Hybrid Indicators for Fast and Sensitive Voltage Imaging


Abstract: Membrane voltage is an important biophysical signal that underlies intercellular electrical communications. A fluorescent voltage indicator is presented that enables the investigation of electrical signaling at high spatial resolution. The method is built upon the site-specific modification of microbial rhodopsin proteins with organic fluorophores, resulting in a hybrid indicator scaffold that is one of the most sensitive and fastest orange-colored voltage indicators developed to date. We applied this technique to optically map electrical connectivity in cultured cells, which revealed gap junction-mediated long-range coupling that spanned over hundreds of micrometers.

Membrane voltage controls many fundamental aspects of cellular physiology. The transmembrane electric field arises from the action of ion-selective channels and pumps, and it acts upon a range of membrane-embedded biomolecules, such as voltage-gated ion channels and receptors.[1] As exemplified in neural and cardiac tissues, membrane voltage is often tightly regulated through intercellular communications such as chemical and electrical synapses.[2–3] Understanding the function of these systems requires recording membrane voltage dynamics with high spatial and temporal resolutions. To this end, various voltage-sensitive fluorescent indicators have been developed, including both synthetic dyes[4] and protein-based sensors.[5] While protein-based sensors are readily targetable to specific cell types, their brightness and photostability are typically worse than synthetic dyes. In the present study, we sought to create a hybrid voltage indicator that combines the merits of small molecules and proteins.

Our method capitalizes on the electrochromic Förster resonance energy transfer (eFRET) mechanism, initially discovered in microbial rhodopsins.[6–8] As shown in Figure 1, membrane voltage fluctuations lead to changes in the absorption spectrum of rhodopsin, which could be probed with a site-specifically ligated organic fluorophore donor (Supporting Information). We called this design FlareFRET, for fluorophore ligation-assisted rhodopsin eFRET.

Figure 1. The FlareFRET design. A) A small molecule fluorophore (yellow star) is site-specifically ligated to rhodopsin (gray). Membrane voltage modulates the acid–base equilibrium of a Schiff base. When the cell undergoes depolarization, Schiff base protonation increases the absorption and leads to fluorescence quenching of the ligated fluorophore. B) Theoretical calculation shows that as baseline FRET efficiency (E_{FRET}) increases, voltage sensitivity increases linearly while the brightness decreases. In the regime of shot-noise limit, the signal-to-noise ratio is optimal when E_{FRET} is 67% (see discussion in the Supporting Information).

We chose PRIME (probe-incorporation mediated by enzyme) labeling technique to construct FlareFRET sensors, because it offered a combination of small tag size, targeting specificity and fast reaction kinetics.[9,10] Fluorophore ligation was achieved via two steps (Figure 2A). In the first step, an engineered lipoic acid ligase mutant (W37V LplA) recognized a 13-amino-acid acceptor peptide (LAP) that was fused to rhodopsin, and ligated a picolyl azide (pAz) moiety to its lysine side chain. Subsequently, an alkyne-derivatized fluorophore was conjugated to pAz via Cu²⁺-catalyzed azide–alkyne [3+2] cycloaddition (CuAAC) reaction.

We started by comparing several rhodopsin candidates for their tolerance of LAP insertions. In human embryonic kidney (HEK) cells, Acetabularia acetabulum rhodopsin (Ace)[11] showed the best membrane trafficking (Supporting Information, Figure S1), which we used for subsequent studies. The proton-pumping activity of Ace was reduced with a single mutation D81N (Supporting Information, Figure S2).[7] We inserted LAP at various locations in the Ace sequence and compared the PRIME labeling efficiency using Cy3 as the fluorescence reporter. As shown in Figure 2B, Cy3...
These sensors all had similar voltage sensitivity in HEK cells (36.0 ± 1.4 %) and Chinese Hamster Ovary (CHO) cells (30.9 ± 1.9 %). Our theoretical model of eFRET voltage sensing predicts a positive correlation between voltage sensitivity and baseline FRET efficiency, $E_{\text{FRET}}$ (see the Supporting Information). To evaluate voltage sensitivity, we used whole-cell patch clamp technique to control the membrane voltage of labeled HEK cells, while simultaneously recording the fluorescence signal under the microscope. As we varied the membrane voltage across the physiologically relevant range between −100 mV and 50 mV, the fluorescence signal from these hybrid voltage indicators changed almost linearly (Figure 3C; Supporting Information, Figure S7 and Figure S8). The highest sensitivity was observed in Ace-L1 conjugated with Cy3 (Ace-L1-Cy3), achieving $-AF/F$ of 35.9 ± 0.8 % (per 100 mV, normalized to F at −70 mV; Supporting Information, Movie S1). This sensor, which we named as Flare1, had similar voltage sensitivity in HeLa (36.0 ± 1.4 %) and Chinese Hamster Ovary (CHO) cells (30.9 ± 1.9 %; Supporting Information, Figure S9 and Table S2). In comparison, Ace2N-2AA-mNeon showed only 9.4 ± 0.6 % fluorescence change under the same condition (Figure 3C; Supporting Information, Movie S1). Both Ace-L1-AF488 (28.2 ± 0.9 %) and Ace-L1-AF594 (23.9 ± 1.3 %) had lower sensitivity than Flare1, whereas Ace-L1-Cy5 had negligible response (5.4 ± 0.6 %). We noted that the maximum emission wavelength of Cy3 coincided with the absorption maximum of Ace.[7] This spectral dependence of voltage sensitivity was expected from the eFRET mechanism.

Voltage sensitivity also depends on the protein scaffold. Ace-N sensors overall are less sensitive than Ace-L1 sensors (Supporting Information, Figure S7 and Table S2). We also explored other site-specific protein labeling techniques, including HaloTag,[11] SNAP-tag,[12] and streptavidin labeling (Supporting Information, Figure S10).[13] These sensors all exhibited lower voltage sensitivity than Flare1 (Supporting Information, Table S2), which we attributed to long donor-acceptor distances owing to the bulky tag size (HaloTag is 34 kDa, SNAP-tag 19 kDa, streptavidin 53 kDa, whereas LAP tag is only 1.6 kDa). We noticed slight hysteresis in the $F$–$V$ response curves of FlareFRET sensors, which likely arises from voltage-induced conformational changes in Ace. Taken together, the above screening efforts identified Flare1 as the most sensitive hybrid voltage indicator.

To quantify the response kinetics, we stepped the membrane voltage between −70 mV and 30 mV, which induced biexponential fluorescence responses in FlareFRET sensors (Figure 3D; Supporting Information, Figure S7). As shown in the Supporting Information, Figure S11 and Table S2, both Ace-L1 and Ace-N sensors responded within milliseconds. Flare1 had a dominating fast component with time constant $\tau = 0.92 ± 0.03$ ms (96.0 ± 0.7 %) during depolarization and $\tau = 1.41 ± 0.04$ ms (91.0 ± 0.7 %) during hyperpolarization. Consistent with a previous report,[7] Ace2N-2AA-mNeon exhibited complex response kinetics, in which a rapid initial overshoot was followed by a slower decay to steady-state fluorescence on a time scale of about 100 ms. The fast and simple response kinetics of Flare1 could simplify data analysis when extracting voltage information from optical traces.

Our theoretical model of eFRET voltage sensing predicts a positive correlation between voltage sensitivity and baseline FRET efficiency, $E_{\text{FRET}}$ (see the Supporting Information). To
verify this model, we calculated FRET efficiencies based on fluorescence lifetime measurements in HEK cells, which have resting membrane voltage of about −40 mV.\[14\] The excited-state decay of Flare1 can be fitted with a bi-exponential function, with a fast component lifetime of 0.177 ± 0.006 ns (81.1%) and a slow component of 0.968 ± 0.041 ns (18.9%); Supporting Information, Figure S12 and Table S3). In the absence of FRET, the lifetime of cell surface-targeted Cy3 is almost two-fold longer (fast: 0.359 ± 0.003 ns, 65.2%; slow: 1.519 ± 0.017 ns, 34.8%). From these data, we calculated \(E_{\text{FRET}}\) of Flare1 to be 56.8% (see the Supporting Information). For comparison, both Ace-N-Cy3 (40.5%) and Ace2N2AA-mNeon (25.1%) had lower \(E_{\text{FRET}}\) than Flare1. Our model also suggests that the signal-to-noise ratio (SNR) is optimal when \(E_{\text{FRET}}\) reaches 67%. Notably, this level of energy transfer efficiency has never been achieved by fluorescent protein-based eFRET sensors,\[6\] which is presumably due to the bulky size of their \(\beta\)-barrel scaffold. On the other hand, Flare1 has FRET efficiency that approaches this optimal range. Taken together, these data agreed with our theoretical model and highlighted the advantage of small tag size in FlareFRET hybrid voltage indicators.

We applied Flare1 to investigate the electrical connectivity in a cell population. Gap junction channels are specialized ion-conducting intercellular connections that enable direct electrical coupling between neighboring cells. These channels are known to contribute to cardiac activity, neuronal signal-
However, we observed greatly reduced neuronal excitability and action potential firing rates (Supporting Information, Figure S15), which is mainly due to the addition of copper. Although these labeling reagents have negligible toxicity in HEK cells (Supporting Information, Figure S6), they appear to be too harsh for neurons. This calls for the development of milder probe conjugation methods.

In summary, we have created a palette of hybrid voltage indicators (Figure 5). Among these, Flare1 represents one of the most sensitive (–ΔF/F = 35.9 % per 100 mV) and fastest (sub-millisecond response) orange-colored fluorescent voltage indicators developed to date. The superior sensitivity of Flare1 has enabled optical mapping of electrical connectivity among cultured cells, which revealed gap-junction-mediated long-range coupling that spanned over hundreds of micrometers. Moreover, the kinetics and dynamic range of Flare1 are sufficient to report simulated action potential spike train waveforms. We envision that FlareFRET sensors will greatly expand the toolbox of voltage imaging, and the next goal is to apply these tools to visualize action potentials in neurons.

Figure 4. Optical mapping of electrical connectivity and simulated action potential waveforms with Flare1. A)–C) Optical mapping of electrical connectivity in HEK cells and HeLa cells with Flare1. Left: HEK cells labeled with Flare1; Middle: Flare1-labeled HEK cells in the presence of gap junction blocker 2-aminoethoxydiphenyl borate (2-APB). Right: HeLa cells labeled with Flare1. A) Representative fluorescence images of cells expressing Flare1. B) Differential interference contrast (DIC) images of the same fields of view. Regions of interest (ROIs) are marked in colored polygons. C) Normalized fluorescence responses to command voltage (a single trial of four –100 mV to 50 mV cycle) for each group. Colored traces correspond to ROIs with the same color code in (B). D) A simulated action potential spike trains (top, black trace) was applied to a Flare1-labeled HEK cell via voltage clamp. Flare1 fluorescence response was simultaneously recorded (bottom, yellow trace). E) Simulated spontaneous action potential waveforms applied to the HEK cell. F) Magnification of the voltage fluctuations under the shaded region shown in (E). G),H) Flare1 fluorescence response to the voltage waveforms in (E) and (F), respectively. Black arrows indicate a subthreshold voltage fluctuation. Scale bars: 50 μm in (A) and (B), 10 μm in (D).

Figure 5. Summary of the voltage indicators reported herein. The horizontal axis represents the maximum emission wavelength, and the vertical axis represents voltage sensitivity in –ΔF/F, normalized to the fluorescence at V_m = –70 mV. The text color represents the approximate fluorescence emission color based on the spectrum. Error bars represent s.e.m.
Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant 21673009, 21727806) and the Peking-Tsinghua Center for Life Sciences. P.Z. was supported by the National Thousand Young Talents Award.

Conflict of interest

The authors declare no conflict of interest.

Keywords: click chemistry · fluorescent probes · FRET · protein engineering · voltage imaging

How to cite: Angew. Chem. Int. Ed. 2018, 57, 3949–3953

Angew. Chem. 2018, 130, 4013–4017


Manuscript received: December 8, 2017
Revised manuscript received: January 23, 2018
Accepted manuscript online: February 13, 2018
Version of record online: March 8, 2018