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The evolving capabilities of rhodopsin-based genetically encoded voltage indicators

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Protein engineering over the past four years has made rhodopsin-based genetically encoded voltage indicators a leading candidate to achieve the task of reporting action potentials from a population of genetically targeted neurons *in vivo*. Rational design and large-scale screening efforts have steadily improved the dynamic range and kinetics of the rhodopsin voltage-sensing domain, and coupling these rhodopsins to bright fluorescent proteins has supported bright fluorescence readout of the large and rapid rhodopsin voltage response. The rhodopsin-fluorescent protein fusions have the highest achieved signal-to-noise ratios for detecting action potentials in neuronal cultures to date, and have successfully reported single spike events *in vivo*. Given the rapid pace of current development, the genetically encoded voltage indicator class is nearing the goal of robust spike imaging during live-animal behavioral experiments.

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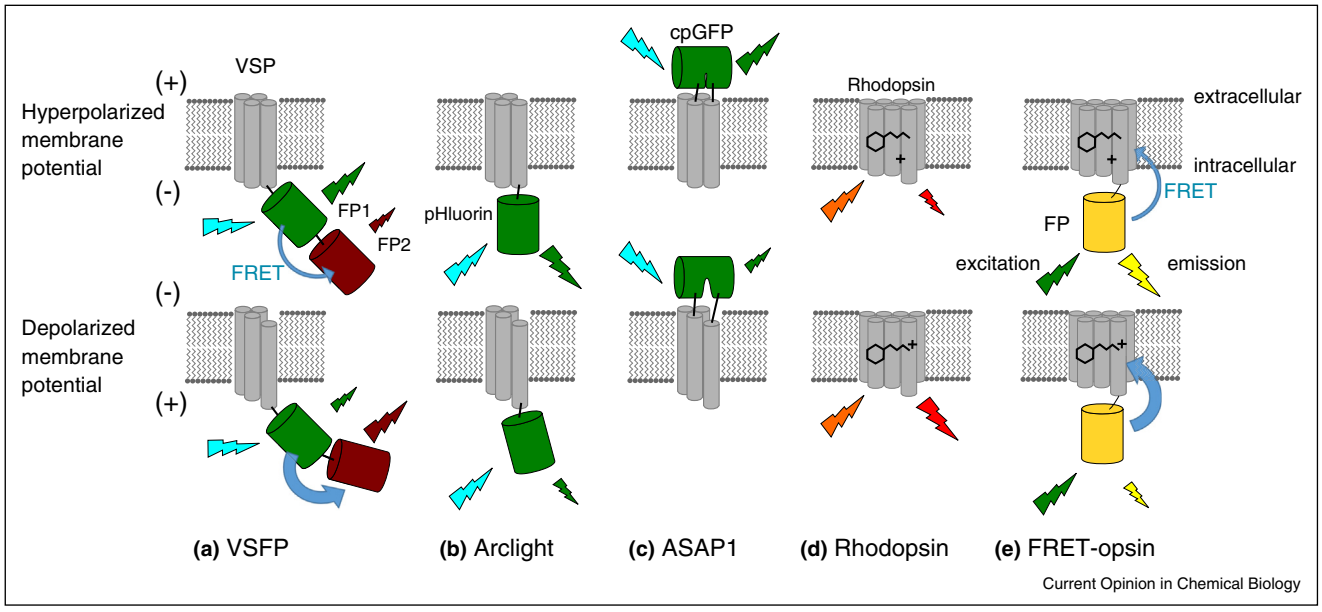
The desire for genetically encoded voltage indicators (GEVIs) that precisely read out the arbitrary spiking activity of multiple genetically targeted neurons stems from the large potential impact that these precise recordings of the spiking code can have at connecting neural activity to animal behavior [1,2]. Although genetically encoded calcium sensors offer similar targeting capabilities and continued development of these sensors has resulted in tools that can report the broad outlines of neural activity with high fidelity [3,4], GEVIs have the potential to read out the spike train with millisecond temporal resolution and map membrane potential hyperpolarizations, capabilities not present with calcium sensors. Voltage sensors developed over the past two decades have demonstrated improving signal-to-noise ratio (SNR) in well controlled culture experiments, but only moderate success when measuring action potentials in live-brain imaging preparations, where noise sources such as scattering, hemodynamics, and general background fluorescence wash out the small sensor response. Here we describe the progress of rhodopsin-based voltage sensors over the past four years, both in developing rhodopsins to serve as highly effective voltage-sensing domains (VSDs), and in developing methods to extract the information from these VSDs with high SNR. Fluorescent protein–rhodopsin fusion sensors represent the leading edge of voltage imaging tools when compared to the other members of the GEVI class, and are on the cusp of reporting neural spiking activity from live animal preparations.

Early generations of GEVIs fused voltage-sensitive phosphatases (VSPs) such as the *Ciona intestinalis* voltage-sensitive domain (Ci-VSD) with fluorescent proteins (FPs). In particular, the voltage sensitive fluorescent protein (VSFP) design attached pairs of FPs that interacted with fluorescence resonance energy transfer (FRET) to one Ci-VSD terminus [5–8] (Figure 1a), or split the pair between the termini [9,10]. The fluorescence ratio of the two FPs thus reported the voltage-sensitive conformation of Ci-VSD with the high brightness of FPs. However, these sensors generally had slow voltage-sensing kinetics (>20 ms) and therefore had only small optical response to neuronal action potentials in the culture setting (~1% $\Delta F/F$). Subsequent engineering of the FP and VSD components resulted in sensors that reported the VSP conformation change with charge sensing FPs or allostery. In particular, Arclight (Figure 1b) [11,12] and ASAP1 (Figure 1c) [13] coupled a pHluorin mutant to Ci-VSD and a circularly permuted GFP to the homologous *Galos galos* VSD (gg-VSD), respectively, and currently represent the best engineered VSP-based sensors in terms of dynamic range (which improves response to long voltage transients) and kinetics (which improves response to short voltage transients) (Table 1).

Archaerhodopsin-3 (Arch) has simultaneously risen as an alternative VSD with voltage-sensitive electronic configurations that modified the protein's absorption spectrum (Figure 1d) [14]. The initial report of Arch voltage-sensitive fluorescence suggested that rhodopsins could serve as VSDs with fast and large dynamic range voltage response. Rational design then improved Arch using site-directed mutagenesis that drew heavily on existing literature detailing how mutations in the homologous bacteriorhodopsin might affect the rhodopsin photocycle

Figure 1



Multiple voltage sensor configurations report membrane voltage using different mechanisms. GEVIs in the past decade of development have used primarily VSPs or rhodopsins as VSDs. **(a)** The VSFP configuration fused FP pairs that interact with FRET to VSPs. During voltage-sensitive conformation changes, the donor (FP1) and acceptor (FP2) of the FRET pair physically shift, leading to enhanced FRET interaction, decreased donor emission, and increased acceptor emission. **(b)** Arclight fused a mutant of pHluorin to VSPs, and the FP readout decreased intensity with voltage depolarization. **(c)** ASAP1 fused a circularly permuted GFP to the extracellular terminals of a VSP. The voltage sensitive conformation manipulated the GFP structure via allostery, and decreased the sensor fluorescence intensity with voltage depolarization. **(d)** Inhibitory rhodopsin proteins derived voltage sensitivity from the Schiff base protonation site within the proton-conduction pathway. During voltage depolarization, protonation of the Schiff base increased, leading to increases in rhodopsin absorption and fluorescence emission at constant excitation power. **(e)** FRET-opsin fusions with bright FPs serving as the FRET donor reported the voltage-sensitive absorption of the rhodopsin FRET acceptor. During voltage depolarization, rhodopsin absorption increased, and the FP fluorescence decreased. Because the FP fluorescence quantum yield was orders of magnitude higher than the rhodopsin fluorescence quantum yield, the FP channel had much higher shot-noise limited SNR than the rhodopsin channel, and optical experiments using FRET-opsin sensors imaged only the FP channel.

[15–20]. These studies mutagenizing the charge centers of Arch within the proton conduction pathway significantly improved the kinetics and voltage sensitivity of the rhodopsin protonation event that supports voltage-sensitive absorption and fluorescence. Specifically, manipulation of the charge center D95 [21•,22•,23,24] eliminated the protein's native photocurrent, while manipulation of the charge center D106 [21•,22•] increased the protein's voltage sensing kinetics. The rational designs improved the sensing dynamic range and kinetics of the Arch photocurrent-knockout mutants, but kinetics remained at ~10 ms, far slower

Table 1

Voltage sensor kinetics and spike detection metrics

Sensor	+ Kinetics		– Kinetics		Experimental excitation wavelength (nm)	$\Delta F/F$ (spike) (%)	Relative brightness	d' (spike)	Bleaching rate % per s	Reference
	τ_{fast} (ms)	P_{fast} (%)	τ_{fast} (ms)	P_{fast} (%)						
Arclight-239	9	50	17	79	488	3	0.2	13	0.6	[11]
ASAP1	2.1	60	2.0	44	488	5	0.1	9	0.3	[13]
QuasAr	0.3	62	0.3	73	640	48	<0.01	NR	NR	[25•]
Archer	<1	NR	NR	NR	655	25–40	<0.01	NR	NR	[26•]
MacQ-mCitrine	2.8	74	5.4	77	500	5	1.0	30	1.3	[28••]
QuasAr-mCitrine	3.1	62	4.8	38	488	~5	NR	NR	NR	[29••]

The '+' and '–' kinetics modeled the voltage sensor's optical responses to depolarizing and hyperpolarizing voltage transients using bi-exponential fits to the experimental data, respectively. In brief, P_{fast} is the percentage of the amplitude associated with the fast component in response to depolarizing and hyperpolarizing voltage transients, and τ_{fast} is the fast time constants of the response to depolarizing and hyperpolarizing voltage transients. NR: not reported.