Title:

Designs and sensing mechanisms of genetically encoded fluorescent voltage indicators.

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Abstract

Neurons tightly regulate the electrical potential difference across the plasma membrane with millivolt accuracy and millisecond resolution. Membrane voltage dynamics underlie the generation of an impulse, the transduction of impulses from one end of the neuron to the other, and the release of neurotransmitters. Imaging these voltage dynamics in multiple neurons simultaneously is therefore critical for understanding how neurons function together within circuits in intact brains. Genetically encoded fluorescent voltage sensors have long been desired to report voltage in defined subsets of neurons with optical readout. In this review, we discuss the diverse strategies used to design and optimize protein-based voltage sensors, and highlight the chemical mechanisms by which different classes of reporters sense voltage. To guide neuroscientists in choosing an appropriate sensor for their applications, we also describe operating trade-offs of each class of voltage indicators.

Introduction

Neurons can encode and transmit information by regulating the electrical field (voltage) across their plasma membrane. Voltage dynamics track both neural inputs and outputs: voltage can be modulated by neurotransmitters released by upstream neurons; in turn, voltage controls whether neurotransmitters will be discharged onto downstream neurons. The central role of voltage as a carrier of neural information thus motivates the development of powerful tools to image voltage transients within individual cells and across large populations. While voltage is most commonly measured with electrodes, recent engineering efforts have substantially improved the ability of protein-based fluorescent sensors to image fast electrical activity in neural tissue. Optical detection of voltage signals with protein-based sensors presents unique opportunities over monitoring voltage with electrodes. First, voltage sensors can image subcellular regions such as dendritic spines or axonal termini that are typically too small to be accessible by standard electrodes. Second, they could enable monitoring of voltage dynamics over thousands or millions of cells. In contrast, electrode arrays have lower spatial resolution given their limited number and density of electrodes. Third, protein-based voltage sensors can restrict visualization to genetically defined cell types of interest, rather than selectively monitoring electrical activity in neurons that happen to be near the recording electrode.

Yet, imaging voltage dynamics with protein sensors also poses several challenges. First, to report on membrane voltage transients, the indicator must be in the plasma membrane, or be tightly coupled to a sensor element in the membrane. As a result, the sensor must hijack the cellular plasma membrane trafficking machinery and avoid accumulating in intermediate organelles such as the endoplasmic reticulum or the Golgi apparatus. Second, voltage transients are often rapid; for example, action potentials last less than a few milliseconds, while neurotransmitter-induced depolarizations typically have time courses of less than tens of milliseconds. Sensors must therefore have sufficiently rapid kinetics and be very sensitive to detect these voltage transients. Finally, voltage indicators must be sufficiently bright and photostable to report voltage dynamics with the required spatiotemporal resolution over the course of an entire experiment. We review different strategies for developing protein-based probes that begin to address these challenges. [We focus on voltage indicators that are fully genetically encoded; voltage-sensitive dyes, and hybrid sensors combining a protein component and a synthetic dye, are reviewed elsewhere \[1,2\].](#)

Sensors exploiting voltage-induced conformational changes in natural voltage sensing domains

In one family of genetically encoded voltage indicators, integral membrane voltage sensing domains (VSDs) are fused to fluorescent proteins from jellyfish or coral. In their native proteins, VSDs either control the opening and closing of ion channels, or the activity of a phosphatase. In all cases, VSDs are composed of four transmembrane helices, with the fourth (S4) containing several positively-charged residues — arginines, or a mixture of arginines and lysines. These residues are sensitive to the electric field, so that S4 moves towards the intracellular or extracellular space upon hyperpolarization and depolarization, respectively. The amplitude of S4 movements is still under debate, with estimates ranging from a 5 to 20 Å translation and a 60 to 120° rotation during action potentials [3,4•,5].

First-generation protein-based voltage indicators coupled a green fluorescent protein (GFP) to full-length voltage-gated ion channels or their isolated VSDs (See [1] for a more comprehensive review). While these sensors exhibited voltage sensitivity when tested in *Xenopus laevis* oocytes [6-8], they were inefficiently expressed at the plasma membrane of mammalian cells [9]. A second generation of voltage sensors, beginning with VSFP2.1 [10••], used a VSD from the voltage-sensitive phosphatase of the sea squirt *Ciona intestinalis* (CiVSD) [11•] . Whereas sodium or potassium channels are assembled from four VSDs and pore domains, CiVSD can exist as a single VSD monomer [12,13]. Many fusions of CiVSD with FPs are efficiently targeted to the plasma membrane, giving rise to new protein-based voltage indicators (Figure 1, left). Some of these novel indicators are based on voltage-sensitive Fluorescence Resonance Energy Transfer (FRET) between two fluorescent proteins (FPs), either in tandem at the

carboxyl terminus of CiVSD [10••,14-16] or each fused to a separate terminus of CiVSD[17,18]. In all cases, the precise mechanism that explains how voltage perturbs FRET is not well understood. Nevertheless, it is plausible that voltage-induced translation and rotation of the S4 helix affects the relative orientation of fused fluorescent proteins, thus modulating FRET efficiency.

While these new CiVSD-based indicators exhibited more efficient plasma membrane targeting than probes based on ion channel VSDs, they still tended to produce some intracellular fluorescent granules, especially during longer-term expression [19,20]. Another limitation of early CiVSD-based FRET voltage sensors was their relatively slow off-kinetics (e.g. 92 ms for VSFP2.3), which hinders accurate reporting of voltage transient durations and discrimination of closely spaced action potentials. Mishina and colleagues replaced segments of CiVSD with homologous sequences from the fast voltage-gated potassium channel Kv3.1 [21•,22]. Some resulting chimeras, e.g. Chimeric Butterfly, accelerated the response kinetics to repolarization (Table 1), while preserving the parental sensor's response amplitude. One effect of the Kv3.1 segments may be to reduce or eliminate VSD relaxation — the slow transition from the active state to a more stable state that occurs upon prolonged depolarization [23,24]. Nevertheless, the sensitivity to voltage of these and other FRET-based voltage indicators remains limited, especially for detection of rapid voltage transients in neurons. **For example, although the FRET sensor Mermaid2 yields the highest dynamic range (~49% in YFP/CFP fluorescence ratio) and fastest kinetics ($\tau_{on, fast} \sim 0.9$ ms) of this sensor group, it only produced 2.6% fractional change in YFP fluorescence in response to action potentials in dissociated neurons [17](Table 1).**

In other indicators, voltage modulated the brightness of a single fluorescent protein fused to the carboxyl terminus of CiVSD [20,25,26]. While most sensors of this design showed limited ($< 2\%$) sensitivity to voltage, an unintended point mutation in a fusion between CiVSD and the pH-sensitive ecliptic pHluorin GFP increased the response amplitude of its voltage response [27••]. Specifically, replacing an alanine with an aspartic acid near the C-terminus of ecliptic pHluorin (A227D) increased this sensor's voltage response by 14-fold to 18.1% over a physiological range (-70 mV to +30 mV). With further engineering, Jin and colleagues increased the sensitivity, brightness and photostability of this sensor, which they named ArcLight. The mechanism by which A227D improves voltage sensitivity is not well understood. It was suggested that voltage might impact fluorescence by altering the putative ability of the pHluorin to associate with the membrane, or by controlling the formation of dimers between the fluorescent proteins of distinct sensor molecules [28]. Like most VSD-based sensors, ArcLight has both a fast and slow fluorescence response to voltage changes (Table 2). However, even the fast components of the response kinetics of ArcLight are appreciably slower (> 9 ms for both depolarization and repolarization) than the duration of action potentials (~ 1 ms [29]). Significant effort has therefore focused on increasing ArcLight's speed so as to improve its ability to detect single and trains of action potentials. Combinatorial analysis of several mutations of CiVSD in ArcLight reduced the magnitude of the slow component, resulting in a new probe, Bongwoori, with more accurate representation of the underlying voltage signal [30]. However, Bongwoori has fast kinetics that are still slower than the timescale of action potentials, and also exhibits large changes in baseline fluorescence during trains of action potentials. In a separate study, CiVSD was replaced with a homologous VSD from chicken, improving ArcLight's kinetics ($\tau_{on} \sim 4$ ms, $\tau_{off} \sim 9$ ms) but reducing its response amplitude from $\sim 35\%$ to $\sim 9\%$ [31].

Circular permutation is a rearrangement whereby the N- and C- termini of a protein are relocated to different sites in the protein [32]. Circularly permuted FPs (cpFPs) may be one way to more directly couple conformational changes in VSDs to fluorescence, to improve either speed or response amplitude. In cpFPs where termini have been brought closer to the chromophore, fluorescence can be sensitive to conformational changes in fused domains: by allostery, these structural changes can perturb the positioning of amino acids near the chromophore that influence its protonation state and thus, its excitation spectra [32]. cpFPs have thus formed the basis of new probes sensitive to a variety of molecules including calcium [33,34]. However, initial designs with cpFPs coupled to the VSD C-terminus produced sensors with low sensitivity to voltage. For example, cpGFP-based sensors produced fast (< 2

ms) but small responses (<2% to 100 mV depolarizations) [33], while cpRFP-based sensors produced responses that were both small and slow [34].

Some of us (F.S.-P., M.Z.) recently reported a new design where the circularly permuted GFP was inserted in an extracellular loop rather than the C-terminus of the VSD [35••]. This loop was predicted to be sensitive to voltage based on crystal structures [4•] and simulations [3] of homologous VSDs. The resulting new probe, Accelerated Sensor of Action Potentials 1 (ASAP1), has high sensitivity to voltage (~18-29%) and kinetics well-matched to action potentials ($\tau_{\text{on, fast}} \sim 2\text{ms}$, $\tau_{\text{off, fast}} \sim 2\text{ms}$). The combination of high dynamic range and rapid kinetics produced larger responses to action potentials compared to other VSD-based sensors [35••, 36••]. While the mechanism of ASAP1 is unknown, it is plausible that voltage-induced conformational changes in the S3-S4 loop [3, 4•] impact the protonation state of the chromophore, changing its absorbance at typical GFP-excitation wavelengths [37].

Sensors exploiting voltage-sensitive photophysical properties of microbial rhodopsins

A surprising development came in 2011 with the demonstration that microbial rhodopsins can be repurposed as voltage indicators (Figure 1, right column). Rhodopsins are light-sensitive membrane proteins composed of 7 transmembrane α -helices; they normally function as light-driven ion pumps, channels or light sensors [38]. They have met broad adoption over the last decade for optical control of neural activity, a technology called optogenetics [39]. The light sensitivity of these proteins comes from the retinal chromophore covalently bound via Schiff-base linkage to a lysine residue in the protein core. In the light-driven proton pump bacteriorhodopsin, light absorption drives all-*trans* retinal isomerization to the 13-*cis* form, initiating a cyclic series of reactions (the photocycle) that ultimately leads to unidirectional translocation of a proton across the membrane. During this photocycle, the retinal Schiff base becomes transiently deprotonated when it donates a proton to an acceptor on the extracellular side (D85). Subsequent reprotonation of the Schiff base from a proton donor on the cytoplasmic side (D96), followed by thermal isomerization back to all-*trans* state resets the cycle.

Deprotonation of the Schiff base causes a dramatic shift in optical properties of bacteriorhodopsin, reducing its light absorption maximum from ~600 nm to ~400 nm [40]. In the D85N mutant, where the Schiff-base proton is more loosely bound, an external electric field can modulate the protonation state of the Schiff base, thus changing its color (absorption) [40]. Cohen and colleagues reasoned that physiological changes in the plasma membrane potential, rather than application of an external electric field, would also cause a shift in the rhodopsin optical properties. To demonstrate rhodopsin-based voltage sensing, they chose the green-absorbing proteorhodopsin D97N mutant, a homologue of bacteriorhodopsin D85N that lacks light-induced proton pumping and displays weak fluorescence upon protonation of the retinal Schiff base. This variant reported voltage fluctuations in bacteria as hypothesized [41••], but did not traffic properly to the plasma membrane in eukaryotic cells [42••].

Improved membrane localization was achieved by using a different opsin: Archaeorhodopsin 3 (also known as Arch), a light-driven proton-pump from *Halorubrum sodomense* that is commonly used to silence neurons [43]. Mechanistic characterization of the Arch photocycle suggests that voltage does not affect the ground state, but instead may modulate equilibrium between two proposed photocycle intermediates, a deprotonated M state and a protonated N state [44]. With the D95N mutation, Arch is able to sense voltage without passing protons, and can report individual action potentials *in vitro* [43]. Like many VSD-based sensors, Arch D95N kinetics are characterized by a fast and a slow component. At 25°C, the response kinetics of Arch D95N are dominated by its slow (30-36 ms) time constant. However, at 35°C, the contribution of the fast component (< 1 ms time constant) increases to ~55% of the response. Further mutations of amino acids that modulate the charge of the Schiff base yielded improved sensitivity and kinetics, increasing the fluorescence response to action potentials by 3-4 fold *in vitro* at room temperature [45]. However, the main drawback of these opsin-derived sensors is their low brightness; for example, the quantum yield of Arch D95N is two to three orders of magnitude lower than EGFP. These

initial Arch-based sensors thus require intense laser illumination *in vitro* and may not be detectable *in vivo*.

The low brightness of rhodopsin-based sensors motivated subsequent protein engineering efforts. A screen of mutant Arch variants identified brighter indicators with improved voltage sensitivity and speed (QuasArs) [46••]. Remarkably, one of the QuasArs surpassed even the speed of wild type Arch, with unprecedented ~0.05 ms response to step changes in voltage. In a different approach, mutations that produced brighter fluorescence in the *Gloeobacter violaceus* rhodopsin were transferred to Arch[47•]. The resulting voltage sensors, named Archers (Arch with enhanced radiance), exhibited high fluorescence, high voltage sensitivity and fast kinetics [48••].

Despite the success at improving their brightness, rhodopsin-based sensors remain dimmer than many VSD-based sensors by ~2 orders of magnitude, limiting their use *in vivo*. An alternative approach leverages the possibility of FRET between a bright fluorescent protein donor fused to a rhodopsin acting as a FRET acceptor [36••,44,49••]. In Arch, depolarization favors protonation of the retinal Schiff base, increasing its absorption of yellow-orange light. Emission from a yellow- or orange-emitting FP would thus be quenched upon depolarization, because the improved overlap between FP emission and rhodopsin absorbance increases FRET. Unlike ratiometric sensors, fluorescence is typically only monitored from the much brighter fluorescent protein, with rhodopsin considered a dark partner. Sensors based on this design are bright enough to detect voltage dynamics in brain slices and in mouse brain [36••,49••]. However, membrane localization remains imperfect, with intracellular aggregates visible in many transfected neurons. Moreover, the response amplitude of the response of the FP is lower than that of the standalone opsin, consistent with suboptimal FRET efficiency (~18% for QuasAr2-Citrine) [36••]. Attempts to improve FRET efficiency by shortening the linker between opsin and fluorescent protein **improved response amplitude but reduced FP brightness, either because of impaired folding and/or higher basal FRET efficiency** [36••]. Although the voltage-dependent kinetics of retinal fluorescence remained unchanged in the context of the fusion (e.g. 1.2 ms for QuasAr2), the response of the attached fluorescent protein was significantly slower (e.g. 4.3 ms for QuasAr2-mOrange2). These slower kinetics may be due to putative slow voltage-dependent changes in distance or orientation — and therefore in FRET efficiency — between the opsin and the attached fluorescent protein.

Future directions

Given the role of voltage for encoding, transforming and propagating information in animal brains, high performance genetically encoded voltage indicators are on the wish lists of many neuroscientists[50]. Interestingly, protein engineers have leveraged not one, but two distinct voltage sensing mechanisms for developing protein-based voltage sensors: voltage-sensitive conformational states, and voltage-sensitive photophysical states. These sensors are beginning to be used to capture neural voltage dynamics *in vivo* in a variety of model systems including worms [48••], flies [51•], and mammals [17-19,22,49••,52,53•,54,55]. **Voltage sensors are also beginning to be used to monitor cardiac electrical activity *in vitro* [56] and in living zebrafish [57,58].**

Many challenges remain. First, the ability to image electrical activity with two-photon laser scanning microscopy — an important technique for deeper tissue penetration and lower out-of-focus fluorescence — has been established for some sensors based on VSDs [53•,55] but not for opsin-based sensors. Second, to be a true replacement of electrodes, voltage indicators should be able to report absolute voltage, not only *in vitro* [59] but also *in vivo*. Finally and more generally, robust *in vivo* imaging of voltage transients at high spatiotemporal resolution and without averaging remains a major challenge for all sensor classes.

Meeting these challenges will be difficult given the complicated photophysics of opsins' photocycle [44] and our incomplete understanding of voltage-driven conformational changes in VSDs. Better mechanistic understanding of the basis of voltage sensing may prove useful in guiding rational improvements. As with the development of genetically encoded calcium indicators, it is also likely that

higher-throughput approaches will play a critical role in improving sensor performance [60,61]. We look forward with great anticipation to new voltage indicators to expand our current toolbox, and to the deployment of current sensors to understand the role of membrane potential changes in microbes, cardiac cells and neurons.

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Table 1 | Properties of selected ratiometric voltage indicators

Sensors ^a	ref. ^b	Dynamic range (% ΔR/R) ^c	Depolarization ^d			Repolarization ^d
			τ _{fast} (ms)	% fast	τ _{slow} (ms)	τ _{off} (ms) ^e
Tandem FRET pair						
VSFP2.3	[21]	~ -13	3.0	26.6	69.2	91.6
VSFP-CR	[15]	~ -12	5.4	ND	59.5	~90 ^f
CiVSD-Kv3.1 chimera (C5)	[21]	~ -7.5	2.1	60.1	36.8	13.4
Non-tandem FRET pair						
VSFP-Butterfly 1.2	[18]	~ -8	1.0	40.9	12.2	89.9
Chimeric Butterfly cyan-yellow	[22]	~ -8	2.1	60	36.7	14.6
Mermaid2	[17]	-48.5	0.92	79	13	10.3

^aAll measurements done at 33-34°C in PC12 cells except Mermaid2 (HEK 293A cells) and VSFP-CR (neurons, 20°C).

^bSource of data unless otherwise noted. ^c $\Delta R/R$ from -70 to +30 mV estimated from data for all sensors except Mermaid2.

^dKinetics were not evaluated using identical voltage steps for all sensors. ^eTime constant obtained by fitting to single exponential decay function. ^fEstimated from data.

Table 2 | Properties of selected non-ratiometric voltage indicators

Sensors ^a	ref. ^b	Dynamic range (% ΔF/F) ^c	Depolarization ^d			Repolarization ^d		
			τ _{fast} (ms)	% fast	τ _{slow} (ms)	τ _{fast} (ms)	% fast	τ _{slow} (ms)
VSDs fused with FP								
Arclight Q239	[27,36]	-35, -31.6	9, 28	50, 30	48, 271	17, 104	79, 61	61, 283
Bongwoori	[30]	~ -16 ^e	8	91	ND	7 ^f	100	
ASAP1	[35]	-17.5, -28.8 ^g	2.1	60.2	71.5	2.0	43.7	50.8
Rhodopsins								
Arch D95N	[44]	60 ^h	< 1	20	36	< 1	80	20
QuasAr1	[46]	32	0.05	94	3.2	0.07	88	1.9
QuasAr2	[46]	90	1.2	68	11.8	80	15.9	15.9
Rhodopsins fused with FP								
QuasAr2-mOrange2	[36]	-10	3.9	27	60	4.3	45	26
QuasAr2-Citrine	[36]	-13.1	3.1	62	21	4.8	38	21
MacQ-mCitrine	[49]	~ -20 ⁱ	2.8 ^j	71 ^j	74 ^j	5.4 ^j	77 ^j	67 ^j

^aAll measurements done in HEK293 cells at 22-25°C, except Bongwoori (34°C) and the first set of values for Arclight (34°C).

^bSource of data unless otherwise noted. ^c $\Delta F/F$ measured from -70 to +30 mV. Values with tildes (~) were estimated from data

^dKinetics were not evaluated using identical voltage steps for all sensors. ^eEstimated from a single representative trace ^fTime constant obtained by fitting to single exponential decay function. ^gFrom Ref. [36]. ^hFrom Ref. [46]. ⁱY. Gong, pers. comm.

^jMeasurements done in neurons. ND = not determined.

Figure 1. Designs for genetically encoded voltage indicators. Examples are shown for different classes of voltage sensors based on voltage-sensing domains (left column) and microbial rhodopsins (right column). The upper half of the figure depicts sensors that require voltage-sensitive FRET between two fluorescent proteins (left) or a fluorescent protein and a rhodopsin (right). The bottom half of the figure shows sensors based on a single chromophore from a green fluorescent protein (left) or a rhodopsin (right). CFP, YFP, GFP and RFP are cyan, yellow, green and red fluorescent proteins, respectively. cpGFP, circularly permuted GFP. Protonation of rhodopsin's retinal Schiff base is depicted as a circled proton (H^+). Intensity of fluorescence emission is depicted using the size of wavy arrows. For FPs, lower fluorescence emission is also shown with greyed-out rather than colored cylinders. Absorption and emission spectra are inspired by Ref. [41,42].

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•• A systematic investigation of voltage sensors based on FRET between fluorescent proteins and a rhodopsin. The authors tried multiple fusions between an engineered Arch rhodopsin and several fluorescent proteins spanning the spectrum from cyan to red emission. The resulting sensors should be bright enough for imaging *in vivo*, but have decreased dynamic range and kinetics compared to rhodopsin-based sensors without fluorescent protein domain.

[37] Haupts U, Maiti S, Schwille P, Webb WW: **Dynamics of fluorescence fluctuations in green fluorescent protein observed by fluorescence correlation spectroscopy.** *Proc Natl Acad Sci U S A* 1998, **95**:13573-13578.

[38] Ernst OP, Lodowski DT, Elstner M, Hegemann P, Brown LS, Kandori H: **Microbial and animal rhodopsins: structures, functions, and molecular mechanisms.** *Chem Rev* 2013, **114**:126-163.

[39] Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K: **Millisecond-timescale, genetically targeted optical control of neural activity.** *Nat Neurosci* 2005, **8**:1263-1268.

[40] Kolodner P, Lukashev EP, Ching YC, Rousseau DL: **Electric-field-induced Schiff-base deprotonation in D85N mutant bacteriorhodopsin.** *Proc Natl Acad Sci U S A* 1996, **93**:11618-11621.

[41] Kralj JM, Hochbaum DR, Douglass AD, Cohen AE: **Electrical spiking in Escherichia coli probed with a fluorescent voltage-indicating protein.** *Science* 2011, **333**:345-348.

•• The first demonstration that microbial rhodopsins can be repurposed as fluorescent voltage indicators. The authors also report surprising spontaneous voltage activity in bacteria.

[42] Kralj JM, Douglass AD, Hochbaum DR, Maclaurin D, Cohen AE: **Optical recording of action potentials in mammalian neurons using a microbial rhodopsin.** *Nat Methods* 2012, **9**:90-95.

•• This study constitutes the first report use of a microbial rhodopsin to image voltage dynamics in neurons. This sensor, Arch(D95N), can track voltage transients in dissociated neurons with high fidelity.

[43] Chow BY, Han X, Dobry AS, Qian X, Chuong AS, Li M, Henninger MA, Belfort GM, Lin Y, Monahan PE, Boyden ES: **High-performance genetically targetable optical neural silencing by light-driven proton pumps.** *Nature* 2010, **463**:98-102.

[44] Maclaurin D, Venkatachalam V, Lee H, Cohen AE: **Mechanism of voltage-sensitive fluorescence in a microbial rhodopsin.** *Proc Natl Acad Sci U S A* 2013, **110**:5939-5944.

[45] Gong Y, Li JZ, Schnitzer MJ: **Enhanced Archaelrhodopsin Fluorescent Protein Voltage Indicators.** *PLoS One* 2013, **8**:e66959.

[46] Hochbaum DR, Zhao Y, Farhi SL, Klapoetke N, Werley CA, Kapoor V, Zou P, Kralj JM, Maclaurin D, Smedemark-Margulies N et al.: **All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins.** *Nat Methods* 2014, **11**:825-833.

•• Impressive protein engineering efforts led to the development of rhodopsin-based indicators with improved kinetics (QuasAr1) or dynamic range (QuasAr2) over the parental protein (Arch). Both indicators are also brighter than Arch, mitigating one of the main limitations of rhodopsin-based indicators. The improved characteristics of QuasAr sensors enabled monitoring of voltage in brain slices.

[47] McIsaac RS, Engqvist MK, Wannier T, Rosenthal AZ, Herwig L, Flytzanis NC, Imasheva ES, Lanyi JK, Balashov SP, Gradinaru V, Arnold FH: **Directed evolution of a far-red fluorescent rhodopsin.** *Proc Natl Acad Sci U S A* 2014, **111**:13034-13039.

• The authors used directed evolution to improve the fluorescence of the microbial rhodopsin Arch, a protein repurposed as a voltage sensor by Adam Cohen and colleagues. Their brightest variant displayed 10-100-fold improved fluorescence over wild-type Arch in purified preparations. The one variant tested in human embryonic kidney (HEK293) cells was also ~4.5-fold brighter than wild-type Arch.

[48] Flytzanis NC, Bedbrook CN, Chiu H, Engqvist MK, Xiao C, Chan KY, Sternberg PW, Arnold FH, Gradinaru V: **Archaelrhodopsin variants with enhanced voltage-sensitive fluorescence in mammalian and Caenorhabditis elegans neurons.** *Nat Commun* 2014, **5**:4894.

- The authors report two Arch-based voltage indicators (Archer1 and Archer2) with improved baseline brightness and voltage response over a previously reported Arch variant. They also demonstrate that Archer1 can detect voltage transients in the worm *Caenorhabditis elegans*.

[49] Gong Y, Wagner MJ, Zhong Li J, Schnitzer MJ: **Imaging neural spiking in brain tissue using FRET-opsin protein voltage sensors.** *Nat Commun* 2014, **5**:3674.

- An interesting example of a voltage sensor based on FRET between a bright fluorescent protein (mCitrine YFP) and a dark rhodopsin. Whereas Arch had been previously used as a template for rhodopsin-based indicators, the authors used Mac, a light-driven proton pump from the fungus *Leptosphaeria maculans*

[50] Peterka DS, Takahashi H, Yuste R: **Imaging voltage in neurons.** *Neuron* 2011, **69**:9-21.

[51] Cao G, Platasa J, Pieribone VA, Raccuglia D, Kunst M, Nitabach MN: **Genetically targeted optical electrophysiology in intact neural circuits.** *Cell* 2013, **154**:904-913.

- A pioneering demonstration of voltage imaging in the fly using a genetically encoded voltage indicator (ArcLight).

[52] Carandini M, Shimaoka D, Rossi LF, Sato TK, Benucci A, Knöpfel T: **Imaging the awake visual cortex with a genetically encoded voltage indicator.** *J Neurosci* 2015, **35**:53-63.

[53] Akemann W, Sasaki M, Mutoh H, Imamura T, Honkura N, Knöpfel T: **Two-photon voltage imaging using a genetically encoded voltage indicator.** *Sci Rep* 2013, **3**:2231.

- Akemann and colleagues demonstrate that the voltage indicator “VSFP-Butterfly” can detect voltage responses in the mouse cortex. Critically, voltage dynamics could be imaged by two-photon microscopy, an important technique for *in vivo* studies that enables imaging with lower autofluorescence and deeper tissue penetration.

[54] Scott G, Fagerholm ED, Mutoh H, Leech R, Sharp DJ, Shew WL, Knöpfel T: **Voltage imaging of waking mouse cortex reveals emergence of critical neuronal dynamics.** *J Neurosci* 2014, **34**:16611-16620.

[55] Ahrens KF, Heider B, Lee H, Isacoff EY, Siegel RM: **Two-photon scanning microscopy of in vivo sensory responses of cortical neurons genetically encoded with a fluorescent voltage sensor in rat.** *Front Neural Circuits* 2012, **6**:15.

[56] Leyton-Mange JS, Mills RW, Macri VS, Jang MY, Butte FN, Ellinor PT, Milan DJ: **Rapid cellular phenotyping of human pluripotent stem cell-derived cardiomyocytes using a genetically encoded fluorescent voltage sensor.** *Stem Cell Reports* 2014, **2**:163-170.

[57] Hou JH, Kralj JM, Douglass AD, Engert F, Cohen AE: **Simultaneous mapping of membrane voltage and calcium in zebrafish heart in vivo reveals chamber-specific developmental transitions in ionic currents.** *Front Physiol* 2014, **5**:344.

[58] Tsutsui H, Higashijima S, Miyawaki A, Okamura Y: **Visualizing voltage dynamics in zebrafish heart.** *J Physiol* 2010, **588**:2017-2021.

[59] Hou JH, Venkatachalam V, Cohen AE: **Temporal dynamics of microbial rhodopsin fluorescence reports absolute membrane voltage.** *Biophys J* 2014, **106**:639-648.

[60] Tsutsui H, Jinno Y, Tomita A, Okamura Y: **Rapid evaluation of a protein-based voltage probe using a field-induced membrane potential change.** *Biochim Biophys Acta* 2014, **1838**:1730-1737.

[61] Park J, Werley CA, Venkatachalam V, Kralj JM, Dib-Hajj SD, Waxman SG, Cohen AE: **Screening fluorescent voltage indicators with spontaneously spiking HEK cells.** *PLoS One* 2013, **8**:e85221

Figure 1