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Small molecule fluorescent voltage indicators for studying membrane potential

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Voltage imaging has the potential to unravel the contributions that rapid changes in membrane voltage make to cellular physiology, especially in the context of neuroscience. In particular, small molecule fluorophores are especially attractive because they can, in theory, provide fast and sensitive measurements of membrane potential dynamics. A number of classes of small molecule voltage indicators will be discussed, including dyes with improved two-photon voltage sensing, near infrared optical profiles for use in *in vivo* applications, and newly developed electron-transfer based indicators, or VoltageFluors, that can be tuned across a range of wavelengths to enable all-optical voltage manipulation and measurement. Limitations and a 'wish-list' for voltage indicators will also be discussed.

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Introduction

The cellular plasma membrane separates the internal cellular environment from external surroundings, enabling the accumulation of nutrients, ions, biomolecules, and genetic material that allows for the existence of life on earth. One particularly important facet of membrane biology is the ability to sequester ionic species in unequal concentrations, setting up electrochemical potential gradients, or membrane voltage (V_{mem}), that cells harness for a variety of physiological roles. Cells that rapidly change their V_{mem} are known as 'excitable' and use V_{mem} changes, on the order of 1–100 ms in duration, to drive their unique physiology. For example, membrane depolarization drives neurotransmitter release in neurons and contraction in myocytes. Mounting evidence points to important roles for V_{mem} in a number of basic physiological processes such as cell division, migration, and differentiation, underlying the centrality of V_{mem} dynamics that stretches far beyond those typically associated with specialized, excitable cells [1].

Tools to assess V_{mem}

Despite the central importance of V_{mem} to cellular physiology, in both excitable and non-excitable cells, methods to monitor V_{mem} dynamics remain limited. Patch clamp electrophysiology remains the 'gold standard' to probe V_{mem}. While patch-clamp methodologies deliver extremely sensitive measurements of V_{mem}, they are highly invasive, limited to cell bodies, and exceptionally low-throughput. Multi-electrode arrays enable parallel electrode recordings, but cannot provide cellular spatial resolution. Imaging approaches represent an attractive solution to interrogating V_{mem}, because they are relatively non-invasive, high throughput, and provide spatial information regarding V_{mem} dynamics that would not be achievable through more traditional methods [2,3].

Voltage indicator challenges

Voltage imaging with fluorescent indicators has been a long-standing goal of the scientific community [2–4]; however, the broad implementation of voltage imaging remains elusive, due in part to the challenge of developing optical tools for sensing V_{mem}. First, because neuronal action potentials are fast, on the order of several ms, optical voltage sensors must be able to respond quickly (sub-millisecond). Furthermore, unlike Ca^{2+} indicators, whose wide-spread implementation represent a triumph of chemical biology, voltage indicators must localize to the plasma membrane in order to sense V_{mem}. This represents both a design challenge (localization) and a limit on the number of dye molecules that can generate useful signal, since the volume of the cytosol dwarfs that of the thin plasma membrane. The double jeopardy of fast biological events and small volumes requires bright, photostable, highly sensitive, and non-disruptive voltage-sensitive dyes. There have been a number of promising approaches put forward for optical voltage sensing, including, more recently, the use of fluorescent proteins [5,6], opsins [7,8], second-harmonic generation [9], and nanomaterials [10,11]. This review will focus on recent [12–14] developments in small molecule fluorescent voltage indicators.

Small molecule approaches to voltage imaging

Voltage imaging with small molecules has a long history. In the early 1970s, Larry Cohen and coworkers screened

thousands of available dyes searching for compounds that display voltage-sensitive optical properties. Merocyanine 540 (Figure 1) displayed voltage-sensitive fluorescence, enabling tracking of action potentials in a giant squid axon [4]. A comprehensive history is available from Cohen himself and acknowledges the myriad contributions made in the early days of voltage imaging [15]. Since that time, a number of strategies for voltage imaging with small molecules have been explored in the search for more sensitive compounds. The dyes can generally be divided into two classes, 'fast' and 'slow' response dyes.

Electrochromic dyes (Figure 2b), often referred to as 'fast' dyes, provide excellent response speed, making them ideally suited to monitoring fast neuronal voltage changes. Voltage sensitivity in electrochromic dyes arises from of a molecular Stark effect, in which the cellular electric field perturbs the energy levels of the chromophore dipole, resulting in fast (fs to ps) shifts in both the absorption and emission profiles. The color change can be used for ratiometric imaging, but the shifts themselves are actually quite small (<10 nm), meaning that maximum voltage sensitivity arises at the edge of the excitation and emission spectrum. Practically speaking, this means that instrumental parameters must be highly tuned to the dye of interest and that the majority of excitation and emission photons are not used, further diminishing an already-starved photon budget.

Among the most widely used fast dyes are the aminonaphthyl-ethylene-pyridinium-propylsulfonate, or ANEPPS dyes, first developed by Leslie Loew and coworkers [16,17]. The 4-carbon and 8-carbon substituted varieties, di-4-ANEPPS and d-8-ANEPPS (Figure 1), respectively, provide fractional changes in fluorescence of approximately 10% per 100 mV. A more water-soluble version, JPW-1114 or di-2-ANEPEQ (Figure 1), features a quaternary ammonium solubilizing group in place of the sulfonate and has found application for single-cell loading in brain slice via a patch pipet [18]. Another class of popular 'fast' dyes have been developed Rina Hildesheim and Amiram Grinvald and are designated with the prefix 'RH'. Oxonols RH 1691 and 1692 (Figure 1) [19,20] are often used as indicators of local field potential for intact brain imaging in primates and rodents [21].

Annulation of styryl dyes give the 'ANellated hemicyaNINE,' or ANNINE, class (Figure 1), which show improved voltage sensitivity on account of a rigidized π-system that enhances the dipole of the chromophore, increasing the molecular Stark effect on the dye [22,23]. More recently, the Loew group introduced a palette of fluorinated styryl dyes that show improved photostability [24•]. In addition to spanning wavelengths ranging from 440 to 670 nm, the dyes have varying degrees of voltage sensitivity, from 10% to 22% per 100 mV. Importantly, they showed improved efficacy under two-photon illumination and enabled voltage imaging in intact heart and brain slice.

In 2014 Bezanilla and co-workers reported the use of indocyanin green, or ICG (Figure 1) as a voltage-sensitive dye. ICG shows a modest 2% decrease in fluorescence intensity upon 100 mV depolarization, but has sufficient signal-to-noise to track action potentials in frog oocytes expressing sodium channels and in rat dorsal root ganglia neurons. In rat brain slices, ICG could monitor field evoked stimuli. Importantly, because ICG is FDA-approved for use in humans and has long-wavelength excitation and emission profiles, this opens up the possibility to use ICG in humans or in deep tissue context [25••].

In a complementary fashion, 'slow' dyes, which are based on voltage-dependent accumulation in (Figure 2a) or redistribution within (Figure 2c) the membrane can display much larger fractional changes in fluorescence (up to 80% $\Delta F/F$ per 100 mV) [26], compared to 'fast' dyes, but suffer from drawbacks in slow response time and prohibitive capacitive load on the membranes. Because the voltage sensing mechanism relies on the redistribution of a lipophilic dye within a lipid bilayer, this molecular diffusion can be much slower than the biological event of interest, making 'slow' voltage dyes unable to track fast action potentials. Additionally, because charged dye molecules move through the membrane at a time scale similar to the underlying biological change, this adds an artificial gating charge or capacitive current that can severely disrupt normal membrane and cellular function [27,28].

While these of 'slow' dyes, which also include positively charged rhodamines, can be used in isolation, they are much more effective when paired with a stationary chromophore that enables ratiometric measurements of voltage via fluorescence-resonance energy transfer, or FRET (Figure 2c). Tsien and Gonzalez established a small molecule FRET-based system mobile, containing a voltage-sensitive oxonol and stationary coumarin FRET pair [26]. This approach has since been elaborated by a number of groups, typically by replacing the stationary fluorophore with a genetically encoded fluorophore, such as green fluorescent protein, or GFP [29]. Optimized versions in which the position of the fluorescent protein relative the membrane are altered show improved sensitivity [28,30,31]. More recent incarnations of this approach couple a mobile, lipophilic anion like dipicrylamine (DPA) as a fluorescence quencher of a membrane-associated fluorophore such as DiO to achieve voltage sensing in a variety of systems [32,33]. The DiO/DPA pair is compatible with two-photon imaging, making it useful for interrogating membrane potential in thick tissues.

Our lab has recently undertaken a program to develop new voltage sensing fluorophores that combine both