Linker length and fusion site composition improve the optical signal of genetically encoded fluorescent voltage sensors

Arong Jung
Jessica E. Garcia
Eunha Kim
Bong-June Yoon
Bradley J. Baker

Linker length and fusion site composition improve the optical signal of genetically encoded fluorescent voltage sensors

Arong Jung,[a] Jessica E. Garcia,[a] Eunha Kim,[a] Bong-June Yoon,[b] and Bradley J. Baker[a,*]
[a]Korea Institute of Science and Technology, Center for Functional Connectomics, Hwarangno 14-gil 5, Seoul 136-791, Republic of Korea
[b]Korea University, College of Life Sciences and Biotechnology, Anam-dong, Seongbuk-gu, Seoul 136-701, Republic of Korea

Abstract. Several genetically encoded fluorescent sensors of voltage were created by systematically truncating the length of the linker sequence between the voltage-sensing domain and the position of the fluorescent protein, Super Ecliptic A227D. In addition to varying the length, the amino acid composition at the fusion site for the fluorescent protein was modified. Both linker length and amino acid composition affected the size and voltage sensitivity of the optical signal. The truncation mutants revealed a potential structural periodicity with a maximum signal three amino acids from the voltage-sensing domain and another maximum 11 amino acids from the voltage-sensing domain. These results confirm that the linker length and composition can fine tune the size and voltage range of the sensor. The potential periodicity suggests that the orientation of the fluorescent protein could be important for improving the signal size implicating dimerization of the fluorescent protein. © *The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI.* [DOI: 10.1117/1.NPh.2.2.021012]

1 Introduction

In order to faithfully represent neuronal activity, a genetically encoded fluorescent voltage sensor (GeFVS) must respond to changes in membrane potential with a rapid and large fluorescent change. A rapid fluorescent change is required since action potentials only last 1 to 2 ms. A large fluorescent change is needed since the total amount of responsive probe is limited to the plasma membrane.

1.1 ArcLight Gives a Large Voltage-Dependent Optical Signal, while Zahra Gives a Rapid Voltage-Dependent Optical Signal

The brightest GeFVS consists of a transmembrane, voltage-sensing domain (VSD) coupled to a cytoplasmic, fluorescent protein (FP) via a linker sequence. Improvements in the speed and size of the optical response to membrane depolarizations have been achieved by modifying different regions of the sensor. ArcLight, a GeFVS with one of the largest optical signals reported, yields a 30 to 40% $\Delta F/F/100$ mV depolarization of the plasma membrane.[1,2] This large signal is due to the combination of a mutation to the FP, Super Ecliptic pHlorin A227D (SE 227D) and the optimization of the linker length between the VSD and the FP. While ArcLight gives a large optical signal, it is also slow with taus >20 ms. ArcLight uses the VSD from the *Ciona* voltage-sensing phosphatase (VSP) gene. Some of the fastest GeFVS published utilize the VSD of the zebrafish VSP (Ref. 3) or the VSD from the

chicken VSP.[1,4] Zahra 1 and Zahra 2 are GeFVS containing the cerulean/citrine FP pair that optically reports changes in membrane potential via *Förster* resonance energy transfer (FRET) with extremely rapid kinetics comparable to voltage-sensing dyes but they have small signal sizes.[3] Both Zahra sensors utilize the VSD of the zebrafish VSP gene. In an effort to improve the kinetics of ArcLight, the *Ciona* VSD was replaced with the VSD from zebrafish or chicken.[1] These ArcLight derivatives exhibited improved kinetics but weaker signals. The FP fusion site for the zebrafish ArcLight was nearly 20 amino acids upstream of the fusion site for the Zahra constructs. To test the effect of linker length on the voltage-dependent optical signal, the FRET pair in the Zahra constructs was replaced with SE 227D. In this report, we demonstrate that the length of the linker alters the voltage sensitivity of the optical response, resulting in a slight increase in the size of the fluorescent change. A structural periodicity of eight amino acids was also observed in the linker sequence. The fluorescent signal increased when the FP was inserted three amino acids downstream of the VSD and again increased when the FP was inserted 11 amino acids downstream of the VSD. In addition, the amino acid composition at the fusion site of the FP also affected the fluorescent response. These results suggest that the orientation of the FP is important for the voltage-dependent optical signal.

2 Materials and Methods

2.1 Probe Design

The Zahra 1 SE (227D) and the Zahra 2 SE (227D) were generated by replacing the FRET FPs with SE 227D. The

*Address all correspondence to: Bradley J. Baker, E-mail: bradley.baker19@gmail.com

polymerase chain reaction (PCR) was used to introduce an upstream Eco RV site and a downstream Xho I site for the introduction of SE 227D, resulting in the amino acid composition of RYR at the fusion site of the FP. Two-step PCR was used to generate the RYR truncated constructs, A1 through A7. A single-step PCR process was used to introduce a Bam H1 site and systematically increase the linker length between the S4 of the VSD and the FP, resulting in the JE series of constructs that contain the GDP amino acid sequence at the FP fusion site. For altering the amino acid composition at the FP fusion site, a two-step PCR process was employed, enabling the site-directed mutagenesis of the probe. All constructs were verified by DNA sequencing (Cosmogenetech, Republic of Korea).

2.2 Transient Expression of GeFVS in Mammalian Cells

HEK293 cells were maintained in Dulbecco's modified Eagle medium (high glucose DMEM; Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, USA) and seeded onto #0 coverslips coated with poly-D-lysine (Sigma, USA). Transient protein expression in HEK cells was done by using Lipofectamine 2000 (Invitrogen, California, USA) according to manufacturer instructions.

Primary hippocampal neurons were prepared from C57BL6/F mice (Koatech, South Korea) on embryonic day 17 as described previously.[5] Dissociated neurons were obtained by digesting hippocampi with 0.05% trypsin (Invitrogen, USA) for 10 min at 37°C. Neurons were then dissociated by mechanical trituration through Pasteur pipettes (Hilgenberg, Germany). Neurons were plated at 5×10^4 cells/ml onto 10 mm #0 coverslips (TED PELLA, New Hampshire, USA) precoated for 3 h with poly-D-lysine (Sigma, Missouri, USA) in 0.1 M Borate buffer (pH 8.5). Neurons were cultured at 37°C in Neurobasal medium (Gibco) supplemented with B-27 (Gibco by Life Tech, USA), 0.5 mM Glutamax-I (Gibco by Life Tech, New York, USA), L-glutamic acid (Sigma, USA), and 0.5% FBS. Transient transfections for neurons were performed at 8 to 10 days *in vitro* (DIV 8-10) using Lipofectamine 2000 (Invitrogen, USA) as per manufacturer's instruction. Neurons were usually tested at DIV 9-11 (16 to 24 h post-transfection).

2.3 Electrophysiology

Transfected HEK293 cells were patched at 33°C and perfused with bath solution containing 150 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM D-Glucose, and 5 mM HEPES (pH 7.4). We used 3 to 5 MΩ glass patch pipettes (World Precision Instruments, Florida) that were pulled by a P-97 micropipette puller (Sutter Instrument Company, California). The pipette solution contained 120 mM K-aspartate, 4 mM NaCl, 4 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM EGTA, 3 mM Na$_2$ATP, and 5 mM HEPES (pH 7.2). Whole-cell voltage-clamp recordings of transfected HEK293 cells were done using an EPC10 amplifier (HEKA, Germany). Electrical activity of cultured hippocampal neurons was recorded in current-clamp mode to evoke action potentials.

2.4 Arc Lamp and Wide-Field Imaging

During the patch-clamp experiments, the cells were imaged on an IX71 microscope with a 60×1.35 numerical aperture oil-immersion lens (Olympus, Japan). Fluorescence excitation

was delivered using a 75 W xenon arc lamp (Cairn, England). All constructs utilize the same FP identified in ArcLight, SE (227D).[2] The filter set used was that reported for ArcLight. The excitation filter was FF02-472/30 (Semrock, USA). The emission filter was FF01-496/LP (Semrock, USA). The dichroic was FF495-Di03 (Semrock, USA). The objective C-mount image was demagnified by an Optem zoom system A45699 (Qioptiq LINOS, USA) and projected onto the e2v CCD39 chip of NeuroCCD-SM 80 pixel × 80 pixel camera (RedShirtImaging, LLC, USA). The imaging apparatus was mounted on a Vibraplane Bench Top vibration isolation platform (Kinetic System, USA; Minus K Technology, USA). The mechanical shutter in the incident light patch was mounted on a separate table. Images were recorded at a frame rate of 1 kfps.

2.5 Laser Illumination

For neuronal recordings, excitation light was from a 473 nm laser (MLL-FN-473-50 mW; Changchun New Industries Opto-electronics Tech. Co., Ltd., China). The laser light was guided into the microscope by a quartz light guide (Till Photonics, USA) and an achromatic epifluorescence condenser (Till Photonics, USA). The dichroic, emission filter, and image projection are as described above. The frame rate was either 1 or 2 kfps as described in Fig. 6.

2.6 Optical-Signal Analysis

Optical-signal recordings were analyzed using Neuroplex (RedshirtImaging, USA) Excel (Microsoft, USA) and Origin 8.6 (OriginLab, USA). The fluorescent traces for constructs expressed in HEK cells were the averages of 24 trials. The fluorescent traces for constructs expressed in neuronal cells were from a single trial. The fluorescent traces are the spatial average of the output of all the pixels receiving light from the cell. For neuronal cells, pixels corresponding to the soma were used to limit the variability of cell size. HEK cells ranged from 30 to 50 μm in length, while neuronal somas ranged from 10 to 15 μm in length. The offline low-pass temporal filtering used to improve the signal-to-noise ratio is indicated in the figure legends. For the kinetics, the optical traces were fitted to a double exponential decay,

$$y = y_0 + A_1 e^{-(t-t_0)/\tau_1} + A_2 e^{-(t-t_0)/\tau_2},$$

where t is time in milliseconds, and a single exponential decay,

$$y = y_0 + A_1 e^{-(t-t_0)/\tau_1},$$

where t is time in milliseconds.

To compare the optical responses that were better fitted to a single exponential decay to those better fitted to a double exponential decay, a weighted tau was calculated as the sum of τ_1 multiplied by the relative amplitude, $A1$, plus τ_2 multiplied by the relative amplitude, $A2$, as defined by the following equation:

$$\tau \text{ weighted} = \tau_1[A1/(A1 + A2)] + \tau_2[A2/(A1 + A2)].$$

The voltage sensitivity was determined by initially fitting individual cell responses to the Boltzmann equation:

$$y = (A1 - A2)/(1 + e(x - x_0)/dx) + A2,$$

where y is $-\Delta F/F$, and x is membrane potential in mV. $A1$ is the minimum value, and $A2$ is the maximum value. x_0 is the membrane potential in mV at half maximal $\Delta F/F$, and dx is the slope at x_0. All traces were then normalized such that $A1 = 0$ and $A2 = 1$. The trials for each construct were then averaged and refitted. The range of the fluorescent voltage response listed in Fig. 5 was calculated by extrapolating the $V_{1/2}$ value to the normalized maximum and minimum fluorescent change using the slope of the Boltzmann fit at $V_{1/2}$, where

$$y = (A2 - A1)/4dx.$$

3 Results

3.1 Replacing the Fluorescent FRET Pairs in Zahra 1 and Zahra 2 with the Fluorescent Protein Super Ecliptic A227D Resulted in a Larger Optical Signal with Slower Kinetics

The position of the FP in Zahra was determined by the sequence conservation at the start of the phosphatase domain. Figure 1 shows the alignment of VSP homologs from several different species. Close inspection of this alignment reveals a rather high degree of conservation that is obscured by the *Ciona* sequence. Since conservation suggests function and this region is the start of the phosphatase domain, the FP was inserted at position Y194 (numbering based on zebrafish amino acid position). The Y194 position showed the least amount of conservation among VSP homologs and may, therefore, tolerate the FP better than other positions in the linker. These new constructs are referred to as Zahra 1 SE (227D) and Zahra 2 SE (227D). For comparison purposes, a GeFVS utilizing the wild-type zebrafish VSD sequence was also tested.

The zebrafish SE (227D) only gives an optical response with depolarization steps of over 150 mV from a holding potential of -70 mV (Fig. 1). Zahra 1 SE (227D) and Zahra 2 SE (227D) give a 3 to 5% $\Delta F/F/100$ mV (Fig. 1). Zahra 1 SE (227D) contains a single mutation in the S4 transmembrane domain (R153Q). Zahra 2 SE (227D) has two mutations to the S4 transmembrane domain, the R153Q also present in Zahra 1 and the I165R mutation, which converts the zebrafish sequence in S4 to that of *Ciona* (Fig. 1). The voltage range of the optical response is quite large for both Zahra 1 SE (227D) and Zahra 2 SE (227D) spanning at least 300 mV from -170 mV to $+130$ mV. While having the deleterious effect of diluting the optical signal over a large voltage range, these mutations enable the probes to respond to more physiologically relevant potentials. The signal sizes for Zahra 1 SE (227D) and Zahra 2 SE (227D) are 6- to 10-fold better than their FRET counterparts, which exhibit a 0.5% $\Delta F/F/100$ mV depolarization.[3] The speed of the optical signal is slightly slower than the original Zahra constructs with the τ_{on} around 6 ms and the τ_{off} closer to 7 ms.

3.2 Truncation of the Linker Length Between the S4 Transmembrane Domain and the Fluorescent Protein Improved the Size of the Optical Signal for Zahra 1 SE (227D)

The zebrafish ArcLight derivative gives an 8% $\Delta F/F/100$ mV,[1] which is almost double the size of the Zahra 1 SE (227D) and Zahra 2 SE (227D) constructs. Since the linker length of the zebrafish ArcLight derivative is shorter than Zahra 1 SE (227D), a series of truncations was performed on Zahra 1 SE (227D) to determine the effect of the linker length on the voltage-dependent optical signal. Seven new constructs designated A1 to A7 are shown in Fig. 2(a). The RYR sequence preceding the FP sequence is due to an Eco RV site used for cloning. As can be seen in Fig. 2(b), the general trend was an increase in the optical signal as the linker length became shorter. This was in part due to the $V_{1/2}$ being shifted to more negative potentials. Another contributing factor for the increase in the signal size was the increase in the slope of the voltage sensitivity for the optical signal. Instead of the optical response ranging over 300 mV, several of these truncated probes exhibited ranges of under 200 mV [Fig. 2(c)]. Truncation of the linker did not significantly affect the speed of the response with the on and off taus ranging from 5 to 7 ms [Fig. 2(d)].

3.3 Amino Acid Composition at the Fluorescent Protein Fusion Site Affects the Size of the Optical Signal

Another difference between the zebrafish ArcLight derivative and Zahra 1 SE (227D) is the amino acid composition at the FP fusion site. This difference is due to the restriction enzyme used to fuse the FP onto the VSD. ArcLight was constructed using a Bam H1 restriction site, which results in a GDP preceding the methionine of the FP.[1,2] The Zahra constructs used an Eco RV site to preserve the RYR sequence at the homologous position in the *Ciona* linker[3] (see Fig. 1). To test whether the amino acid composition at the fusion site affected the optical signal, 22 novel constructs were created, which systematically truncated the linker by a single amino acid. In these linker truncations designated JE01 through JE22, a GDP preceded the FP [Fig. 3(a)]. Note that JE22 contains the same linker sequence as Zahra 1 SE (227D) except that the RYR sequence has been replaced with the GDP sequence at the fusion site. JE05 is equivalent to the zebrafish ArcLight derivative.[1]

Many of the truncations (but not all) give a stronger optical signal. Once again, this is partly due to a shift in the voltage sensitivity of the construct [Fig. 3(c)]. Plotting the signal strength for a 100 mV depolarization step versus linker length revealed a repeating unit consisting of eight amino acids [Fig. 3(d)]. JE03 gave the largest signal of $\sim$12% $\Delta F/F/100$ mV. Subsequent increases in the linker length resulted in a steady decline of the optical signal to 6% $\Delta F/F/100$ mV for JE10. JE11 jumps to 9% and subsequent amino acid additions again result in a steady decline of the optical signal. The effect of the truncations on speed was marginal with all truncations ranging from 5 to 8 ms [Fig. 3(e)].

3.4 Amino Acid Composition at the FP Fusion Site Affects the Voltage-Dependent Optical Signal

While shortening the linker length improved the signal size, the restriction enzyme used to fuse the FP onto the linker also contributed to differences in the optical signal. Figure 4 compares five linker lengths that differ only at the fusion site. The overlapping traces are shown with the standard error of the mean in faded color. For the longer linker lengths tested, the GDP amino acid sequence at the fusion site yielded a larger signal than the RYR amino acid sequence, while the optical signal for RYR construct plateaued at lower voltage steps. For the shortest linker length comparing the GDP to RYR fusion site, the RYR
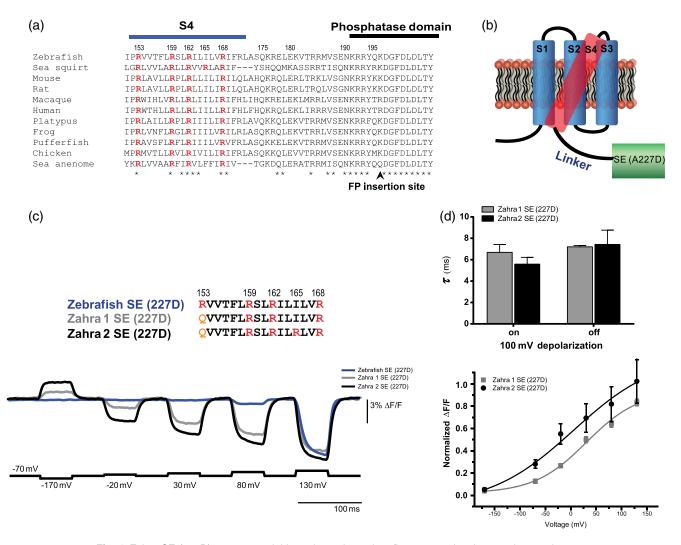
Fig. 1 Zahra SE (227D) constructs yield a voltage-dependent fluorescent signal over a large voltage range. (a) Partial amino acid alignment of the voltage-sensing phosphatase from the voltage-sensing domain (VSD) to the phosphatase domain for several species. The arrow denotes the fusion site for the fluorescent protein (FP), SE 227D. (b) Schematic diagram of the genetically encoded fluorescent voltage sensor. (c) Representative optical responses to a series of voltage steps from Zebrafish SE (227D), Zahra 1 SE (227D), and Zahra 2 SE (227D). The S4 sequence of the VSD showing the R153Q mutation for Zahra 1 SE (227D) and the R153Q/I165R mutations for Zahra 2 SE (227D). (d) The on and off taus for both Zahra 1 SE (227D) and Zahra 2 SE (227D) as well as the voltage range of the fluorescent signal.

construct plateaued at a lower voltage step and yielded a slightly stronger optical signal for the 50 mV depolarization step. Comparison of the zebrafish ArcLight derivative (JE05 – GDP fusion sequence) to the RYR fusion sequence did not exhibit a difference.

To determine if a single amino acid in the fusion sequence was responsible for the different voltage-dependent optical signals, 10 new constructs that varied the fusion site amino acid sequence were tested. Since the RYR versus GDP differences were slightly more pronounced in the longer linker constructs, the linker length of the JE18 construct was used to compare the effects of modifying the three amino acids at the FP fusion site (Fig. 5). Mutating the first position of the fusion site from GDP to RDP shifted the $V_{1/2}$ from 27 ± 5 to 3 ± 1 mV. Mutating the middle position from GDP to GYP also shifted the $V_{1/2}$ to 6 ± 3 mV. Changing the final position of GDP to GDR resulted in less of a shift to 19 ± 1 mV. Converting RYR to the GYR fusion sequence had the most dramatic effect on signal size and

speed. The GYR fusion sequence gave the largest optical signal for a 100 mV depolarization ($13 \pm 1\%$ $\Delta F/F$). This GYR construct was also the slowest. This was due to the presence of a slow component not seen in the other constructs. The GYR fusion sequence was the only construct that exhibited kinetics better fit by a double exponential. The fast component of GYR was 5 ± 1 ms accounting for $51 \pm 2\%$ of the total optical amplitude. The slow component for the GYR fusion sequence was 49 ± 2 ms.

3.5 Truncated Constructs Are Able to Optically Resolve Action Potentials in Hippocampal Neurons

Truncated constructs were transfected into dissociated hippocampal neurons that were subjected to current clamp to generate action potentials. Figure 6 shows a comparison of zebrafish ArcLight to that of A6 and A7. A 50 mW laser was used to image action potentials as the 75 W xenon arc lamp was
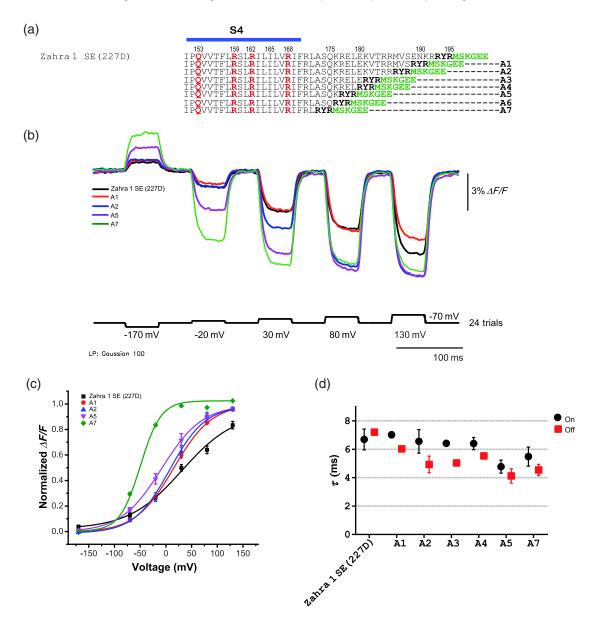
(a)

(b)

(c)

(d)

Fig. 2 Truncations to the linker segment between the VSD and the FP in Zahra 1 SE (227D) shift the voltage sensitivity of the optical response. (a) Partial amino acid sequences of the Zahra 1 SE (227D) and the A1–A7 linker truncation series of constructs from the S4 domain to the FP domain. The red amino acids depict the R153Q mutation and the positive arginine residues in S4. The FP fusion site sequence is in bold. The FP sequence is in green. (b) Representative fluorescent traces in response to a series of voltage steps from several truncation constructs compared to Zahra 1 SE (227D). (c) Boltzmann fit of the normalized optical response of several A truncation series constructs and the Zahra 1 SE (227D) construct. (d) The on and off time constants for 100 mV depolarization step shows little change in the kinetics of the optical signal.

unable to optically resolve action potentials from subthreshold depolarizations (data not shown). All three probes are capable of giving rapid optical responses to a train of action potentials in a single trial. Given the speed of the optical signal, the sampling frequency was doubled to 2000 fps. Even though the higher sampling rate decreases the signal-to-noise ratio, A6 is still able to optically resolve action potentials in an unfiltered trace.

4 Discussion

4.1 Honoring Our Mentor

In 2003, one of the authors (B.J.B.) joined Larry Cohen's lab to generate a transgenic mouse expressing a GeFVS. The goal was to measure the output of mitral cells in the olfactory bulb. That project proved to be about a decade too early as those probes failed to work in mammalian cells.[6] Rather than being discouraged, Larry gathered the laboratories competing to develop these probes and convinced them to work together in order to avoid repeating the same failures over and over. One of those labs solved the trafficking problem by utilizing the newly discovered VSD from the *Ciona* VSP.[7] Another quantum leap was made by the joint efforts of the Pieribone and Cohen labs. A point mutation in a stable cell line resulted in a 15-fold increase in the optical signal of a probe containing a single FP. Optimizing the position of the FP improved the optical signal to 30 to 40% $\Delta F/F/$ 100 mV depolarization and was named ArcLight.[1,2]

In this issue, to honor our mentor and friend, we present our efforts to understand how the linker sequence affects the optical signal of the fast zebrafish probes first developed in the Cohen lab.[3] By replacing the fluorescent proteins in the Zahra probes with SE 227D, a better signal-to-noise was achieved with a slightly slower optical response. Since ArcLight was improved by varying the linker length, we employed the same strategy. None of the truncations vastly improved the speed of the response. However, the size of the optical signal and the voltage range of the fluorescent response were affected by varying the linker length and the amino acid composition of the FP fusion site.

4.2 R153Q Mutation in the Zebrafish S4 Domain Extends the Range of the Voltage-Dependent Optical Signal to 300 mV

The Zahra 1 SE (227D) and Zahra 2 SE (227D) probes exhibit a gradual decrease in fluorescence as the membrane potential is
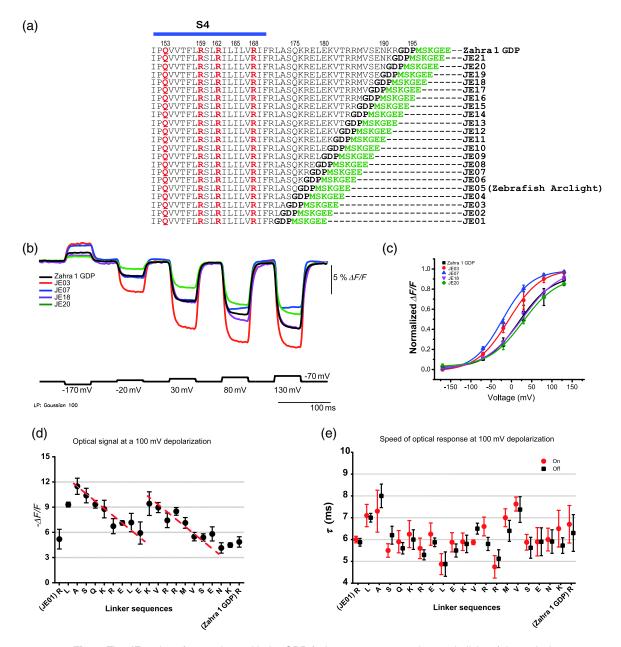

Fig. 3 The JE series of truncations with the GDP fusion sequence reveals a periodicity of the optical signal as the linker length changes. (a) Partial amino acid sequences of the Zahra 1 SE (227D) and the JE linker truncation series of constructs from the S4 domain to the FP domain. The red amino acids depict the R153Q mutation and the positive arginine residues in S4. The FP fusion site sequence is in bold. The FP sequence is in green. JE05 is the same as zebrafish ArcLight.[1] (b) Representative fluorescent traces from several JE truncation constructs in response to a series of voltage steps compared to Zahra 1 SE (227D). (c) Boltzmann fit of the normalized optical responses from several JE truncation series compared to the original length of the Zahra 1 SE (227D) construct. (d) The fluorescent signal for a 100 mV depolarization step plotted versus linker length. (e) The on and off time constants for 100 mV depolarization step.
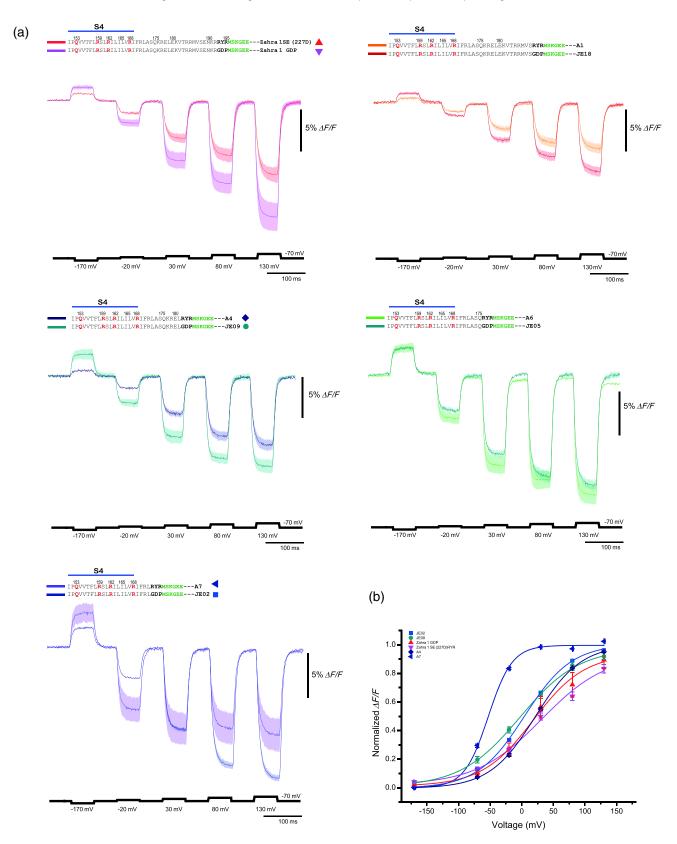
(a)

Fig. 4 Comparison of the A series of truncations (RYR fusion site) and the JE series of truncations (GDP fusion site). (a) Fluorescent responses from two constructs with the same linker length that differ at the fusion site are overlaid. The dark color is the average of at least three cells. The faded color is the standard error of the mean. (b) The Boltzmann fit of the voltage-dependent optical signals for the traces shown in (a).

(a) IP**Q**VVTFL**R**SL**R**ILILV**R**IFRLASQKRELEKVTRRMVS**XXX****MSKGEE**

Amino acid composition

(b)

(c)

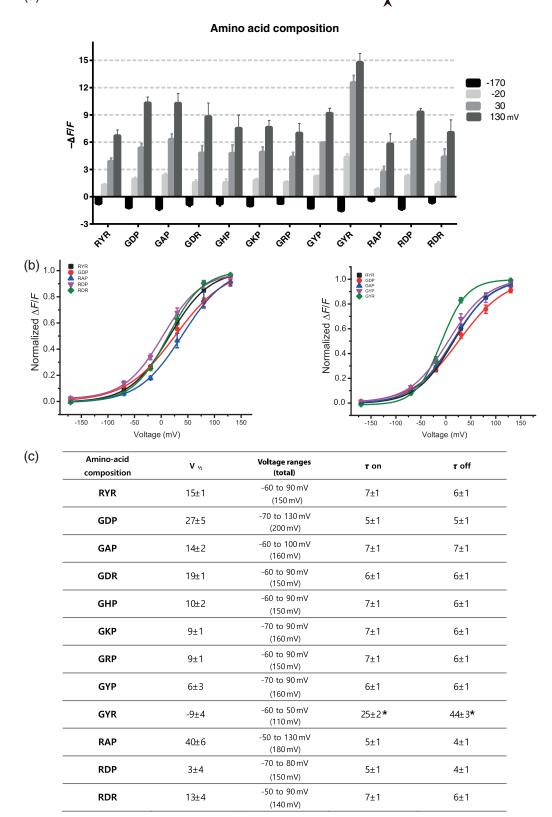| Amino-acid composition	V ½	Voltage ranges (total)	τ on	τ off
RYR	15±1	-60 to 90 mV (150 mV)	7±1	6±1
GDP	27±5	-70 to 130 mV (200 mV)	5±1	5±1
GAP	14±2	-60 to 100 mV (160 mV)	7±1	7±1
GDR	19±1	-60 to 90 mV (150 mV)	6±1	6±1
GHP	10±2	-60 to 90 mV (150 mV)	7±1	6±1
GKP	9±1	-70 to 90 mV (160 mV)	7±1	6±1
GRP	9±1	-60 to 90 mV (150 mV)	7±1	6±1
GYP	6±3	-70 to 90 mV (160 mV)	6±1	6±1
GYR	-9±4	-60 to 50 mV (110 mV)	25±2 *	44±3 *
RAP	40±6	-50 to 130 mV (180 mV)	5±1	4±1
RDP	3±4	-70 to 80 mV (150 mV)	5±1	4±1
RDR	13±4	-50 to 90 mV (140 mV)	7±1	6±1

Fig. 5 Changing the FP fusion site amino acid composition alters the $V_{1/2}$ and total voltage range of the optical signal. (a) The fluorescent response from several different probes containing the same linker length but differing at the FP fusion site (XXX). The FP sequence is in green and the positive charges in S4 are red. (b) Boltzmann fits of the constructs in A. (C) The $V_{1/2}$, voltage range, and time constants for a 100 mV depolarization step are listed for the constructs in A. * denotes the one construct where the kinetics of the optical response were better fit by a double exponential. The weighted time constants are shown for the GYR fusion site construct.

varied from −70 to +130 mV. The wild-type zebrafish VSD fused to SE (227D) results in a probe that requires a strong depolarization of 150 mV to illicit an optical signal but has a much steeper voltage sensitivity than the Zahra probes [Fig. 1(c)]. The R153Q mutation in S4, therefore, is responsible for dramatically reducing the slope of the voltage sensitivity and expands the range of the fluorescence response to over 300 mV for the Zahra probes. The R153Q mutation exhibited a similar effect on the enzymatic activity of the zebrafish VSP.[8] Unfortunately, this also dilutes the signal since the maximal fluorescent signal has not changed.

4.3 Altering the Linker Length Between the VSD and the FP Changed the Voltage Sensitivity of the Probe

The original Zahra 1 and 2 FRET probes also exhibited a very large voltage range. Replacing the FRET pair with a single FP did not change the range of the voltage-dependent optical signal even though the carboxyl-terminal cytoplasmic region was reduced by over 200 amino acids. However, truncations to the region between the VSD and the FP improved the optical signal in part by reducing the range of the optical response
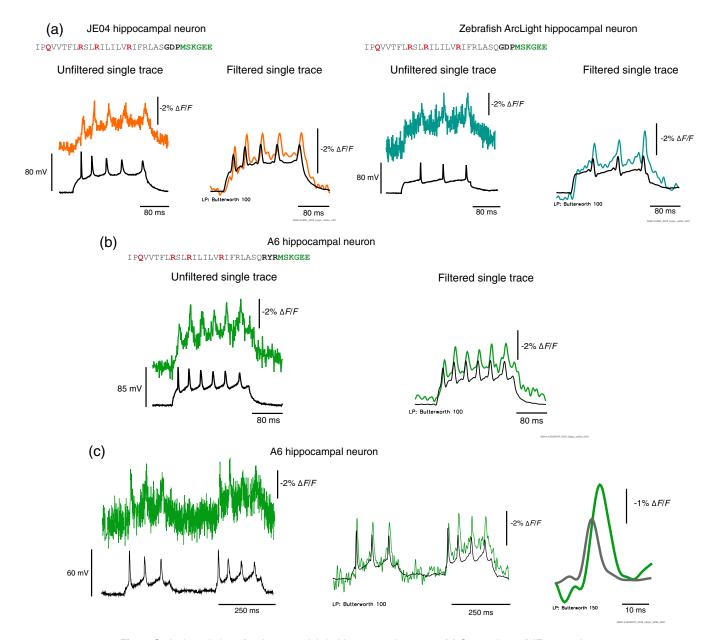

Fig. 6 Optical resolution of action potentials in hippocampal neurons. (a) Comparison of JE04 to zebrafish ArcLight. Orange is fluorescent trace for JE04. Cyan is fluorescent trace of zebrafish ArcLight. Black trace is voltage. Both probes have the GDP amino acid sequence at the FP fusion site and are capable of rapidly responding to action potentials. The increased noise in the zebrafish ArcLight trace is probably due to variation in the expression of the probe and is not a real difference. (b) An A series construct having the RYR sequence at the FP fusion site is also capable of rapidly responding to action potentials. (c) A recording at 2 kHz frame rate of action potentials by the A6 probe. Green is the fluorescent trace. Black is the voltage trace. The shorter exposure time results in an increase in the noise, but the action potential can still be resolved in the unfiltered trace.

[Fig. 2(c)]. Moving the FP closer to the VSD shifted the $V_{1/2}$ to more negative potentials. Reducing the linker length also steepened the slope of the voltage response.

4.4 Orientation of the FP Potentially Affects the Voltage-Dependent Fluorescent Signal

Systematically truncating the linker length revealed a potential structural component [Fig. 3(d)] in the linker sequence. The fluorescent signal of the truncated probes exhibited a repeated pattern as the linker length was extended from the S4 of the VSD. JE03 is three amino acids downstream of the VSD and gave the largest fluorescent signal for a 100 mV depolarization step. As the linker length was extended one amino acid at a time, the fluorescent signal dissipated from ∼12 to 6% $\Delta F/F/$ 100 mV for JE10, which links the FP 10 amino acids downstream of the VSD. JE11 exhibits a slight jump in the fluorescent from 6 to 9%. Subsequent amino acid additions again result in the dissipation of the fluorescent signal. This periodicity and the effects of the amino acid composition at the FP fusion site suggest that the orientation of the FP is important for the voltage-dependent optical signal.

4.5 Dimerization of the FP May Contribute to the Voltage-Dependent Fluorescent Change of GeFVS

The mechanism of how the movement of S4 causes the fluorescence change is not well understood. If there is a structural component in the linker affecting the orientation of the FP, then that would implicate a quenching partner. Two potential partners stand out, the plasma membrane or the intermolecular dimerization of the FP to another sensor molecule. It is not easy to understand how the interaction with the plasma membrane would result in the quenching of the fluorescent signal. However, the dimerization of the FP could be the mechanism since a vast majority of the mutations that created the pH-sensitive Super Ecliptic pHlorin reside at the GFP dimerization interface.[9] The A227D in Super Ecliptic, which vastly enhances the voltage-dependent optical signal of ArcLight, is also close to the dimerization interface.[2]

4.6 Faster Speed of the Zebrafish VSD-Based Sensors Enables the Resolution of Action Potentials Firing at Higher Frequencies

The zebrafish ArcLight and two truncated versions of Zahra 1 SE (227D) were able to optically resolve action potentials from hippocampal neurons. The signal size and kinetics for all three sensors were very similar (Fig. 6). To better resolve an action potential optically, the fluorescent sampling needs to be in the submillisecond range. Figure 6 compares the optical trace from a neuron sampled at 1000 fps to a neuron sampled at 2000 fps.

By reducing the exposure time, the signal-to-noise ratio has been decreased. However, the probe was still capable of resolving single action potentials without the need for offline filtering.

Of course, these probes need to have a faster and larger optical response to membrane potential changes. At least significant improvements in the size of the optical signal have been achieved over early optical recordings.[10,11] So we will continue to employ the lessons Larry has taught and continues to teach us to make better probes for imaging the voltage changes that occur during neuronal activity.

References

1. Z. Han et al., "Fluorescent protein voltage probes derived from ArcLight that respond to membrane voltage changes with fast kinetics," *PLoS One* **8**(11), e81295 (2013).
2. L. Jin et al., "Single action potentials and subthreshold electrical events imaged in neurons with a fluorescent protein voltage probe," *Neuron* **75**(5), 779–785 (2012).
3. B. J. Baker et al., "Genetically encoded fluorescent voltage sensors using the voltage-sensing domain of Nematostella and Danio phosphatases exhibit fast kinetics," *J. Neurosci. Methods* **208**(2), 190–196 (2012).
4. F. St-Pierre et al., "High-fidelity optical reporting of neuronal electrical activity with an ultrafast fluorescent voltage sensor," *Nat. Neurosci.* **17**(6), 884–889 (2014).
5. G. Banker and K. Goslin, "Developments in neuronal cell culture," *Nature* **336**(6195), 185–186 (1988).
6. B. J. Baker et al., "Three fluorescent protein voltage sensors exhibit low plasma membrane expression in mammalian cells," *J. Neurosci. Methods* **161**(1), 32–38 (2007).
7. D. Dimitrov et al., "Engineering and characterization of an enhanced fluorescent protein voltage sensor," *PLoS One* **2**(5), e440 (2007).
8. M. I. Hossain et al., "Enzyme domain affects the movement of the voltage sensor in Ascidian and Zebrafish voltage-sensing phosphatases," *J. Biol. Chem.* **283**(26), 18248–18259 (2008).
9. G. Miesenbock, D. A. De Angelis, and J. E. Rothman, "Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins," *Nature* **394**(6689), 192–195 (1998).
10. L. B. Cohen and B. M. Salzberg, "Optical measurement of membrane potential," *Rev. Physiol. Biochem. Pharmacol.* **83**, 35–88 (1978).
11. B. M. Salzberg, A. L. Obaid, and F. Bezanilla, "Microsecond response of a voltage-sensitive merocyanine dye: fast voltage-clamp measurements on squid giant axon," *Jpn. J. Physiol.* **43**(Suppl. 1), S37–41 (1993).

Biographies of the authors are not available