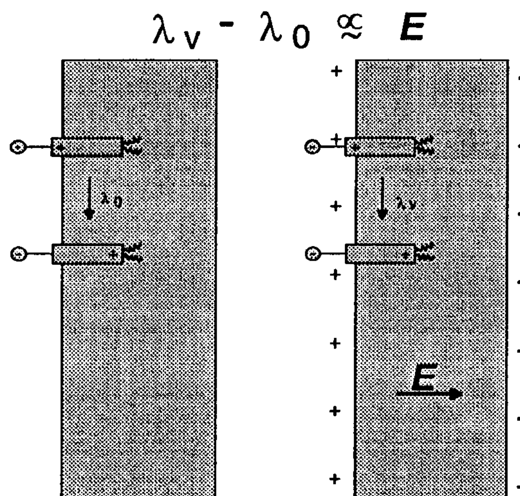


Figure 1. Principals for the design of an electrochromic dye for sensing membrane potential. The chromophore undergoes a large change in dipole moment upon excitation. It is oriented in the membrane so that this changing dipole is parallel to the intramembrane electric field.

It is important to understand that the diverse biological preparations and applications of the potentiometric dye technology require a large repertoire of dyes tailored to each preparation and experimental protocol. We had originally hoped that it might be possible to design a universal potentiometric dye (3) and this goal has been partially realized with the compound di-4-ANEPPS (5). However, it has become clear that the variable physical/chemical characteristics of biological preparations and the optical instrumentation used to study them in different laboratories have belied the practicality of this goal. A large number of dyes have been synthesized in this laboratory (6,7) as well as in the laboratories of Alan Waggoner and Amiram Grinvald (8-11); many of these are commercially available, mainly through Molecular Probes, Inc. There is a continuing need, however, to customize fast potentiometric dyes to the needs of individual experiments. It also follows that it is necessary to have an appreciation of the physical/chemical properties of the existing dyes in order to properly choose and use them for multisite optical recording or digital imaging experiments. Table 1 depicts some of the variations on the naphthylstyryl dyes that have been designed to meet specific needs.

In general, the sensitivities of a potentiometric dye to changes in membrane potential varies from preparation to preparation. For dyes with potential-dependent absorbance changes, the magnitude of the relative change in the optical signal depends on the degree of staining and the thickness of the preparation, so it is difficult to devise a figure of merit. The situation is somewhat better for fluorescence since the entire optical signal usually arises from stained membrane and a relative change may be more meaningful; but even for fluorescence, the signal may be contaminated by fluorescence from non-excitable cells that have been also stained in the preparation. L.B. Cohen has suggested the signal to noise ratio (S:N) in the squid axon preparation as a means to compare the sensitivities of different dyes. His laboratory has published data on S:N ratios for over 1000 dyes (12-14). In this laboratory, we screen all newly synthesized dyes on a voltage-clamped model membrane and determine both the relative change in transmitted light and fluorescence for a 100 mV step (2,15). The best fast fluorescent dyes show relative changes of ca. 10% for a 100 mV potential step. In general, there is a good correlation between the S:N on the squid axon and the corresponding relative change on the model membrane but some dyes show totally different behavior in the 2 systems (16); this was traced to differences in the dye's ability to permeate through the glia surrounding the axon and to the ability of some dyes to flip across the axon membrane but not that of the model membrane.

Table 1. Properties of Selected Potentiometric Dyes

Name	Other Names	Comments	ABS (nm)	EM (nm)	Structure
JPW-211	Di-4-ANEPPS	Versatile fast dye. May need Pluronic F127 to solubilize. Obtain from Molecular Probes, Inc.	502	723	
JPW-1114	Di-2-ANEPEQ	More soluble analog of di-4-ANEPPS. Best potentiometric dye for microinjection. Will also show great tissue penetration when applied externally, but may wash out quickly.	529	725	
JPW-1153	Di-8-ANEPPS	More slowly internalized than di-4-ANEPPS. Needs Pluronic to stain. Wash away with pluronic then pluronic-free buffer.	500	705	
JPW 1259		Chiral ribose group enhances non-linear optical properties in oriented assemblies.	502	712	
JPW-1294	Di-4-ANEPPQ	Doubly cationic relative of di-4-ANEPPS. Good tissue penetration for thick preparations.	514	715	
JPW-2045	Di-8-ANEPPQ	Potentiometric dye for neuronal tracing and long-term studies.	513	717	
JPW-2066	Di-18:2-ANEPPS	di-linoleyl-ANEPPS, oil soluble. May be microinjected to stain er (ala Mark Terasaki). Potential-dependence not yet established experimentally, but it should work based on precedent.	497	707	

The dye that has been the most popular is di-4-ANEPPS (Figure 1 and Table 1). We recommend that investigators use this compound as a first try because it gives the most consistent results with good sensitivity. In our own work (5,17,18) it was demonstrated that this compound can measure membrane potential in squid axon, red blood cells, guinea pig and sheep heart, lipid vesicles, and a variety of cells in culture (others have published papers employing this dye in at least 10 additional preparations). We have also used this dye to establish the method of dual wavelength ratiometric potential measurements (18). This idea is based on the potential-dependent spectral shift of the ANEP chromophore (15). It gives the same advantages as the more familiar ratiometric dyes for calcium, obviating problems of variable staining and dye bleaching. Most importantly, it permits the imaging of variations in electrical potential laterally along the surface of a single cell (19).

A problem with di-4-ANEPPS is that for some cells it can become rapidly internalized. If the dye lines the inner and outer leaflets of the membrane bilayer, the response to voltage will cancel out. This is a good example of how an experimental difficulty prompted a redesigned fluorescent probe. We discovered that dyes with longer hydrocarbon tails (counterintuitively) are less likely to flip across the bilayer and become internalized. We therefore synthesized di-8-ANEPPS, which contains 2 octyl chains

instead of the butyl chains on di-4-ANEPPS. It is poorly soluble in water so staining of cells requires Pluronic F127, a macromolecular surfactant. Di-8-ANEPPS is now also commercially available and has been employed to follow membrane potentials over long timecourses. It can also be used for ratiometric imaging (19,20).

Of course, other applications are not compatible with the low solubility of either di-4- or di-8-ANEPPS. In a collaboration with Dejan Zecevic of the University of Zagreb, which calls for intracellular injection to label a single neuron in a complex preparation, a dye was required which has high water solubility to minimize injection volume (the solubility of di-4-ANEPPS is only 0.3 μM), will remain internalized, and still have sufficient membrane affinity to bind to the cytosolic face of the plasma membrane. The answer (21) was di-2-ANEPEQ, which contains a pair of ethyl chains at one end and a double positive charge at the other.

We have also prepared indicators with very long hydrocarbon chains for investigators wishing to combine potentiometric studies with neuronal tracing; the dye is applied to one end of a neuron and its water-insolubility prevents it from spreading anywhere but along the membrane of that one cell. In Table 1, di-8-ANEPPQ is an example of such a dye. In addition to the long hydrocarbon chains, the opposite end of the molecule contains a doubly positive charge to inhibit flipping of the dye through the membrane bilayer. This is of great concern because of the very long durations of neuronal tracing experiments.

The dyes may be used to image variations in the electrical properties along the surface of a cell membrane by using them in conjunction with digital fluorescence microscopy. The challenge is to find a way to correct or normalize away contributions to the fluorescence signal resulting from variations in the concentration of dye or the complex morphology of the cell surface and thereby extracting the dyes's response to its electrical environment. This is achieved by employing a dual wavelength ratiometric scheme (18) that is most easily understood by reference to Figure 2. Because the spectrum undergoes a

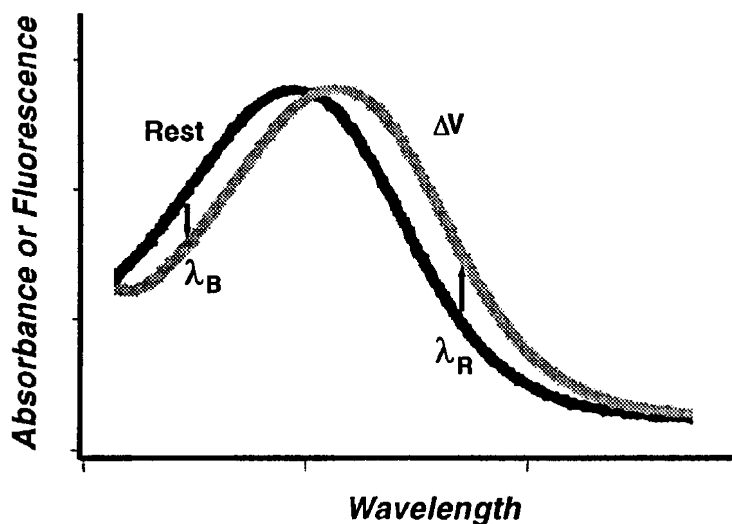


Figure 2.The spectra of styryl dyes are shifted in the presence of electric fields comparable to those found in biological membranes. By ratioing the fluorescence at the red and blue wings of the spectrum, the membrane potential may be monitored without regard to uneven dye distribution.

shift, the fluorescence goes down on the blue wing of the spectrum and up on the red wing. Any variation in concentration or volume dye would cause changes in the same direction at both wavelengths. So a ratio of the two wavelengths normalizes away everything but the dye's response to the intramembrane electric field. This idea was used to study the electric field induced variations in cell membrane potential (19) and allowed us to discover intrinsic variations in intramembrane electric field in different regions of a differentiated neuronal cell (22).

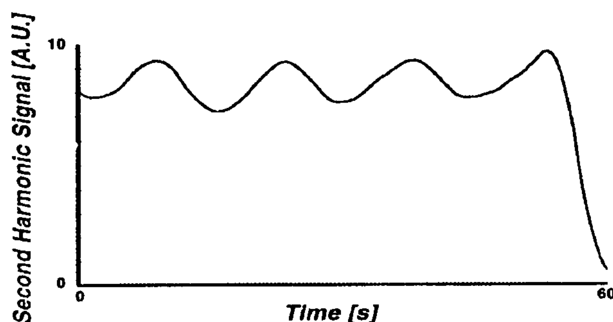


Figure 3. Modulation of SHG Signal from a JPW1259-Stained Lipid Bilayer by 60mV Membrane Potentials. Square wave pulses are applied with periods of 8s at +30mV and 8s at -30 mV. The time course of the optical response is distorted by the timeconstant of the electronics. The experiment is described in Bouevitch et al. (1993) Biophys. J. 65:672-679.

Finally, one of the most exciting findings has been that these dyes all display significant non-linear optical properties when deposited as monolayers (23) or when bound to one surface of a biological membrane (24). The best of the dyes for this purpose is JPW1259 (Table 1) which has a chiral sugar moiety appended to the pyridinium nitrogen. Furthermore, the second harmonic signal displayed by these dyes is itself sensitive to membrane potential. Figure 3 illustrates the voltage-dependent modulation of the second harmonic signal from a model lipid bilayer membrane stained with JPW1259,; details may be found in Bouevitch et al., 1993 (24). The amplitude of the modulation is linearly dependent on the membrane potential and in unpublished work with the laboratory of A. Lewis, we have shown that physiological responses from living cells can be monitored with this technique.

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