Measuring Membrane Voltage with Fluorescent Proteins

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Measuring signal transduction in large numbers of cells with high spatial and temporal resolution is fundamental to studying information processing in the nervous system. DNA-encoded sensors have an advantage in that they can be introduced into an organism noninvasively and targeted to specific brain regions, cell types, or subcellular compartments. A variety of chimeric proteins that report transmembrane voltage have been developed. The prototype sensor, FlaSh, is a green fluorescent protein fused to a voltage-sensitive K+ channel, where voltage-dependent rearrangements in the channel induce changes in the protein’s fluorescence. Subsequent sensors have refined this basic design using a monomeric voltage-sensing phosphatase domain from Ciona intestinalis and pairs of fluorescent proteins to produce a larger fluorescent signal. These sensors and their uses are discussed here.

OPTICAL APPROACHES TO STUDYING MEMBRANE VOLTAGE

Fluorescent Dyes as Sensors

Fluorescent indicator dyes, and calcium indicators in particular, have revolutionized our understanding of cellular physiology. The many available membrane voltage dyes can be roughly divided into two categories: Faster (millisecond-range) dyes with a small response, and slower dyes with a large response (Apell and Bersch 1987; Loew et al. 1992; Sjulson and Miesenböck 2007). Typically, the slower dyes depend on partitioning of the dye across the membrane, and the faster dyes use a redistribution of intramolecular charge. Although calcium-indicator fluorescence can change manyfold on binding calcium, in practice, fast voltage probes often show fluorescence changes of <10% per 100 mV, the size of the largest physiological voltage signal in neurons (Homma et al. 2009).

Recently, fast dyes that are excited in the far-red have been introduced, and hybrid systems consisting of a fixed fluorophore and a small mobile quencher or a Förster resonance energy transfer (FRET) partner have seen more use (González and Tsien 1997; Salama et al. 2005; Wuskell et al. 2006; Bradley et al. 2009). However, apart from the signal size, a general drawback for using organic dyes for neuroscience applications is that they cannot be targeted directly to a specific cell population. In the absence of targeting, optical measurements in neural tissue usually cannot distinguish whether a signal originates from activity in neurons or glia, nor can it determine which types of neurons are involved. Moreover, signals that occur in small numbers of cells are often lost in the noise.

Protein-Based Sensors

Because protein-based sensors are DNA-encoded, they can be placed under the control of cell-specific promoters, introduced in vivo or in vitro using gene transfer techniques, and even targeted to specific subcellular compartments. In general, these sensors are chimeras between a signal transduction...
protein fragment (detector) and a fluorescent protein (reporter). Whether based on a single fluorescent protein or a FRET pair, they typically depend on a movement or change in orientation to alter fluorescence. Voltage reporters depend on a protein with a membrane voltage-induced conformational change, such as a voltage-gated K⁺ or Na⁺ channel.

Protein-based sensors also have been developed for a variety of biologically important molecules, such as Ca²⁺ and cAMP (Nagai et al. 2001; Miyawaki 2003; Knöpfel et al. 2006). FlaSh, a protein-based voltage reporter, was one of the first protein-based sensors (Siegel and Isacoff 1997); since its creation much effort has been devoted to producing better reporters. Here, we describe FlaSh as the prototypical voltage sensor and review subsequently developed voltage reporters (see Table 1).

**FLUORESCENT SHAKER (FlaSh) K⁺ CHANNEL: A SENSOR TO MEASURE CHANGES IN TRANSMEMBRANE POTENTIAL**

For FlaSh, a modified green fluorescent protein (GFP) was fused in-frame at a site just after the sixth transmembrane segment (Fig. 1A) of the voltage-activated Shaker K⁺ channel (Tempel et al. 1987; Chalfie et al. 1994; Guerrero et al. 2002). To prevent FlaSh from loading target cells with additional potassium current, a point mutation was engineered into the pore of the channel to prevent ion

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<tr>
<th>TABLE 1. Properties of protein-based fluorescent voltage sensors</th>
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<tr>
<td>Properties&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Sensor</td>
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<td>Fluorescent protein(s)</td>
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<td>Insertion point</td>
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<td>Dynamic range</td>
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<td>Reported change (100 mV)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Activation time constant&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Excitation wavelength</td>
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<sup>a</sup>Data for FlaSh and SPARC were collected in oocytes. The remaining data were collected in various mammalian cell lines.

<sup>b</sup>First-generation sensors use multimeric proteins as their voltage transducers and target the plasma membrane poorly in mammalian cells. Second-generation sensors rely on the Ci-VSP VSD and show improved localization.

<sup>c</sup>Reported voltage changes are steady-state values and typically correspond to the slow fluorescence response. Ratios are given for constructs with two fluorescent proteins.

<sup>d</sup>Because the response is nonlinear, activation time constants vary depending on the voltage range. Some can also include multiple variants, and are thus best used to characterize constructs as either fast or slow.

<sup>e</sup>Siegel and Isacoff (1997) and Guerrero et al. (2002).

<sup>f</sup>Ataka and Pieribone (2002).

<sup>g</sup>Sakai et al. (2001) and Knöpfel et al. (2003).

<sup>h</sup>Dimitrov et al. (2007) and Lundby et al. (2008).

<sup>i</sup>Tsutsui et al. (2008).

<sup>j</sup>Mutoh et al. (2009).
conduction but preserve the channel’s gating rearrangements. The voltage-dependent structural rearrangements in the channel are transmitted to GFP, resulting in a fluorescence change. Correlating closely with the voltage-dependent rearrangements of Shaker, the dynamic range of FlaSh is approximately −50 mV to −30 mV, following the sigmoidal relationship between gating charge and voltage and corresponding to suprathreshold voltages for action potential initiation. This voltage range can be shifted in a predictable manner in the negative direction by mutating the S4 voltage sensor (Guerrero et al. 2002).

The speed of the fluorescence change depends on the version of GFP used, ranging from a time constant of hundreds of milliseconds in the case of yellow fluorescent protein (YFP), which tracks the process of slow inactivation, to ~10 msec in the case of enhanced GFP (eGFP) tracking activation at room temperature (Guerrero et al. 2002). Although the temporal amplification does make apparent events that would otherwise be difficult to detect, these rates are too slow to distinguish multiple rapid events such as neuronal spiking, even at modest frequencies. For a train of brief pulses, the integral of the fluorescence response is constant at frequencies of ≤20 Hz.

**IMPROVED VOLTAGE-SENSING FLUORESCENT PROTEINS**

Several variants fusing a voltage sensor to a fluorescent protein have been designed to improve on the characteristics of FlaSh. The voltage-sensing fluorescent protein 1 (VSFP1) introduced a truncated...
(i.e., consisting of S1–S4 of the pore region, with S5 and S6 removed) voltage-sensing domain from the potassium voltage-gated channel, Shab-related subfamily, member 1 (Kv2.1) fused with a cyan fluorescent protein (CFP)–YFP FRET pair connected to each other by a short linker (Fig. 1C; Sakai et al. 2001; Knöpfel et al. 2003). The placement of the two fluorescent proteins adjacent to each other in the amino acid sequence separated by a short (and presumably inert) linker is distinct from other protein-based sensors that rely on FRET, such as calcium sensors like Cameleon or TN-XL in which the moving part (i.e., the calmodulin or troponin calcium-binding sensor domain) links the CFP donor to the YFP acceptor (Miyawaki et al. 1997; Mank et al. 2006). However, this short passive linker donor–acceptor design does create an effective, detectable fluorescent change and has been used in subsequent VSFPs. It has been hypothesized that the FRET change results from a change in the angle between the two fluorescent proteins (Fig. 2C,D; Sakai et al. 2001).

SPARC (sodium channel protein-based activity reporting construct) (Fig. 1B), another of the first generation of protein-based voltage sensors, uses a complete Na,1.4 sodium channel as its sensor, with GFP inserted in the intracellular loop between the second and third domains (Ataka and Pieribone 2002). The voltage-dependent fluorescent change is small but rapid, corresponding to the gating-charge movement characteristic of sodium channels.

The two biggest obstacles to using these proteins in neuroscience are the small fluorescence change (a few percent or less for a 100-mV step) and, more importantly, their poor membrane targeting in mammalian cells. These chimeras suffer from poor localization: The majority of the proteins never make it to the plasma membrane, staying instead within the cells, where they contribute to the fluorescence background (Baker et al. 2007). The fact that most of the initial experiments on protein-based voltage indicators were performed in oocytes delayed the recognition of this problem. In retrospect, this is not surprising, considering that the fluorescent proteins are linked so closely to the membrane in a region crowded by four channel subunits and (in the cases of the potassium channel-based Shaker and Kv2.1 constructs) multiple fluorescent proteins (Fig. 1).

The discovery of the C. intestinalis voltage-sensing phosphatase (Ci-VSP), in which a voltage-sensing domain (VSD) is connected to a cytoplasmic enzyme, enabled the construction of a new class of voltage-sensing proteins (Fig. 1D; Murata et al. 2005). Replacing ion channel VSDs with that of Ci-VSP has two advantages. First, Ci-VSP already couples its VSD to a cytoplasmic protein that is sensitive to its voltage-sensing motion. Second, because Ci-VSP is a monomer, fluorescent protein fusions do not face potential steric clashes between multiple fluorescent proteins in the crowded juxtamembranous space of a channel. Indeed, Ci-VSPs localize more efficiently to the cell membrane in mammalian cells (Dimitrov et al. 2007).

First among this second generation of proteins is the VSFP2 family (Dimitrov et al. 2007; Lundby et al. 2008). Several variants of Ci-VSP-based sensors have been developed that, like VSFP1, use a pair

![FIGURE 2. Possible mechanisms for voltage-dependent fluorescence changes. The (A) resting and (B) depolarized membrane of a single-FP sensor. The force exerted by the motion of the voltage-sensing domain destabilizes the fluorescent protein and causes a decrease in fluorescence. The (C) resting and (D) depolarized membrane of a FRET-based sensor. (D) When the cell depolarizes, the angle between the chromophore dipoles changes, increasing FRET efficiency. This decreases the observed donor emission and increases acceptor emission. For clarity, only the charged S4 helix of the voltage-sensing domain is shown.]
of adjacent fluorescent proteins with varying lengths of linker that connect the VSD to the phosphatase of Ci-VSP (Mutoh et al. 2009). The sensors incorporate the charge neutralization of the first S4 arginine (R217Q) to shift the voltage range negatively into the physiological range (Dimitrov et al. 2007; Kohout et al. 2008). The majority of the voltage-dependent fluorescence change occurs between −80 mV and 0 mV, with a sigmoidal relationship between fluorescence and voltage. VSFP2 has been shown to respond to physiological neuronal membrane signals (Dimitrov et al. 2007). Finally, in a detour from paired fluorescent protein designs, VSFP3.1 consists of the Ci-VSP VSD coupled to a single CFP. Like the SPARC construct, this provides a faster response at the cost of a reduced fluorescence change (Lundby et al. 2008).

The voltage sensor Mermaid borrows the VSFP design of adjacent fluorescent proteins and currently produces the largest reported fluorescence change (Tsutsui et al. 2008). Mermaid replaces the classical CFP–YFP donor–acceptor pair with mUKG and mKOκ (monomeric umi-kinoko green and kusabira orange, respectively), new green and orange GFP-like proteins that have excellent spectral overlap for FRET. Large fluorescence changes can be observed by examining the emission ratio of the two fluorophores, in some cases >10% per 100 mV. Like VSFP2, the voltage-dependent fluorescence change occurs between −80 mV and 0 mV, with a sigmoidal relationship between fluorescence and voltage. Although the magnitude of fluorescence response has improved greatly since the first generation of sensors, like those earlier proteins these still require hundreds of milliseconds to reach their maximum response to a voltage change (Baker et al. 2008; Mutoh et al. 2009). Nevertheless, it is possible with Mermaid to optically detect single slow cardiac action potentials in cultured cardiac myocytes and even low-frequency neuronal action potentials.

**MECHANISM OF FLUORESCENCE CHANGE**

Most of the protein-based optical voltage sensors have two components to their voltage response: a fast, small change followed by a slower, larger change (Lundby et al. 2008; Villalba-Galea et al. 2009). The fast change closely corresponds to the gating charge movement of the voltage sensor, but it is typically <1% (ΔF/F). Not present in constructs like SPARC, but prevalent in Ci-VSP constructs and FlaSh, the slow component can be an order of magnitude larger. It has been proposed that this derives from the relaxation of the voltage-sensing domain with prolonged depolarization (Villalba-Galea et al. 2009). However, for both components of the response, the mechanism by which the enclosed chromophore of the fluorescent protein is influenced by structural rearrangements remains unclear. It is possible that the movement of the voltage-sensing domain destabilizes the fluorescent protein, decreasing its fluorescence perhaps by increasing chromophore exposure to solvent (Fig. 2). In Mermaid and VSFPs, the FRET effect could result from changes in the angle between the two chromophores (Sakai et al. 2001).

**EXPERIMENTAL SETUP AND APPLICATIONS**

The improved Ci-VSP-based optical voltage sensors are optimally used with a microscope configured for emission ratio fluorescence imaging. Excitation is performed at a single wavelength appropriate to the FRET donor. A first dichroic mirror separates the emission and excitation light. A second dichroic mirror in the light path then separates the emissions of the donor and acceptor. Two detectors simultaneously record the changes at the respective wavelengths. With a camera as the detector, commercial beamsplitters can allow the collection of both wavelengths on a single charge-coupled device. For faster recording, photomultiplier tubes provide an efficient detector. For most reported values, the ratio of the two wavelengths is used, with corrections made for background fluorescence (Tsutsui et al. 2008; Mutoh et al. 2009).

Currently, the only physiological results from these fluorescent voltage sensors have come from their developers. Investigators have successfully detected action potentials when it is possible to...
average across multiple sweeps, such as replayed voltage traces (Dimitrov et al. 2007). A single application of voltage-sensing fluorescent proteins in natively excitable cells has been presented (Tsutsui et al. 2008). The Mermaid sensor was used in two types of cultured cells: rat cardiomyocytes and cortical neurons. In both cases, a single sweep was sufficient to observe membrane activity. The Mermaid construct was transfected, and after a few days of expression, imaging was conducted using separate charge-coupled cameras for each fluorescent protein wavelength. The spontaneous cardiomyocyte depolarization cycle was clearly visible with Mermaid (~22% ratio change) (Fig. 3A,B). In cortical neurons, field stimulation triggered firing in cells in which synaptic transmission was blocked pharmacologically. Fluorescence responses to both a single induced action potential (~1.5%) and a pulse train (~5%) were detected. As might be expected, the magnitude of the fluorescence ratio change was larger for the longer, larger events (Fig. 3C,D).

**DISCUSSION AND CONCLUSION**

Since the introduction of FlAsH, improvements to voltage-sensing fluorescent protein sensors have been somewhat slow. In comparison, methods for genetically targeting and optically stimulating neurons (a complementary set of techniques) have developed much more rapidly (Zhang et al. 2007; Gorostiza and Isacoff 2008; Miesenböck 2009). Protein-based voltage sensors still have yet to be adopted widely as neuroscience tools. Concerns such as capacitive load from VSFP VSDs remain secondary to another limitation shared with chemical voltage dyes: The fast fluorescence response is small relative to the noise (Sjulson and Miesenböck 2007; Akemann et al. 2009); protein-based sensors do not yet approach the signal-to-noise ratio seen with calcium indicator dyes. Additionally, although genetically encoded subcellular targeting is promising, organic dyes still localize to the plasma membranes of homogeneous cultured cells more uniformly than do even second-generation VSFPs. In more heterogeneous systems or in experiments long enough for dye internalization to be a problem, cell-specific targeting of genetically encoded sensors is more advantageous.

Improvements to these sensors have focused largely on modifying the voltage-sensing domain and the linker that connects it to the fluorescent protein. There has been some success in tuning these...
sensors through point mutations, primarily by using already well-characterized mutations. Further advancement will require a more detailed model of how GFP fluorescence is affected by conformational change in the voltage-sensing domain. Difficulties crystallizing membrane proteins make it unlikely that this will be clarified by analysis of crystal structures (e.g., as in the case of the mechanism for the GCaMP calcium sensor) (Wang et al. 2008; Akerboom et al. 2009). Still, a better understanding of the mechanism might allow for more rational design approaches, including tuning the fluorescent protein to better represent the state of the voltage-sensing domain, an aspect of the sensor that has not yet been examined systematically.

With the goal of capturing high-speed events, one reasonable focus might be to increase the size of the fast fluorescence response known to correspond to the gating charge movement. This could be explored by placing greater emphasis on smaller voltage changes that fall within the dynamic range of the voltage sensor, instead of on large fluorescence changes that might correspond to a slower, secondary movement of the voltage-sensing domain. Perhaps mutations that destabilize the fluorescent protein would yield a better sensor. Alternatively, new classes of fluorescent proteins could provide new components for these sensors (Shu et al. 2009). Also, fusion proteins are not the only approach to genetically targeted fluorescent voltage reporting. A hybrid system combining GFP with the small, mobile quencher dipicrylamine has also been described (Chanda et al. 2005; Sjulson and Miesenböck 2008). Although under some conditions this system can detect single action potentials in cultured neurons, a purely genetically encoded sensor would be superior for longer studies, as well as for investigating locations that dipicrylamine cannot reach easily.

In vivo systems benefit the most from DNA-encoded sensors, particularly heterogeneous ones such as the brain where many different cell types are present. By resolving targeting, the signal-to-noise ratio for a phenomenon present in a subpopulation of cells can be greatly increased. The signaling events that govern development, sensory transduction, learning, and memory require simultaneous observation of events occurring in multiple cells, and thus optical techniques are preferable to direct electrical measurement. Although work remains to optimize protein-based voltage reporters as neuroscience tools, fusing VSDs to fluorescent proteins produces sensitive and accurate reporters of membrane voltage; new developments will doubtless provide the detection levels and speed necessary to capture fast neuronal events.

ACKNOWLEDGMENTS

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