Video Article

Imaging Membrane Potential with Two Types of Genetically Encoded Fluorescent Voltage Sensors

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Abstract

Genetically encoded voltage indicators (GEVIs) have improved to the point where they are beginning to be useful for *in vivo* recordings. While the ultimate goal is to image neuronal activity *in vivo*, one must be able to image activity of a single cell to ensure successful *in vivo* preparations. This procedure will describe how to image membrane potential in a single cell to provide a foundation to eventually image *in vivo*. Here we describe methods for imaging GEVIs consisting of a voltage-sensing domain fused to either a single fluorescent protein (FP) or two fluorescent proteins capable of Förster resonance energy transfer (FRET) *in vitro*. Using an image splitter enables the projection of images created by two different wavelengths onto the same charge-coupled device (CCD) camera simultaneously. The image splitter positions a second filter cube in the light path. This second filter cube consists of a dichroic and two emission filters to separate the donor and acceptor fluorescent wavelengths depending on the FPs of the GEVI. This setup enables the simultaneous recording of both the acceptor and donor fluorescent partners while the membrane potential is manipulated via whole cell patch clamp configuration. When using a GEVI consisting of a single FP, the second filter cube can be removed allowing the mirrors in the image splitter to project a single image onto the CCD camera.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53566/>

Introduction

The major focus of this paper is to demonstrate the optical imaging of changes in membrane potentials *in vitro* using genetically encoded fluorescent proteins. Imaging changes in membrane potential offers the exciting possibility of studying the activity of neuronal circuits. When changes in membrane potential result in a fluorescence intensity change, each pixel of the camera becomes a surrogate electrode enabling noninvasive measurements of neuronal activity. For over forty years, organic voltage-sensitive dyes have been useful for observing the changes in membrane potential¹⁻⁴. However, these dyes lack cellular specificity. In addition, some cell types are difficult to stain. Genetically encoded voltage indicators (GEVIs) overcome these limitations by having the cells to be studied specifically express the fluorescent voltage-sensitive probe.

There are three classes of GEVIs. The first class of GEVI uses the voltage-sensing domain from the voltage-sensing phosphatase with either a single fluorescent protein (FP)⁵⁻⁹ or a Förster resonance energy transfer (FRET) pair¹⁰⁻¹². The second class of sensors uses microbial rhodopsin as a fluorescent indicator directly¹³⁻¹⁵ or via electrochromic FRET^{16,17}. The third class utilizes two components, the genetic component being a membrane anchored FP and a second component being a membrane bound quenching dye¹⁸⁻²⁰. While the second and third classes are useful for *in vitro* and slice experiments^{19,20}, only the first class of sensors are currently useful for *in vivo* analyses⁶.

In this report we will demonstrate the imaging of membrane potential using the first class of GEVIs (**Figure 1**) *in vitro*. This first class of voltage sensors is the easiest to transition to *in vivo* imaging. Since GEVIs utilizing a voltage-sensing domain fused to a FP are about 50-fold brighter than the rhodopsin class of sensors, they can be imaged using arc lamp illumination rather than requiring an extremely powerful laser. Another consequence of the disparity in brightness is that the first class of GEVIs can easily exceed the auto-fluorescence of the brain. The rhodopsin-based probes cannot. The third class of sensor is just as bright as the first class but requires the addition of a chemical quencher which is difficult to administer *in vivo*.

We will, therefore, demonstrate the acquisition of a probe with a single FP (Bongwoori)⁸ and a probe consisting of a FRET pair (Nabi 2)¹². The FRET constructs in this report are butterfly versions of VSFP-CR (voltage-sensitive fluorescent proteins - Clover-mRuby2)¹¹ consisting of a green fluorescent donor, Clover, and a red fluorescent acceptor, mRuby2, named Nabi 2.242 and Nabi 2.244¹². The introduction to these types of recordings should give researchers a better understanding of the type of information GEVIs can provide.

Figure 1. Two Types of Genetically Encoded Voltage Indicators (GEVIs) Imaged in This Report (A) A mono FP based GEVI having a trans-membrane voltage-sensing domain and a fluorescent protein. (B) A FRET based GEVI comprised of a trans-membrane voltage-sensing domain, a FRET donor and acceptor. [Please click here to view a larger version of this figure.](#)

Protocol

Ethics statement: The animal experiment protocol was approved by the Institutional Animal Care and Use Committee at KIST animal protocol 2014-001.

1. Equipment Setup

1. Imaging setup

1. Place an inverted fluorescence microscope on a vibration isolation table. Use a high magnification (60X oil immersion lens with 1.35 numerical aperture) objective lens and a filter cube equipped with a dichroic mirror and filters suitable for the fluorescent proteins used for the voltage imaging.

Note: This setup uses an inverted microscope in order to employ objectives with higher NA, but upright microscopes can also be used. Indeed, the inverted microscope is only for probe development since higher numerical aperture (NA) objective lens collects more light than the one with a low NA thereby improving the signal to noise ratio (SNR; can be described as the peak optical signal divided by the standard deviation of the baseline fluorescence) of the recording. For non-developers, we would recommend an upright microscope for applications such as slice or *in vivo* recordings.

2. Prepare a light source, e.g., Xenon arc lamp, equipped with a mechanical shutter for efficient epifluorescence wide-field imaging. Direct the light to the microscope via a light guide mount. The light will pass through the excitation filter and is reflected by the first dichroic mirror in the filter cube. Align the light to illuminate the specimen evenly with maximized light intensity over the field of view²¹. Note: Traditionally, a 75 watt arc lamp was used since higher wattage light bulbs create larger but not brighter illumination fields. Lasers can be used but are restricted to a single wavelength. LEDs are becoming brighter and may indeed be the light source of choice offering multiple wavelengths and not requiring a mechanical shutter.

3. Mount the two CCD cameras to the fluorescence microscope as shown in **Figure 2D**.

Note: The first CCD camera has high spatial resolution and is used for identification of a cell expressing the GEVI in the plasma membrane to be tested. For imaging changes in membrane potential, ensure that the second CCD camera has a high frame rate such as 1,000 frame per sec (fps). Use a dual port camera adapter (**Figure 2D-(3)**) to switch the imaging pathway between the two CCD cameras. In this setup, a demagnifier is used to fit the image from the objective lens onto the CCD chip in the second CCD camera.

4. Install an image splitter between the first (slow) and second (fast) CCD cameras for imaging of FRET based GEVI. Insert a filter cube having a dichroic mirror (560 nm) and two emission filters (520 nm/40 & 645 nm/75) in the image splitter. This will result in two fields of view, one for the donor fluorescence and the other representing the acceptor fluorescence. Remove this second filter cube when imaging a GEVI with a single FP.

Note: The single FP based GEVI tested in this method is Bongwoori which uses the FP, super ecliptic pHluorin A227D (SE A227D). The excitation filter (472 nm/30), emission filter (497 nm/long pass) and the dichroic mirror (495 nm) were selected based on its excitation and emission spectra⁵. Generally, the excitation and emission filters should have the largest overlap with each spectrum of the fluorophore to acquire a bright image while the dichroic mirror blocks any excitation light transmitted through the emission filter. The selection of filters and dichroic mirrors for the FRET pair¹¹ based GEVI recording follows the same principle except that it necessitates a second filter cube in the image splitter for concurrent observation of donor and acceptor fluorescence. The first filter cube placed in the microscope filter box needs to have an excitation filter (475 nm/23) for Clover. Then the emitted fluorescence from both FPs will be transmitted to the second filter cube through the first dichroic mirror (495 nm). The second filter cube has a dichroic mirror (560 nm) and two emission filters (520 nm/40 for Clover and 645 nm/75 for mRuby2) for each FP thus separating the fluorescence from two different FPs.

		Single FP based GEVI (Bongwoori)	FRET pair based GEVI (Nabi 2.42 & Nabi 2.44)
First filter cube placed in the microscope	excitation filter	472 nm/30	475 nm/23
	dichroic mirror	495 nm	495 nm
	emission filter	497 nm/long pass	-
Second filter cube placed in the beam splitter	dichroic mirror	-	560 nm
	emission filter 1	-	520 nm/40
	emission filter 2	-	645 nm/75

Table 1. Two Different Filter Sets Used for a Single FP Based GEVI and a FRET Based GEVI Recordings

2. Vibration isolation
 1. Do not mount any equipment with moving components on the vibration isolation table. Ensure that the cables that are attached to non-isolated equipment are loose to avoid vibration of the sample.
3. Patch clamp chamber
 1. Seal the bottom of the patch clamp chamber with a thin #0 cover glass since the working distance of the objective is relatively small. This is a disadvantage of the inverted microscope setup.
Note: As a tradeoff of having a high NA objective lens, the working distance decreases. In order to place the specimen within the very short working distance, the cover glass and the coverslip (step 2) need to be as thin as possible.
4. Temperature control
 1. Ensure that the bath solution flows through a temperature controller to maintain the patched-cells around 33 °C throughout the experiment.
5. Equipment connections
 1. Connect the patch clamp amplifier and the mechanical shutter of the light source to the Bayonet Neill-Concelman (BNC) command box of the high speed CCD camera. This will enable the imaging software to control the amplifier, camera, and the light source simultaneously.
Note: The electrical and imaging components need to be synchronized in order to provide for simultaneous electrical and optical recordings of electrical activity.

Figure 2. Equipment Setup for Voltage Imaging with GEVIs The workflow following the light path, (A) 75W Xenon arc lamp, (B) the excitation light from the arc lamp is filtered by the excitation filter and then reflected by the first dichroic mirror before it reaches to the specimen stage, an inset at the top right corner shows the whole cell configuration, (C) the slow speed CCD camera is used to aid both choice of a cell and patch clamp, (D) the image acquisition part; (1) the high speed CCD camera, (2) the image splitter for both FRET pair and mono FP GEVIs, (3) the demagnifier to fit the image onto the CCD chip in the high speed CCD camera, (4) the dual port camera adapter to switch the imaging pathway and (5) the slow speed CCD camera with high spatial resolution for identification of the cell to patch, (E&F) the image acquisition with a single-FP based GEVI (E) and a FRET-based GEVI (F). [Please click here to view a larger version of this figure.](#)

2. Expression of GEVIs

1. In Human Embryonic Kidney 293 cells
 1. Culture Human Embryonic Kidney 293 (HEK 293) cells with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a CO₂ incubator (5% CO₂). Use a tissue culture dish with 100 mm diameter and 20 mm height. Start the culture with 2×10^3 - 6×10^3 cells/cm² and incubate until they reach 80-90% confluency for subsequent transfection.
 2. On the day of transfection, aspirate the cell media and gently wash once with Dulbecco's phosphate buffered saline. Disperse the HEK 293 cells with 0.25% trypsin-EDTA solution for 1-2 min, aspirate the solution and gently tap the side of the dish to detach the cells. Add fresh DMEM (10% FBS) and dissociate the clumped cells by pipetting. Determine the cell concentration by using a hemocytometer.
 3. Place 0.08 - 0.13 mm thick, 10 mm diameter coverslips pre-coated with poly-L-lysine in a 24-well tissue culture plate. Seed the HEK 293 cells on coverslips at 1×10^5 cells / cm² cell density.
Note: Since HEK 293 cells are capable of forming gap junctions with each other, the seed number needs to yield isolated cells.
 4. Transiently transfect the cells with an appropriate gene construct encoding a GEVI by using a lipofection reagent following the manufacturer's instruction.
Note: The gene constructs used for this step utilized pcDNA3.1 (Bongwoori) and pUB2.1 (Nabi 2.242) as backbone vectors. The amount of DNA used was 100 ng for each coverslip; however, transfection conditions need to be empirically determined for each GEVI to be tested.
2. In dissociated mouse hippocampal primary neurons
 1. Dissect hippocampi from embryonic day 17 C57BL6/N mice and then dissociate the hippocampal neurons as previously described^{22,23}.
 2. Plate the dissociated neurons onto poly-D-lysine coated 0.08-0.13 mm thick, 10 mm diameter coverslips at 1×10^5 cells/ml cell density. Incubate the cells at 37 °C in CO₂ incubator (5% CO₂).
 3. Transfect the dissociated neurons transiently at 6 - 7 days *in vitro* (DIV) with a gene construct encoding a GEVI by using a calcium phosphate transfection reagent as previously described²⁴.
Note: The gene constructs used for this step utilized pcDNA3.1 (Bongwoori) and pUB2.1 (Nabi 2.244) as backbone vectors. The amount of DNA used was 1 µg for each coverslip. Again, transfection conditions need to be empirically determined for each GEVI tested.
3. Check the expression level prior to voltage imaging
 1. Take the tissue culture plate from the CO₂ incubator and observe the fluorescence from the transfected cells under an epifluorescence microscope.
 2. After confirming the fluorescence from the membrane, transfer the coverslip to the patching chamber for the voltage imaging in section 3.
Note: The voltage imaging is conducted 16 - 24 hr post transfection. However, as different fluorescent proteins have different maturation times, some GEVIs need more time in post transfection. For instance, the FRET pairs containing mRuby2 in this protocol may take longer to express since the maturation of mRuby2 takes significantly longer than Clover¹¹. The fluorescence of both donor and acceptor should be checked.

3. Voltage Imaging Protocol

1. Choose a healthy cell with good membrane expression
 1. Using microscopy, find a healthy cell (**Figure 4B**) that shows strong membrane localized fluorescence compared to internal fluorescence. Try not to patch rounded cells. Circular cells are either dividing or dying and are difficult to patch. Apply a test pulse of 5.0 mV in amplitude and 5.0 msec in duration to aid subsequent patch clamp experiments.
2. Prepare the cell for voltage imaging
 1. After choosing a cell to patch, place the patch clamp pipette above the cell. Bring the pipette down until it gently touches the cell membrane. At this step, observe 1 MΩ - 2 MΩ rise in membrane resistance on the patch clamp software²⁵.
NOTE: Occasionally, a good giga-ohm seal can occur simply by touching the cell membrane. As long as the giga-ohm seal is stable, it should be OK.
 2. Establish a giga-ohm seal by gently applying negative pressure through the pipette. Set the pipette potential at a desired holding potential.
 3. While maintaining the giga-ohm seal, image the cell with the high speed CCD camera and focus it to the membrane area.
 4. Rupture the cell membrane by applying pulses of suction by mouth or syringe gently to make a whole cell configuration²⁶.
 5. Image the patch clamped cell under a whole cell clamp configuration according to the experimental design by using an imaging software
 1. Start the imaging software. Click [ACQUIRE] - [SciMeasure Camera] menu to open up the 'CCD ACQUIRE' page.
 2. Create a new data file to save the images.
 3. Click [ANALOGUE OUTPUT] and then [Read an ASCII] to open up a pulse protocol file to conduct the imaging. Click on 'Average the internal repetitions' if the data needs to be averaged. Then close the 'ANALOGUE OUTPUT' page.
Note: This pulse protocol needs to be designed and saved as a '.txt' file according to the purpose of each experiment prior to this step.
 4. Set specific acquisition parameters from the 'CCD ACQUIRE' page. Input the specific values for 'Number of frames for acquisition' and 'Number of trials'.
Note: The number of frames is determined by the speed of the acquisition and the timing of the pulse protocol. For instance, if imaging at 1 kHz, 1,000 frames equals 1 sec of recording time.

5. Click [TAKE DATA (Optics + BNC)] button to start the recording. While the voltage imaging is taking place, watch the oscilloscope window from the patch clamp software to ensure stable whole cell configuration throughout the recording.
 Note: To test the GEVI's responsive voltage range, signal size in $\Delta F/F$ value or temporal resolution throughout the physiological voltage range, conduct a whole cell voltage clamp experiment with a pulse protocol having stepped voltage pulses ranging from -170 mV to 130 mV. To determine the GEVI's performance in resolving action potentials evoked from cultured primary neurons, image the cell under a whole cell current clamp configuration.

4. Data Acquisition

1. Calculation of $\Delta F/F$
 1. Start the imaging software. Click [FILE] - [Read Data File] to open up a data file to be analyzed. Observe the cell image in its Resting Light Intensity (RLI) on the right side
 Note: The software averages the first 5 frames to determine RLI of the recording.
 2. Click on 'Show BNCs' to bring the current and voltage values measured to the screen.
 3. Change the page mode from 'RLI frame' to 'Frame subtraction' to utilize the frame subtraction function to identify pixels with responsive optical signals. This is the true power of imaging since every pixel can be examined for potential changes in fluorescence.
 4. Select two time points (F_0 and F_1) for subtraction. Identify which area of the cell is showing signals responsive to membrane potential change.
 Note: The SNR for the frame subtraction images can be improved by selecting multiple frames for each time point to temporally average the signal. The software subtracts the average light intensity taken from selected frames and calculates the $F_1 - F_0$ values (ΔF). Usually one chooses a time point acquired at the holding potential and another time point taken during the stimulation period.
 5. Designate the pixels that need to be analyzed by dragging or clicking each pixel with the computer mouse. Observe the graphical representation of the average fluorescence intensity from the selected pixels on the left side of the software window. **Figures 4B & 5B** show representative images from the frame subtraction analysis.
 6. Divide the subtracted pixels by the RLI (F_0). Click [Divide by RLIs] to acquire $\Delta F/F$ values
2. Export data
 1. Calculate $\Delta F/F$ values for each voltage step to further analyze the GEVI's voltage sensing properties. Use these values to draw graphs such as $\Delta F/F$ versus voltage for subsequent data analyses.
 2. Remove current and voltage graphs by unclicking 'Show BNCs' menu. Go to [OUTPUT] - [Save Traces As Displayed (ASCII)] to export the fluorescence trace in an ASCII file format for curve fitting analysis by standard graphing software.

5. Data Analysis

1. Voltage sensing property of the GEVI
 1. Draw the fluorescence change versus voltage graph (F-V) by plotting the $\Delta F/F$ values versus voltage in a data analysis program. Fit the curve to a Boltzmann function (**Table 2**) to determine the voltage range of the optical signal by clicking [Analysis] - [Fitting] - [Sigmoidal fit] - [Open dialog], and then choose Boltzmann function.
 Note: Fitting to a function makes it possible to characterize the voltage sensing properties of different voltage probes or to compare their performances in different cells. The Boltzmann function can be used for the probes tested in this paper because the F-V curves from these probes show sigmoidal pattern.
 2. Normalize each $\Delta F/F$ value by using the initial and final values (A_1 and A_2) calculated by the function.
 Note: In the Boltzmann equation, the minimum is A_1 and the maximum is A_2 . For normalization, use a spreadsheet application software to adjust the $\Delta F/F$ values by defining A_1 as zero and A_2 as one. Average the normalized $\Delta F/F$ values and calculate standard error for statistical analysis.
 3. Replot the normalized $\Delta F/F$ values versus voltage as previously described in section 5.1.1).
2. The speed of the optical response
 1. Open the ASCII file acquired from step 4.2.2) with a data analysis program and plot the $\Delta F/F$ trace versus time.
 2. Click on 'Data selector' in the data analysis software and select one time point corresponding to the beginning of a stepped voltage pulse and a second time point when the optical signal has reached the steady-state. Fit this range to both a single and double exponential decay function by clicking [Analysis] - [Fitting] - [Exponential fit] - [Open dialog], and choose an exponential decay function. Report the better fit.
 Note: By fitting to the single or double exponential decay functions, the quantified time constants represented by τ can be acquired. This will help experimenters compare the kinetics of their measurements done with different voltage indicators. The fluorescence trace might show fast and slow components that can be described with a double exponential decay function. In this case, the calculation will result in two time constants with different amplitudes.
 3. Calculate the weighted τ constants to compare the kinetics of probes exhibiting two temporal components to probes with a single component.
 Note: A weighted tau is calculated as the sum of τ_1 multiplied by the relative amplitude, A_1 , plus τ_2 multiplied by the relative amplitude, A_2 , as defined by the following formula; $\tau_{weighted} = \tau_1(A_1/(A_1 + A_2)) + \tau_2(A_2/(A_1 + A_2))$

Representative Results

Transiently transfected cells can exhibit significant variation in fluorescence intensity and the degree of plasma membrane expression. Even on the same coverslip some cells will have varying levels of internal fluorescence. This is most likely due to the amount of transfection agent absorbed by the cell. Occasionally, too much expression causes the cell to experience the unfolded protein response resulting in apoptosis²⁷ (bright, rounded cells, with high internal fluorescence). The experimenter is therefore cautioned to test both bright cells and dim cells with as little internal fluorescence as possible since internal expression creates a non-responsive fluorescence that lowers the signal to noise ratio (SNR).

Another consideration is the maturation rate of chromophores. **Figure 3** shows disparity in the fluorescence of the acceptor chromophore (mRuby2) which has a longer maturation time than the donor chromophore (Clover).

Figure 3. Confocal Images of HEK 293 Cells Transiently Transfected with a FRET Based GEVI, Nabi2.242. (A) Confocal images of an HEK cell showing membrane localized fluorescence from FRET donor and acceptor, (B) Confocal images showing a potential consequence of slow maturation of mRuby2. All four cells exhibit Clover fluorescence while only two exhibit mRuby2 fluorescence. [Please click here to view a larger version of this figure.](#)

The images were acquired by a confocal microscope. For the FRET donor, Clover, 488 nm laser was used for excitation and the emission was captured with 525/50 nm bandpass filter. The FRET acceptor, mRuby2, was illuminated with 561 nm laser for excitation and 595/50 nm bandpass filter was used for emission. The samples for confocal imaging were fixed with 4% paraformaldehyde/sucrose solution in phosphate buffered saline adjusted at pH 7.4 and then mounted with an anti-fade reagent.

Once the transfection conditions are optimized, the next source of variation comes from determining the region of interest to be analyzed. Using frame subtraction to identify the regions of the cell with the highest fluorescence change is often used to maximize the $\Delta F/F$ value. An alternative is to select all of the pixels receiving light from the cell. This increases the number of pixels resulting in the reduction of noise, but decreases the signal size since non-responsive internal fluorescence is included. Both methods are fine as long as the experimenter remains consistent.

Figure 4A shows the fluorescence change of an HEK cell expressing a single-FP based GEVI, Bongwoori. This is a typical fluorescence change in response to stepped voltage pulses. From this data one can plot voltage sensitivity of the GEVI and determine the on and off τ constants at different voltages by fitting to a single or double exponential decay function. 16 trials are typically averaged when imaging HEK cells to improve the SNR of small voltage steps and to detect any potential bleaching during the recording. Those probes that give a robust signal at 100 mV can be tested in dissociated hippocampal neurons (**Figure 4C**). The fluorescence trace from the hippocampal neuron is a single trial.

Figure 4. Voltage Imaging with a Single FP Based GEVI Expressed in HEK 293 Cells and Hippocampal Primary Neurons. (A) $\Delta F/F$ trace of a single FP based GEVI, Bongwoori, showing responses to stepped voltage pulses recorded at 1 kHz with a high speed CCD camera. (B) An HEK 293 cell imaged with the high speed CCD camera; (left) Resting Light Intensity (RLI) of a cell expressing Bongwoori & (right) the frame subtraction image indicating the pixels where fluorescence change was observed. (C) Optical recording of induced action potentials from mouse hippocampal primary neurons expressing Bongwoori. The action potentials were evoked under whole cell current clamp mode. The $\Delta F/F$ trace was selected from the pixels correlated to the soma. [Please click here to view a larger version of this figure.](#)

A representative fluorescence readout of the donor and acceptor chromophores is shown in **Figure 5A**. The polarity of the fluorescence change is opposite for the donor and acceptor enabling ratiometric analysis to reduce correlated noise sources, e.g., movement. For example, movement correlated noise will exhibit a change in fluorescence in the same direction for both donor and acceptor fluorescence. While the FRET based probe is capable of ratiometric imaging, it is often better to only analyze the brighter chromophore. This is because the dimmer chromophore can substantially increase the noise of the recording. **Figure 5B** shows this effect. The frame subtraction in **Figure 5B** from the brighter Clover clearly shows where the optical signal is in the cell. In contrast, the frame subtraction of mRuby2 fluorescence shows higher degrees of noise throughout the cell. Therefore, only the donor signal of Clover is shown in a hippocampal neuron in **Figure 5C**.

Concepts	Equations	Remark
Fractional fluorescence change ($\Delta F/F$)	$\Delta F/F = \left(\frac{F_1 - F_0}{F_0} \right) * 100$	F_1 = light intensity measured at a time point, F_0 = light intensity measured at holding potential
Boltzmann function	$y = \frac{(A_1 - A_2)}{1 + e^{(V - V_{1/2})/dx}} + A_2$	$y = \Delta F/F$, V = membrane potential in mV, $V_{1/2}$ is the membrane potential in mV at half-maximal $\Delta F/F$, A_1 = the minimum value, A_2 = the maximum value, dx = slope
Double exponential decay function	$y = y_0 + A_1 e^{-(t - t_0)/\tau_1} + A_2 e^{-(t - t_0)/\tau_2}$	τ_1, τ_2 = time constants, A_1, A_2 = amplitudes, t, t_0 = two time points, y_0 = offset

Table 2. Equations

Discussion

The nervous system uses voltage in several different ways, inhibition causes a slight hyperpolarization, synaptic input causes a slight depolarization and an action potential results in a relatively large voltage change. The ability to measure changes in membrane potential by GEVIs offers the promising potential of analyzing several components of neuronal circuits simultaneously. In this report we demonstrate a fundamental method for imaging changes in the membrane potential using GEVIs.

A major key for imaging changes in voltage is the efficient expression of the GEVI in the plasma membrane. Intracellular expression creates a non-responsive fluorescence that reduces the SNR of the probe. Optimizing the transfection conditions vastly improves the consistency of the optical measurements. Indeed, when testing a novel GEVI it is advisable to also test a known probe to ensure that differences in optical activity are due entirely to the new probe and not the conditions of the cells.

Figure 6 shows the effect of internal fluorescence in decreasing the sensitivity of the voltage imaging, $\Delta F/F$ value. **Figure 6B** shows ΔF values an HEK cell expressing the single-FP based GEVI, Bongwoori. Trace 2 comes from region 2 (blue color) where relatively bright internal fluorescence is seen in **Figure 6A**. This trace has similar level of ΔF value as trace 1. However, in **Figure 6C** where the traces in **Figure 6B** were divided by RLI values for $\Delta F/F$ values, the trace 2 drops significantly due to the bright internal fluorescence which decreases $\Delta F/F$ values. Fluorescence trace 3 has a very small ΔF but also has a very small RLI value (F). The result is a misleading $\Delta F/F$ signal that has a substantial increase in the noise of the recording. This example was included to demonstrate the ΔF is also important. A large change in $\Delta F/F$ is not helpful if F is very low to begin with.

The use of an image splitter enables the concomitant measurement of two wavelengths onto a single CCD camera. This greatly assists the measurement of FRET dependent fluorescent changes for probe development and reduces the cost of the setup. However, due to chromatic aberration, these two images will be slightly out of focus necessitating the need for an apochromatic objective. The more light the better SNR, so choosing objectives with the highest numerical aperture also improves fluorescence imaging. In addition, one can use stronger light sources such as light emitting diodes (LED) or laser to increase SNR. LEDs do not require a mechanical shutter.

In this report we show the optical signals from a single FP GEVI and a FRET-based GEVI. The main advantage of a FRET-based GEVI is that ratiometric imaging enables the reduction of correlated noise due to respiration and blood flow *in vivo*. The opposite polarity of the fluorescent signals confirms a real change in membrane potential. However, since the fluorescence intensities of the two chromophores often vary significantly, the SNR will also vary. This confounds the ratiometric analysis and limits its usefulness²⁸. There can also be a FRET-independent signal²⁹. It is therefore sometimes better to use only the brighter fluorescent chromophore when using FRET to analyze changes in membrane potential.

Voltage imaging is more challenging than calcium imaging due to the speed and variability of the changes in membrane potential compared to calcium imaging which measures the calcium flux. The membrane potential changes in very fast time scales compared to the calcium ion flux, and subthreshold events in neurons cannot be measured with calcium imaging³⁰. Furthermore, the voltage probe must be in the plasma membrane to be effective which reduces the amount of probe available to optically report changes in the membrane potential. Consequently, the number of photons that can actually arrive at the CCD chip is relatively few. This requires the need for a high speed and low read-noise camera with high quantum efficiency and a probe that elicits a high SNR upon changes in membrane potential. By imaging cells *in vitro*, researchers will better understand the potential benefits and pitfalls of GEVIs before attempting *in vivo* measurements.

Disclosures

The authors have nothing to disclose.

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