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# Absolute Spectroscopic Determination of Cross-Membrane Potential

**Benjamin Ehrenberg[1,3] and Leslie M. Loew[2]**

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Spectroscopic determination of the cross-membrane electric potential has been used for more than 20 years. This method, which usually employs absorption or fluorescence measurements, allows for a rapid and noninvasive study of the electrical properties of the membranes of cells and liposomes. However, the usual fluorescence techniques preferably allow monitoring changes in the potential on triggerable or excitable membranes, and not the absolute value of the potential. They also do not provide means for measuring the potential on single cells. This paper reviews three methods that solve these issues. Nernstian dyes which partition between intra- and extracompartmental volumes enable a fluorescence microscopic determination of a single cell and even a single organelle. Dual-wavelength ratiometric recording from membrane-staining dyes also provides means for measuring the field on a single cell. Resonance Raman probes provide a spectroscopic method with a natural internal standard for the absolute measurement of membrane potential.

**KEY WORDS:** Membrane potential; Nernst potential; resonance Raman; surface potential.

## INTRODUCTION

The electrical profile on a biological membrane is composed of a few components along an axis vertical to the membrane's surface. The surface-to-bulk potential difference, or Gouy–Chapman potential, is established by charged groups at the surface of the membrane [1]. It affects the binding of charged molecules to the membrane and their accumulation near the surface of the membrane. The electric potential difference between the two bulk solutions on the opposing sides of the membrane is usually simply defined as the "cross-membrane potential." Its main component results from concentration gradients of ions across the membrane and their selective permeability under steady-state conditions. This component is given by the Goldman–Hodgkin–Katz (G-H-K) equation [2]. These two types of electric potential differences set, together, a difference in the electric field between the opposing surfaces of the membrane, commonly termed the "microscopic membrane potential."

*The cross-membrane.* G-H-K potential is defined in the following way:

$$\Delta\psi = \frac{RT}{F} \cdot \ln \frac{\sum P^{+}_{i,out} + \sum P^{-}_{i} \cdot C^{-}_{i,in}}{\sum P^{+}_{i} \cdot + \sum P^{-}_{i} \cdot C^{-}_{i,out}} \quad (1)$$

where $C_{in}$ and $C_{out}$ are the concentrations of the permeable monovalent anions and cations in the two compartments separated by the membrane, and $P_i$ are the permeability coefficients of the ions. F and R are the Faraday and gas constants and $T$ is the temperature. In the simple case when the permeability of the membrane to one of the ions is much greater than to the other ions, for example, in the presence of an ionophore with a high

[1] Department of Physics, Bar Ilan University, Ramat Gan 52-900, Israel.
[2] Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06030.
[3] To whom correspondence should be addressed.



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specificity to one ion, one reverts to the simpler Nernst equation:

$$\Delta\psi = -\frac{RT}{zF} \cdot \ln \frac{C_{in}}{C_{out}} \quad (2)$$

*The surface potential.* $\psi_s$, is defined through the following equation:

$$\sinh\left(\frac{ze\psi_s}{2kT}\right) = A\sigma C^{-0.5} \quad (3)$$

where it can be seen that this potential is determined by the density of charges at the surface of the membrane, $\sigma$, and by the electrolyte's concentration, $C$, and its charge, $z$. A is a constant. An important result of this equation is that $\psi_s$ can be abolished by using high electrolyte concentrations and thus the contributions of the surface and cross-membrane potentials to the microscopic membrane potential can be delineated.

## MEASURING MEMBRANE POTENTIALS

Employing microelectrodes for measuring the cross-membrane potential is feasible. It is, however, limited to those cells that are large enough to be impaled by such electrodes. Even then, the drastically perturbing effect of permeation by an electrode has to be considered in each single cell. Surface potentials cannot be assessed directly by electrodes, because this potential difference exists over the so-called Debye layer, between the surface of the membrane and the bulk of the solution, a distance of usually less than 100 Å. It was thus desirable to employ the smallest probing devices, namely, molecules, to probe membrane potentials.

Probably the first application of a spectroscopic probing molecules to measure a membrane electric potential difference was that of Tasaki *et al.* [3]. They stained different nerve cells with the dye ANS and were able to show transient changes in its fluorescence intensity during passage of an electric potential. Soon thereafter, many existing, and newly synthesized, dye molecules were tested for their response to a cross-membrane potential through their absorption, fluorescence, or optical activity [4]. The response mechanisms are numerous, including potential-driven repartitioning of the dye between the outside and the inside bulks or the aqueous and the lipid phases; dimerization and aggregation at, or near, the surface; and spatial rearrangement of the dye in the membrane. These mechanisms require molecular movements and are therefore considered slow, some taking of the order of >> milliseconds. Electrochromism, which is the direct interaction of the molecular electronic levels with the electric field, otherwise known as the Stark effect, is instantaneous but is usually reflected as a small absorption or fluorescence change, not more than a few percent. The slow mechanisms, on the other hand, can demonstrate changes of more than 50% per 60-mV potential change. A 98% change in the fluorescence intensity at a −170-mV potential was reported [5]. A description of the various dyes and their mechanisms can be found in many review articles; the reader is referred to that by Loew [6].

All these spectroscopic probing methods have in common one critical drawback: They can sense only changes in the electric field, and not its absolute value. This is the case when one measures light transmission or fluorescence intensity, where the intensity value cannot be easily used as an absolute measure of the potential. The intrinsic nature of a fluorescence measurement makes a quantitative comparison of fluorescence intensities from different samples, a nontrivial task. This is especially pronounced when the expected change in the fluorescence is small. Thus, it is common that triggerable changes in the potential are the measurable quantity, while the absolute value is not known. However, there is a need for a method that could yield the electric potential itself and this article reviews a few such innovative methods.

## ABSOLUTE DETERMINATION OF MEMBRANE POTENTIAL

In this review article we dwell solely on the cross-membrane, Nernst component of the electric field on a biological membrane. The reasons for this choice are that, first, this is the parameter which is usually studied by electrophysiologists; second, the other component, namely, the surface potential, can easily be abolished by adding electrolytes and so the Nernst potential can be studied separately; and third, a measurement by spectroscopic probes usually leads automatically to an absolute determination of the surface potential. The need for an absolute approach is felt when dealing with the Nernst potential.

Currently, there are three existing spectroscopic methods for measuring the absolute value of the bulk-to-bulk cross-membrane potential. Two of them are based on fluorescence techniques, while the third method is based on resonance Raman spectroscopy.

### Fluorescent Nernstian Dyes

The first method is based on the fact that the distribution of a permeant cationic fluorescent dye between

two compartments, separated by a membrane, is governed by the Nernst equation [Eq. (2)], where $\Delta\psi$ may originate from ion gradients or from the activity of electrogenic pumps. Thus, such a dye will tend to accumulate in the negatively charged compartment and have stronger fluorescence originating from that compartment. This is to be expected when using such a "Nernstian" dye with cells whose resting potential is usually inside-negative. A $-59$ mV potential difference will cause an accumulation of the dye in the cell to a concentration that is 10 times larger than the outside, medium, concentration. Theoretically, the total measured fluorescence does not change compared to a case of the same sample with a different membrane potential or even zero potential. The fluorescence intensity is spatially nonhomogeneous and this can be measured by fluorescence microscopy.

What are the requirements from such a dye? (*i*) It should be a water-soluble molecular ion with a minimal affinity for the membrane. Naturally, some membrane binding is unavoidable, since the dye is required to be able to permeate through the membrane for equilibration and thus must be soluble in the lipid phase to some extent. Thus, a dye with a low hydrophobicity, having a positive charge that is delocalized over a large $\pi$ system, is preferential. (*ii*) It should have a high fluorescence quantum yield, first, for easier measurement under the microscope and, second, to minimize the yield of intersystem crossing and the possibility of its undergoing photosensitized reactions. (*iii*) The dye probe must be nontoxic to the studied cells. (*iv*) The fluorescence intensity must be linear with the dye's concentration, i.e., the dye should not exhibit nonlinear effects such as aggregation, and its concentration must be kept low.

This method of "Nernstian" dyes is very similar to that of employing radioactively labeled amphiphillic ions, such as tetraphenylphosphonium, which were developed by Kaback and co-workers [7]. That method requires the separation of the cells from the bathing medium and a knowledge of the intracellular volume, so that the measured radioactivity can be converted to concentration and used in Eq. (2). In contrast, the fluorescence method directly reports the concentration without a need for a separation process. This allows for an *in situ* measurement of the potential as well as time-dependent monitoring of changes in this potential. The major advantages of this fluorometric method are that individual cells can be studied under the fluorescence microscope, yet by employing new imaging and digital processing devices, many cells that are contained in an imaging frame can be viewed and analyzed simultaneously.

Given the simplicity of the microfluorometric method, two corrections are needed for accurate measurements. First, the uptake and binding of the dye by the cell by potential-independent mechanisms must be evaluated. This is achieved by a similar measurement with the cells in the depolarized state and is reflected in a deviation of the ratio of the fluorescence inside and outside the cell from unity. Second, when the fluorescence is measured in a small intracellular volume unit, through a small aperture in the microscope's image plane, some intensity is emanating from aqueous layers above or below the object cell. This depth-of-field problem, which is more severe when employing an objective with a low numerical aperture, can be corrected by using, as a standard, a dye that does not partition into the cell, such as a dextran-bound fluorophore.

In the first reports of this method, Ehrenberg, Loew, and co-workers have employed a few existing, as well as synthesized, dyes to measure the resting potentials of HeLa cells, mouse macrophages, and neutrophil cells [8,9]. The cationic dyes rhodamine 6G, amethyst violet, and di-O-$C_1$(3) all reported resting potential values of the above cells within the range given in the literature. The methyl and ethyl esters of tetramethylrhodamine (TMRM and TMRE, respectively) were synthesized to make them less hydrophobic than rhodamine 6G, and indeed, their potential-independent binding to the cells was 10 to 20 times less than that of rhodamine 6G. TMRM was later applied to measure the potential on chick embryo muscle myotubes and neuroblastoma cells [10]. The time for equilibration across the membrane varies somewhat with cell type, over a range of 20 s–3 min. Because of their high brightness, the dyes can be used at low enough concentrations so that dye aggregation is insignificant—even in the mitochondria, where the dye might be concentrated up to 10,000-fold. The dyes are quite resistant to photobleaching and photodynamic effects [11].

Confocal microscopy was employed recently in conjunction with Nernstian dyes. This method affords rapid, high-resolution, quantitative three-dimensional imaging microscopy, helps with the depth-of-field issue, and makes it much easier to measure many cells in a single image [11,12]. Recently, digital image deconvolution techniques have been used to correct wide-field images for out-of-focus blur [13]. This approach, together with computer modeling of mitochondrial TMRE fluorescence intensity, has permitted the measurement of the absolute value of the potential across the membrane of individual mitochondria *in situ* in a single living cell [14].

The dye rhodamine 123 has been used, in a similar manner, as a stain of mitochondria [15]. The staining is based on the dye being a strong fluorophore with a de-

localized positive charge and on the mitochondria having a high, inside negative, membrane potential, of the order of −180 mV. Thus, many mitochondria that are difficult to detect, stand out very clearly following this Nernstian uptake of rhodamine-123. A recent report by Hahn *et al.* employed a cyanine dye with a photofixable nitrophenylazide moiety. By this method, the dye that distributes into cells according to the Nernst equation [Eq. (2)] remains there permanently and the staining is not lost upon cell fixation [16].

### Dual-Wavelength Fluorescence Ratiometry

Among the numerous dyes that stain the cell membrane and give fast responses to membrane potential changes, the class of styryl dyes, which usually contain a *p*-dialkylaniline linked to the 4 position of a pyridinium nucleus, via one or more double bonds, has provided a number of sensitive fluorescent probes of membrane potential [17–20]. A styryl dye, di-4-ANEPPS, in which the aniline is replaced with a dialkylaminonaphthalene moiety, displays the largest relative fluorescence response of any of the probes tested on the spherical lipid bilayer model membrane [20]. The wavelength dependence of the transmittance and fluorescence changes indicate simple shifts of the absorption, excitation, and emission spectra. This would be predicted when electrochromism is the mechanism of response. This dye probe was used to determine the electric field-induced membrane potential in a number of cell types [21–23]. In general, this probe and some of its analogues appear to be useful potentiometric indicators in a variety of biological preparations [24–26].

As demonstrated for red cells [26], for neuroblastoma cells [27], and earlier for lipid vesicles and HeLa cells [28], the dyes can be used in a dual-wavelength ratiometric mode to measure membrane potential. This relies on the potential-dependent wavelength shift of the excitation spectrum—probably an invariant but not exclusive component of the dye response. This mode adds another level of usefulness to the dye because it permits monitoring of long-term potential changes where dye bleaching or removal during perfusion changes the absolute level of fluorescence but not the dual-wavelength ratio [29]. It also obviates single-cell measurements of potential variation along the cell surface by dual-wavelength imaging [27,30]; variable staining along the irregular membrane of most cells precludes single-wavelength measurements.

The details of the protocols involved vary with the cell type and the mode of data collection, i.e., single cells in a microscope [27,28] or bulk suspensions in a fluorometer [26,28]. In general, however, the cells are stained in the cold for 10 min and then excess dye is removed by repeated washings. Data are acquired by rapidly switching excitation wavelengths between 450 and 530 nm and collecting the emission at 610 nm; these wavelengths may be adjusted to optimize the signal in a given setup. After background subtraction, the data from the two wavelengths are ratioed and the values compared to a calibration obtained by setting the cross-membrane potential to the $K^+$ diffusion potential with the $K^+$-selective ionophore valinomycin.

### Resonance Raman Spectroscopic Probes

In resonance Raman spectroscopy, inelastically scattered light is frequency-shifted relative to the exciting laser beam by the energy gap of a vibrational level. Thus, although it is an equivalent energy-loss mechanism, it is usually overwhelmed by strong fluorescence. However, in cases when fluorescence does not mask the Raman scattering, the latter provides a wealth of information [31]. Usually, a spectrum is composed of a few vibrational bands. When the studied sample contains more than one dye, the set of bands of each one of them is resonance-enhanced to a different extent according to the location of the laser-excitation wavelength, within the absorption band of the specific dye. Thus, in all the studies mentioned later, the relative intensity of two vibrational modes changes as a result of a membrane potential. This measurable ratio serves therefore to assess the potential directly.

There were a few studies in which the unique properties of resonance Raman scattering were utilized for the absolute measurement of cross-membrane potential [32]. The first use of a resonance Raman molecular probe to monitor membrane potential was by Carey and coworkers, who employed the natural pigment neurosporene to measure the diffusion potential in chromatophores of *R. sphaeroides* [33]. These workers have shown that the excitation profiles of the two prominent vibrational bands, namely, the -C=C- and the =C–C= stretching modes, were not identical. Since the carotenoids exhibit a potential-dependent absorption spectrum shift, the intensity of these two Raman bands changed in opposite direction upon an induction of a diffusion potential. The importance of this result is that the ratio of the intensities was directly proportional to the membrane potential, and thus, the value of the potential could be established from a Raman spectrum, following a calibration of the spectral response. This study did not demonstrate, however, the functional dependence of the Raman response on the magnitude of the electric field.

The carotenoids are a natural probe for resonance Raman studies, because of their extremely low fluorescence quantum yield, because of their strong Raman scattering cross section and their natural abundance in many biological membranes, or in turn, their good membrane binding capacity. Szalontai measured the kinetics of the evolution of membrane potential on cell envelopes containing the pigment bacteriorhodopsin, by measuring the resonance Raman intensities of the $\nu_1$ and $\nu_2$ bands of native carotenoids [34]. Johnson *et al.* added extrinsic β-carotene to follow the build up of the electric potential on lipid vesicles, into which they reconstituted bacteriorhodopsin [35]. The change in the relative intensity of the above-mentioned Raman bands was of the order of 20%, which is a distinct and easily measurable effect, even with a weak spectroscopic phenomenon such as Raman scattering. In a recent study on sciatic nerve cells it was shown that the intensity of the $\nu_1$ band depended almost linearly on a $K^+$ potential that was set on the cells' membrane. In this study the intensity of the Raman band was normalized to the Raman band of water, at 3400 $cm^{-1}$, which serves as an *in situ* internal standard [36].

Another recent study by Ehrenberg and Pevzner demonstrated the feasibility of employing a well-established fluorescent probe, merocyanine 540, as a resonance Raman probe of cross-membrane potential [37]. It was demonstrated that the relative intensity of two vibrational modes depended, linearly, on the $K^+$ diffusion potential. The effect was explained as resulting from potential-driven vertical displacement of the dye in the lipid bilayer. Quinaldine red, an amphiphillic cation, was shown to be taken up by energized bacterial cells, and as a result of aggregation in the cells, Raman bands shifted and changed in intensity [38].

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