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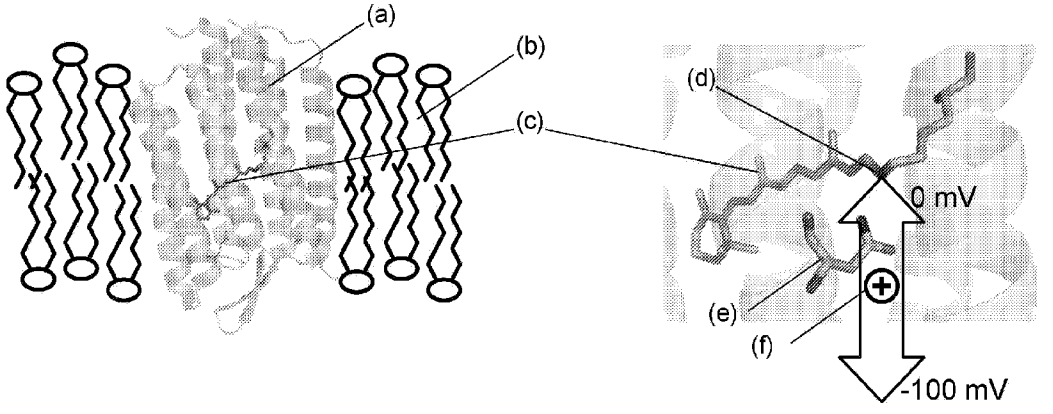
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(54) Title: OPTOGENETIC PROBES FOR MEASURING MEMBRANE POTENTIAL



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The invention provides methods, cells and constructs for optical measurement of membrane potential. These methods can be used in cells that are not accessible to presently available methods using electrodes. The methods can be directed to, for example, high-throughput drug screening assays to determine agents that can affect membrane potential of a target cell.

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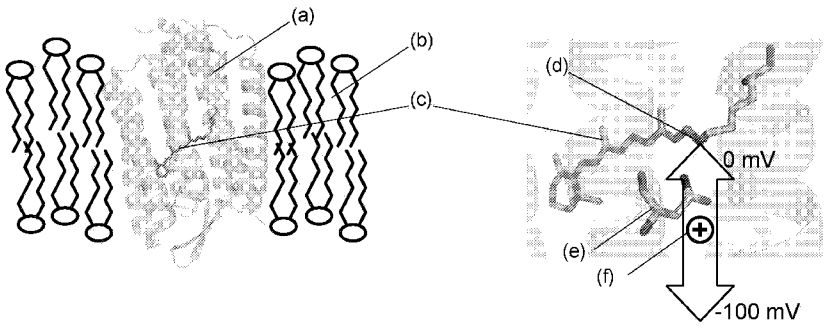
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(54) Title: OPTOGENETIC PROBES FOR MEASURING MEMBRANE POTENTIAL



(57) Abstract: The invention provides methods, cells and constructs for optical measurement of membrane potential. These methods can be used in cells that are not accessible to presently available methods using electrodes. The methods can be directed to, for example, high-throughput drug screening assays to determine agents that can affect membrane potential of a target cell.

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OPTOGENETIC PROBES FOR MEASURING MEMBRANE POTENTIAL

FIELD OF THE INVENTION

[0002] The field of the invention relates to methods, constructs, and compositions for optically measuring electrical potential across a phospholipid bilayer.

BACKGROUND

[0003] Membrane-enclosed biological structures can support a voltage difference between the inside and the outside of the membrane. This voltage, also called membrane potential, serves a variety of biological functions, including carrying information (e.g., in neurons), acting as an intermediate in production of ATP (e.g., in bacteria and mitochondria), powering the flagellar motor (e.g., in bacteria), and controlling transport of nutrients, toxins, and signaling molecules across the cell membrane (in bacteria and eukaryotic cells).

[0004] In spite of its fundamental biological role, membrane potential is very difficult to measure. Electrophysiology involves positioning electrodes on both sides of the membrane to record voltage directly. Electrophysiological experiments are slow to set up, can only be performed on one or a few cells at a time, cannot access deeply buried tissues (e.g., in vivo), do not work for cells that are too small (e.g. bacteria) or are enclosed in a hard cell wall (e.g. yeast), or are motile (e.g., sperm) cannot be applied to long-term measurements, and usually damage or kill the cell under study.

[0005] Accordingly, novel methods for measuring membrane potential are needed.

SUMMARY OF THE INVENTION

[0006] Described herein are methods of harnessing microbial rhodopsins as optical sensors to detect voltage across phospholipid layers. We have discovered thatour novel system allows us to optically measure membrane potential of a cell or membrane bound cellular compartment, such as intracellular organelles and artificial cells or other lipid membrane bound structures.

[0007] The methods comprise expressing a microbial rhodopsin in the cell or cellular organelle, exposing the cell to a light source and detecting the emitted fluorescence from the microbial rhodopsin, wherein the intensity of the emitted fluorescence reflects membrane potential. The method allows measurement of membrane potential without the use of electrodes. The method further allows monitoring membrane potential changes in response to external stimulus or stimuli. This is important not only for research but also, for example, if one wants to screen candidate agents for their capacity to affect membrane potential, e.g., in drug screens.

[0008] Also provided are cells expressing microbial rhodopsins and modified microbial rhodopsins as well as nucleic acids constructs encoding modified archaerhodopsins useful in

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measuring membrane potential and changes thereof in eukaryotic cells. In some embodiments, the optical sensors described herein have their endogenous ion pump activity reduced or inhibited partially or substantially completely compared to the native microbial rhodopsin protein. This permits the optical sensors to sense voltage but not to participate in altering voltage through establishing ionic gradients. The detection of the voltage and its changes can be visualized and measured using optical systems.

[0009] The present invention is based, at least in part, on the discovery that microbial rhodopsin proteins, such as archaerhodopsins or proteorhodopsins and modified versions thereof having reduced ion pumping activity (compared to the natural microbial rhodopsin protein from which it is derived) can be used as optical sensors to sense voltage across membranes in a cell. That is, the microbial rhodopsin and the modified microbial rhodopsin proteins can be used to measure membrane potential of a cell and changes in the membrane potential. The constructs and methods can also be used for *in vivo* imaging of organs and organisms, such as a zebrafish, that could not be studied due to electrode size constraints. This is important not only in research but also for screening novel candidate agents for their capacity to affect membrane potential in cells.

[0010] We have developed Proteorhodopsin Optical Proton Sensor (PROPS), which function primarily in bacterial cells, and a family of archaerhodopsin-based fluorescent voltage-indicating proteins (VIPs) that also function in mammalian cells, including neurons and human stem cell-derived cardiomyocytes. The VIPs are based on voltage indicators derived from Archaerhodopsin 3 (Arch) and its homologues. These proteins indicate electrical dynamics with sub-millisecond temporal resolution and sub-micron spatial resolution. Using VIPs, we demonstrated non-contact, high-throughput, and high-content methods for measuring by using optical detection of electrical dynamics in mammalian cells and tissues.

[0011] The optical sensors described herein are not constrained by the need for electrodes and permit electrophysiological studies to be performed in e.g., subcellular compartments (e.g., mitochondria) or in small cells (e.g., bacteria). The optical sensors described herein can be used in drug screening, research settings, and for *in vitro* and *in vivo* imaging of voltage changes in both eukaryotic and prokaryotic cells.

[0012] We describe voltage indicator proteins and constructs expressing such proteins. The constructs have optionally a cell-type specific promoter that turns on when the cells are differentiated, e.g., to neuronal cells, such as neurons, or to cardiac cells, such as cardiomyocytes, purkinje cells, or sinusoidal cells. The constructs may further include targeting signals such as mitochondrial targeting signals to direct the voltage indicator protein to a desired membrane location. We provide cells and cell lines transiently and stably expressing these proteins, including human stem cells, such as induced pluripotent cells (iPSC) or embryonal stem cells (ESC), neural progenitor cells and neural cells, and cardiac progenitor cells and cardiac myocytes. We also describe methods for screening drugs using the described voltage indicator proteins.

[0013] The cells, whether they be prokaryotic or eukaryotic cells, used in the methods of the invention are typically engineered to express the microbial rhodopsin or the modified microbial rhodopsin as they do not naturally express the microbial rhodopsin protein that is used in the methods of the invention.

[0014] Accordingly, in one embodiment, the invention provides a method for measuring membrane potential in a cell expressing a nucleic acid encoding a microbial rhodopsin protein, the method comprising the steps of (a) exciting, in vitro, ex vivo or in vivo, at least one cell comprising a nucleic acid encoding a microbial rhodopsin protein with light of at least one wave length; and (b) detecting, in vitro, ex vivo or in vivo, at least one optical signal from the at least one cell, wherein the level of fluorescence emitted by the at least one cell compared to a reference is indicative of the membrane potential of the cell.

[0015] In some aspects of any embodiment or aspect of the invention, the microbial rhodopsin protein is a modified microbial rhodopsin protein with reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived.

[0016] In some aspects of any embodiment or aspect of the invention, the microbial rhodopsin protein is a member of a proteorhodopsin family of proteins.

[0017] In some aspects of any embodiment or aspect of the invention, the microbial rhodopsin protein is a member of an archaerhodopsin family of proteins.

[0018] In some aspects of any embodiment or aspect of the invention, the at least one wave length is a wave length between λ = 594-645. Range of wave length between λ = 630-645nm can also be used.

[0019] In some aspects of any embodiment or aspect of the invention, the cell is a prokaryotic cell. The prokaryotic cell can be Gram negative or Gram positive. The prokaryotic cell can be pathogenic or non-pathogenic.

[0020] In some aspects of any embodiment or aspect of the invention, the cell is a eukaryotic cell.

[0021] In some aspects of any embodiment or aspect of the invention, the eukaryotic cell is a mammalian cell.

[0022] In some aspects of any embodiment or aspect of the invention, the eukaryotic cell is a stem cell or a pluripotent or a progenitor cell.

[0023] In some aspects of any embodiment or aspect of the invention, the eukaryotic cell is an induced pluripotent cell.

[0024] In some aspects of any embodiment or aspect of the invention, the eukaryotic cell is a neural cell.

[0025] In some aspects of any embodiment or aspect of the invention, the eukaryotic cell is a cardiomyocyte.

[0026] The cells can be cultured in vitro, ex vivo or the cells can be part of an organ or organism. Exemplary cells include bacteria, yeast, a plant cell, and a cell from vertebrate and non-vertebrate animal. In some embodiments, the eukaryotic cells are human cells. In some embodiments, the eukaryotic cells are non-human cells. In some embodiments, the cells do not naturally express the microbial proteorhodopsin used in the methods.

[0027] In some aspects of any embodiment or aspect of the invention, the method further comprises a step of transfecting, in vitro, ex vivo or in vivo, the at least one cell with a vector comprising the nucleic acid encoding the microbial rhodopsin protein. The cells can be transfected transiently or stably.

[0028] In some aspects of any embodiment or aspect of the invention, the nucleic acid encoding the microbial rhodopsin protein is operably linked to a cell-type specific promoter.

[0029] In some aspects of any embodiment or aspect of the invention, the nucleic acid encoding the microbial rhodopsin protein is operably linked to a membrane-targeting sequence.

[0030] In some aspects of any embodiment or aspect of the invention, the membrane-targeting sequence is a plasma membrane targeting sequence.

[0031] In some aspects of any embodiment or aspect of the invention, the membrane-targeting sequence is a subcellular compartment-targeting sequence.

[0032] In some aspects of any embodiment or aspect of the invention, the subcellular compartment is selected from a mitochondrial membrane, an endoplasmic reticulum, a sarcoplastic reticulum, a synaptic vesicle, an endosome and a phagosome.

[0033] In some aspects of any embodiment or aspect of the invention, the microbial rhodopsin gene is operably linked to a nucleic acid encoding an additional fluorescent protein or a chromophore.

[0034] In some aspects of any embodiment or aspect of the invention, the at least one additional fluorescent protein is a protein capable for indicating ion concentration in the cell.

[0035] In some aspects of any embodiment or aspect of the invention, the at least one additional fluorescent protein capable for indicating ion concentration is a calcium indicator.

[0036] In some aspects of any embodiment or aspect of the invention, the fluorescent protein capable for indicating ion concentration is a pH indicator.

[0037] In some aspects of any embodiment or aspect of the invention, the fluorescent protein is capable of undergoing nonradiative fluorescence resonance energy transfer to the microbial rhodopsin, with a rate of energy transfer dependent on the membrane potential.

[0038] In some aspects of any embodiment or aspect of the invention, brightness of the fluorescent protein is insensitive to membrane potential and local chemical environment, and thereby serves as a reference against which to compare the fluorescence of the microbial rhodopsin

[0039] In some aspects of any embodiment or aspect of the invention, the method further comprises steps of exciting, in vitro, ex vivo or in vivo, the at least one cell with light of at least a first and a second wavelength; and detecting, in vitro, ex vivo, or in vivo, the at least first and the second

optical signal resulting from the excitation with the at least the first and the second wavelength, which is different from the at least first wave length, from the at least one cell.

[0040] In some aspects of any embodiment or aspect of the invention, the at least second wave length is between λ = 447-594 nm.

[0041] In some aspects of any embodiment or aspect of the invention, the method further comprises a step of calculating the ratio of the fluorescence emission from the microbial rhodopsin to the fluorescence emission of the fluorescent protein to obtain a measurement of membrane potential independent of variations in expression level.

[0042] In some aspects of any embodiment or aspect of the invention, the method further comprises the step of exposing, in vitro, ex vivo, or in vivo, the at least one cell to a stimulus capable of or suspected to be capable of changing membrane potential.

[0043] In some aspects of any embodiment or aspect of the invention, the stimulus a candidate agent. In some embodiments, at least one candidate agent is administered. In some embodiments a combination of at least two candidate agents are administered simultaneously or in series.

[0044] In some aspects of any embodiment or aspect of the invention, the stimulus is a change to the composition of the cell culture medium.

[0045] In some aspects of any embodiment or aspect of the invention, the stimulus is an electrical current.

[0046] In some aspects of any embodiment or aspect of the invention, the method further comprises the step of measuring, in vitro, ex vivo or in vivo, the at least one optical signal at least at a first and at least at a second time point.

[0047] In some aspects of any embodiment or aspect of the invention, the at least first time point is before exposing the at least one cell to a stimulus and the at least second time point is after exposing the at least one cell to the stimulus.

[0048] In some aspects of any embodiment or aspect of the invention, the method further comprises a step of measuring the ratio of fluorescence between the optical signals from the exposure to the at least first wave length and the at least second wave length.

[0049] In some aspects of any embodiment or aspect of the invention, the method comprises use of a plurality of cells. For example in a high-throughput assay format. For example, in such embodiments, a plurality of cells expressing the microbial rhodopsin proteins can be exposed to a number of candidate agents, such as drug candidates, and screened for the candidate agents' ability to affect the membrane potential of the cell.

[0050] In some aspects of any embodiment or aspect of the invention, the eukaryotic cell is a human cell. In some embodiments, the eukaryotic cell is a non-human cell.

[0051] In another embodiment, the invention provides an isolated and purified nucleic acid encoding a modified member of an archaerhodopsin family of proteins with reduced ion pumping

activity compared to a natural member of an archaerhodopsin family of proteins from which it is derived.

[0052] In some aspects of any embodiment or aspect of the invention, the modified member of an archaerhodopsin family of proteins with reduced ion pumping activity compared to a natural member of an archaerhodopsin family of proteins from which it is derived comprises a mutated proton acceptor proximal to the Schiff Base.

[0053] In some aspects of any embodiment or aspect of the invention, the isolated and purified nucleic acid is operably linked to a nucleic acid encoding a membrane-targeting sequence.

[0054] In some aspects of any embodiment or aspect of the invention, the membrane-targeting sequence is a subcellular membrane-targeting sequence.

[0055] In some aspects of any embodiment or aspect of the invention, the subcellular membrane is a mitochondrial membrane, an endoplasmic reticulum, a sarcoplastic reticulum, a synaptic vesicle, an endosome or a phagosome.

[0056] In some aspects of any embodiment or aspect of the invention, the isolated and purified nucleic acid is operably linked to a cell-type specific promoter.

[0057] In some aspects of any embodiment or aspect of the invention, the isolated and purified nucleic acid is operably linked to at least one nucleic acid encoding an additional fluorescent protein or a chromophore.

[0058] In some aspects of any embodiment or aspect of the invention, the additional fluorescent protein is green fluorescent protein or a homolog thereof.

[0059] In some aspects of any embodiment or aspect of the invention, the isolated and purified nucleic acid is operably linked to a nucleic acid encoding a fluorescent protein capable of undergoing fluorescence resonance energy transfer to the microbial rhodopsin, with the rate of energy transfer dependent on the membrane potential.

[0060] In some aspects of any embodiment or aspect of the invention, the isolated and purified nucleic acid further comprises a vector.

[0061] In some aspects of any embodiment or aspect of the invention, the vector is a viral vector, such as a lentiviral vector or an adeno-associated virus (AAV) vector.

[0062] In another embodiment, the invention provides a kit comprising the isolated and purified nucleic acid as described above. The nucleic acid may be provided in a buffer solution or in a dried, such as lyophilized form in a suitable container. In some embodiments the kit further comprises buffers and solutions for performing the methods or the assays of the invention. Based on the description provided in the specification, a skilled artisan will be able to pick and choose the appropriate reagents for such a kit. The kit may comprise one or more transfection agents, one or more buffers, one or more cell culture media, and one or more containers, such as cell culture plates or arrays, to perform the methods and assays of the invention. Instruction manuals comprising instructions for performing the assay may also be included with the kits.

[0063] In another embodiment, the invention provides an isolated cell comprising a nucleic acid encoding a microbial rhodopsin protein. The cell is typically engineered to express the microbial rhodopsin protein.

[0064] In some aspects of any embodiment or aspect of the invention, the microbial rhodopsin protein is a modified microbial rhodopsin protein with reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived.

[0065] In some aspects of any embodiment or aspect of the invention, the modified microbial rhodopsin protein comprises a mutated proton acceptor proximal to the Schiff Base.

[0066] In some aspects of any embodiment or aspect of the invention, the microbial rhodopsin is a member of a proteorhodopsin family.

[0067] In some aspects of any embodiment or aspect of the invention, the microbial rhodopsin is a member of a archaerhodopsin family.

[0068] In some aspects of any embodiment or aspect of the invention, the cell is a eukaryotic cell.

[0069] In some aspects of any embodiment or aspect of the invention, the cell is a prokaryotic cell. In some embodiments, the prokaryotic cell is a Gram positive cell. In some embodiments, the prokaryotic cell is a Gram negative cell. In some embodiments, the prokaryotic cell is a pathogenic cell.

[0070] In some aspects of any embodiment or aspect of the invention, the modified microbial rhodopsin gene is operably linked to a promoter.

[0071] In some aspects of any embodiment or aspect of the invention, the promoter is a cell-type specific promoter.

[0072] In some aspects of any embodiment or aspect of the invention, the nucleic acid encoding the modified microbial rhodopsin protein is operably linked to a membrane-targeting nucleic acid.

[0073] In some aspects of any embodiment or aspect of the invention, the nucleic acid encoding the modified microbial rhodopsin protein is operably linked to a nucleic acid encoding at least one additional fluorescent protein or a chromophore.

[0074] In some aspects of any embodiment or aspect of the invention, at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

[0075] In some aspects of any embodiment or aspect of the invention, the at least one additional fluorescent protein is a fluorescent protein capable of undergoing fluorescence resonance energy transfer to the microbial rhodopsin, with a rate of energy transfer dependent on the membrane potential.

[0076] In some aspects of any embodiment or aspect of the invention, the at least one additional fluorescent protein is a fluorescent protein whose brightness is insensitive to membrane potential and local chemical environment

[0077] In some aspects of any embodiment or aspect of the invention, the cell further comprises a nucleic acid encoding a fluorescent protein capable of indicating ion concentration in the cell.

[0078] In some aspects of any embodiment or aspect of the invention, the cell is a stem cell, a pluripotent cell, or an induced pluripotent cell, or differentiated or undifferentiated progeny thereof.

[0079] In some aspects of any embodiment or aspect of the invention, the differentiated cell is a neuron.

[0080] In some aspects of any embodiment or aspect of the invention, the differentiated cell is a cardiomyocyte.

[0081] In one embodiment, the invention provides a kit comprising a cell or a plurality of cells of as described above in a suitable cell culture medium and a container. The kit may comprise frozen cells in a suitable medium. Other reagents, such as one or more buffers, one or more cell culture media, and one or more containers may be included in the kit. The kit may also include instructions for the methods of using the cells in the methods as described herein.

[0082] In another embodiment, the invention provides a method of making an engineered cell for optical measurement of membrane potential comprising the steps of transfecting a cell with a nucleic acid encoding a microbial rhodopsin protein. The transfection may be a transient transfection or a stable transfection.

[0083] In some embodiments, the cell is a prokaryotic cell. The prokaryotic cell may be Gram positive or Gram negative. In some aspects, the prokaryotic cell is a pathogenic bacterium. The cell can also be a stem cell, a pluripotent cell, a differentiated cell or an immortalized cell or a cell line. The cell can be an isolated cell or a part of an organ or an organism, such as a zebrafish or a non-human embryo or a human embryo.

[0084] In one aspect of this embodiment, and any aspect of this embodiment, the microbial rhodopsin protein is a modified microbial rhodopsin protein.

[0085] In one aspect of this embodiment, and any aspect of this embodiment, the nucleic acid encoding the microbial rhodopsin protein is operably linked to the differentiated cell type-specific promoter.

[0086] In one aspect of this embodiment, and any aspect of this embodiment, the nucleic acid encoding the microbial rhodopsin protein is operably linked to at least one additional gene encoding a fluorescent protein or a chromophore.

[0087] In one aspect of this embodiment, the at least one additional gene encoding a fluorescent protein is a green fluorescent protein or a homolog thereof.

[0088] In one aspect of this embodiment, and any aspect of this embodiment, the nucleic acid encoding the microbial rhodopsin protein is operably linked to a fluorescent protein capable of indicating ion concentration in the cell.

[0089] Exemplary nucleic acids and nucleic acid constructs are provided throughout this specification and their nucleic acid sequences are provided in the accompanying Sequence Listing.

Typically, the modified microbial rhodopsins are modified to at least reduce the ion pumping activity of such proteins by mutating the proton acceptor proximal to the Schiff Base. However, the invention is not intended to be limited to these examples, as similar optical measurement is possible for any number of the existing microbial rhodopsin proteins. Any such protein may be used in the methods of the invention and any such protein may also be modified to reduce its ion pumping activity.

BRIEF DESCRIPTION OF THE FIGURES

[0090] Figure 1 shows a mechanism of voltage sensitivity in the D97N mutant of Green Proteorhodopsin. Left: Green Proteorhodopsin (a) spans a lipid bilayer membrane (b). Right: Close-up showing a chromophore, retinal (c), covalently linked to the protein backbone via a Schiff Base (d). Aspartic acid 97 in the wild-type structure has been mutated to asparagine (e) to decrease the pKa of the Schiff Base from the wild-type value of > 12 to the value 9.8 and to eliminate the proton-pumping photocycle. A change in the voltage drop across the membrane changes the local electrochemical potential for a proton (f) to reside on the Schiff Base, and thereby changes the acid-base equilibrium. The absorption spectrum and fluorescence of the retinal depend on the state of protonation of the Schiff Base: the protonated form is fluorescent, the deprotonated form is not. The voltage across the membrane is determined by measuring the fluorescence.

[0091] Figures 2A-2D show that GPR D97N is a transmembrane protein that shows highly photostable and environmentally sensitive fluorescence. *E. Coli* cells expressing GPR D97N were excited at a wavelength of 633 nm and imaged via fluorescence emission of GPR D97N between 660 – 760 nm. The protein is localized in the cell periphery as expected for a transmembrane protein; Figure **2A** shows visible absorption spectra of GPR D97N in whole *E. coli* at pH 7 and pH 11; Figure **2B** shows fluorescence emission spectra of purified GPR D97N protein solubulized in octyl-glucoside at pH 7 and pH 11; Figure **2C** shows photobleaching curves of GPR D97N and the organic dye Alexa 647 (Molecular Probes) under identical illumination conditions. GPR D97N is more photostable than any other known fluorescent protein under comparable illumination intensities. Figure **2D** shows pH titration of the Schiff base as monitored by visible absorption, in the wild-type and D97N mutants of GPR. The pKa of the D97N mutant is 9.8 and the pKa of the wild-type protein is > 12.

[0092] Figure 3 shows fluorescence brightness of GPR D97N as a function of pH *in vivo* and *in vitro*. In both cases the fluorescence decreases at high pH due to the deprotonation of the Schiff Base. The pKa in *E. coli* is measured in cells whose membrane has been made permeable to protons via addition of Carbonyl cyanide 3-chlorophenylhydrazone (CCCP). This treatment is necessary because the proton binds to the Schiff Base from the cytoplasmic side and in the absence of CCCP the cells maintain a nearly constant cytoplasmic pH in the presence of swings in the external pH. The pKa in the cells and in the purified protein differ due to the local environmental effects in the cell.

[0093] Figures 4A and 4B show fluorescence blinking in *E. coli* expressing GPR D97N. Figure **4A** shows a film strip of three *E. coli* at pH 7.5. Each exposure is 100 ms. The brightness of

individual cells varies with time. Figure **4B** shows a blinking pattern from a single cell at pH 7.5. Each trace is a 50 second (s) record of the intensity. The time after the start of the experiment is indicated on the right. Cells continued to blink throughout the 1 hour experiment. The same cell shows fast blinks (0 and 7 minutes), slow blinks (28 minutes), and 'ringing' behavior (18 minutes).

Figure 5 shows a map of the plasmid containing Archaerhodopsin 3(Arch3, also referred to in some instances herein as Ar-3) as described in the syntheticbiology web site.

[0095] Figures 6A-6E show that Arch is a fluorescent voltage indicator. Figure **6A** shows a model of Arch as a voltage sensor. pH and membrane potential can both alter the protonation of the Schiff base. The crystal structure shown is bacteriorhodopsin; the structure of Arch has not been solved. Figure **6B** shows absorption (solid line) and fluorescence emission (Em, see, dashed line) spectra of purified Arch at neutral and high pH. Figure **6C** top shows a HEK cell expressing Arch, visualized via Arch fluorescence. Figure **6C** bottom shows a pixel-weight matrix regions of voltage-dependent fluorescence. Scale bar 10 μm. Figure **6D** shows fluorescence of Arch as a function of membrane potential. The fluorescence was divided by its value at -150 mV. Figure **6E** shows dynamic response of Arch to steps in membrane potential between -70 mV and +30 mV. The overshoots on the rising and falling edges were an artifact of electronic compensation circuitry. Data were an average of 20 cycles. Inset shows that step response occurred in less than the 0.5 ms resolution of the imaging system.

[0096] Figures 7A-7D show optical recording of action potentials with Arch3 WT. Cultured rat hippocampal neuron expressing Arch-GFP were imaged via fluorescence of GFP. Arch fluorescence was shown in cyan and regions of voltage-dependent fluorescence were shown in red. Figure **7A** shows whole-cell membrane potential determined via direct voltage recording (bottom, dotted line) and weighted Arch3 fluorescence (top, solid line) during a single-trial recording of a train of action potentials. Inset shows an averaged spike response for 269 events in a single cell, showing voltage (dotted line) and fluorescence (solid line). Figure **7B** shows recording of multiple spike-trains from a single cell. Current injections (shown in black dotted line) of 200 pA were applied to a neuron expressing Arch WT. Action potentials (shown in grey line) were readily detected via fluorescence over multiple rounds of current injection. Figure **7C** shows sub-cellular localization of an action potential. We took an image of a field of neuronal processes expressing Arch and created a weight matrix indicating pixels, shown as images in the Figure, whose fluorescence co-varied with the recorded potential in red, overlaid on the time-average Arch fluorescence which was shown in cyan. We detected sub-cellular regions within the electrically active cell. The figure shows a timecourse of an action potential determined via fluorescence (F) on the top graph (averaged over n = 100 spikes) corresponding to each of the regions indicated. Also shown is the electrical recording of the action potential (V, bottom graph). Figure **7D** shows heterogeneous dynamics of an action potential within a

single neuron, computed from an average of n = 33 spikes. The region indicated by the arrow in the pixel map (see also graph) lags behind the rest of the cell by ~1 ms (black arrows). Scale bar 5 μm.

[0097] Figures 8A-8D demonstrate that Arch D95N shows voltage-dependent fluorescence but no photocurrent. Figure **8A** shows photocurrents in Arch 3 WT and Arch 3 D95N mutant, expressed in HEK cells clamped at V = 0. Cells were illuminated with pulses of light at λ = 640 nm, 1800 W/cm^2. Figure **8B** shows that Arch D95N fluorescence increased 3-fold between -150 mV and +150 mV, with nearly linear sensitivity from -120 to +120 mV. Inset shows a map of voltage sensitivity. Scale bar 5 μm. Figure **8C** shows that the step response comprised a component faster than 500 μs (20% of the response) and a component with a time constant of 41 ms. Figure **8D** shows that Arch D95N provided highly accurate estimates of membrane potential, clearly resolving voltage steps of 10 mV, with a noise in the voltage estimated from fluorescence of 260 $\mu V/(Hz)^{1/2}$ over timescales < 12 s.

[0098] Figures 9A-9C show optical recording of action potentials with ArchD95N. Figure **9A** shows electrically recorded membrane potential of a neuron expressing Arch WT, subjected to pulses of current injection and laser illumination (I = 1800 W/cm2, λ = 640 nm). Illumination generated sufficient photocurrent to suppress action potentials when the cell was near threshold. Grey bars indicate laser illumination. Figure **9B** is same as Figure **9A** in a neuron expressing Arch D95N, showing no effect of illumination on spiking or resting potential. We showed a neuron expressing Arch D95N, showing Arch D95N fluorescence (shows in cyan in the experiment), and regions of voltage-dependent fluorescence (shown in red in the experiment). Figure **9C** shows whole-cell membrane potential determined via electrical recording (bottom, voltage line) and weighted ArchD95N fluorescence (top, fluorescence line) during a single-trial recording of a train of action potentials.

[0099] Figure 10 shows existing genetically encoded fluorescent voltage indicators classified according to their sensitivity and speed—the two key parameters that determine the performance of an indicator. VSFPs, FLARE and SPARC represent indicators based on fusions of GFP homologues to membrane proteins. The exemplary proteins we have developed are the Proteorhodopsin Optical Proton Sensor (PROPS), Arch3 WT, and Arch3 D95N, shown on the upper right. PROPS functions in bacteria, while Arch3 WT and Arch3 D95N function in mammalian cells. Note the logarithmic axes. Microbial rhodopsin-based voltage indicators are much faster and far more sensitive than other indicators.

[00100] Figures 11A-11D show optical recordings of action potentials in a single HL-1 mouse cardiomyocyte expressing Arch3 D95N - eGFP. Action potentials were recorded for up to 1000 s, with no signs of phototoxicity. This experiment is the first quantitative measurement of cardiac action potentials with a genetically encoded voltage indicator. We showed an overlay showing fluorescence of Arch D95N and GFP in a Arch D95N-GFP fusion. Figure **11A** shows a

comparison of the action potential determined from patch clamp recording (dashed line) and fluorescence (solid line). Figures **11B-11D** show optical recordings of the action potentials in a single HL-1 cell over increasingly long intervals. Data in **11D** have been corrected for photobleaching.

[00101] Figure 12 shows optical recordings of action potentials in human induced pluripotent stem cell (iPS)-derived cardiomyocytes, expressing Arch3 D95N – eGFP. Human induced pluripotent stem cells (hiPSC) were provided by Cellular Dynamics Inc. Cells were plated into MatTek dishes coated in 0.1 % gelatin at a density of 20, 50, or 75 thousand cells per square centimeter. These conditions showed cells that were sparse and did not beat spontaneously (20K), a confluent monolayer that did beat spontaneously (50K), and a dense monolayer (75K). iPS cells were plated and maintained for 48 hours in plating medium, and thereafter fed every 48 hours with maintenance medium (both from Cellular Dynamics). iPS cells were transfected using Mirus LT-1 according to the manufacturer directions. To a tube containing 20 uL of OPTI-MEM®, we added 200 ng of DNA and 1.2 uL of the LT-1 transfection reagent. The DNA mixture was incubated at room temperature for 20 minutes. Fresh maintenance medium was added to the iPS cells during the incubation and the DNA mixture was added dropwise over the plate. Cells were imaged 48 to 96 hours after transfection. We observed synchronized beating of adjoining cells, indicating that VIPs can probe intercellular conduction. We recorded for more than 10 minutes continuously, with little phototoxicity. Within the population of cells we observed cells with action potentials matching those of ventricular, atrial, and nodal cells, as expected for this population. Addition of drugs led to changes in the action potential waveform that matched changes reported by conventional patch clamp. Cell 1fluorescence information is indicated as a solid line and Cell 2 fluorescence as a dashed line on the photos fluorescence vs. time.

[00102] Figure 13 illustrates constructs of fused VIPs with GFP-homologue proteins to develop a series of improved voltage indicators. The length of each bar indicates the length of the protein sequence or linker region. The color indicates the color of the fluorescence of the corresponding protein. All constructs were constructed with both Arch WT and Arch D95N backbones. The sequences for the constructs are provided in the Sequence Listing as follows:

pADD247/248	SEQ ID NO: 48
pADD286/287	SEQ ID NO: 53
pADD292/293	SEQ ID NO: 50
pADD294 (D95N only)	SEQ ID NO: 54
pADD297/300	SEQ ID NO: 51
pADD259/298	SEQ ID NOS: 55 and 52, respectively
Pfck:Arch3(WT/D95N)-EGFP	SEQ ID NOS: 56-57, respectively
pADD269/270	SEQ ID NO: 49
Pcmv:Arch3(WT/D95N)-GCaMP	SEQ ID NOS: 58-59, respectively

[00103] Figures 14A-14B illustrate mechanism of ssFRET. Figure 14A shows that when the Schiff Base (SB) on the retinal is protonated, the absorption spectrum of the retinal (solid) overlaps with the emission spectrum of the GFP (dotted), and the fluorescence of the GFP is quenched. However, the retinal itself is fluorescent in this state. Figure 14B shows that when the SB is deprotonated, the GFP fluorescence (solid line) becomes de-quenched and the retinal fluorescence vanishes.

[00104] Figure 15 shows distance dependence of ssFRET signal. As the distance between the mOrange2 and the Arch chromophores decreased, the magnitude of the ssFRET signal increased.

[00105] Figure 16 shows a step response of Arch 3 fluorescence and mOrange fluorescence in pADD294. Here the mOrange2 signal has been inverted to facilitate comparison with the Arch 3 signal. The similarity of the timecourses is consistent with modulation of the mOrange2 fluorescence via ssFRET. The voltage step is from -70 mV to +30 mV.

[00106] Figures 17A-17C show pH-dependent spectra of Arch 3 WT and D95N. Figure 17A shows Arch WT absorption at neutral (bold line) and high (this line) pH. At neutral pH, Arch absorbed maximally at 558 nm. Fluorescence emission (dashed line) was recorded on 2 μM protein solubilized in 1% DM, with λexc = 532 nm. Figure 17B shows Arch D95N spectra under the same conditions as in Figure 17A. The absorption maximum was at 585 nm. Figure 17C shows absorption spectra that were recorded on purified protein between pH 6 – 11. Singular Value Decomposition of absorption spectra between 400 – 750 nm was used to calculate the fraction of the SB in the protonated state as a function of pH. The result was fit to a Hill function to determine the pKa of the SB.

[00107] Figure 18 shows frequency response of Arch 3 WT. A chirped sine wave with amplitude 50 mV and frequency from 1 Hz – 1 kHz was applied to a HEK cell expressing Arch 3 WT (wild type). Membrane potential $\hat{V}_{FL}$ was determined from fluorescence and the Fourier transform of $\hat{V}_{FL}$ was calculated. The uptick at 1 kHz is an artifact of electronic compensation circuitry. Inset: power spectrum of noise in $\hat{V}_{FL}$, under voltage clamp at constant V = 0 mV shows a shot-noise limited noise floor of 470 μV/(Hz)1/2 at frequencies above 10 Hz. The noise figures reported here are specific to our imaging system and serve primarily as an indicator of the possible sensitivity of Arch 3.

[00108] Figure 19 shows sensitivity of Arch 3 WT to voltage steps of 10 mV. Whole-cell membrane potential determined via direct voltage recording, V, (bolded black line, showing step-like line on the graph) and weighted Arch 3 fluorescence, $\hat{V}_{FL}$, (solid narrower line showing serrations on the graph).

[00109] **Figure 20 shows that arch reports action potentials without exogenous retinal.** We made an image of 14 day in vitro (DIV) hippocampal neuron imaged via Arch 3 fluorescence with no exogenous retinal. Electrical (bolded solid black line) and fluorescence (non-bolded line, showing serrated line in the graph) records of membrane potential from the neuron during a current pulse. Action potentials are clearly resolved.

[00110] **Figure 21 shows a frequency response of Arch D95N,** measured in the same manner as for Arch 3 WT (Fig. 18).

DETAILED DESCRIPTION OF THE INVENTION

[00111] The present invention is based, at least in part, on the discovery that microbial rhodopsin proteins or modified microbial rhodopsin proteins that have reduced ion pumping activity, compared to the natural microbial rhodopsin protein from which they are derived, can be used as an optically detectable sensor to sense voltage across membranous structures, such as in cells and sub-cellular organelles when they are present on the cell membrane. That is, the microbial rhodopsin proteins and the modified microbial rhodopsin proteins can be used to measure changes in membrane potential of a cell, including prokaryotic and eukaryotic cells. The optical sensors described herein are not constrained by the need for electrodes and permit electrophysiological studies to be performed in e.g., subcellular compartments (e.g., mitochondria) or in small cells (e.g., bacteria). The optical sensors described herein can be used in methods for drug screening, in research settings, and in *in vivo* imaging systems.

Microbial Rhodopsins: Design of Optical Voltage Sensors

[00112] Microbial rhodopsins are a large class of proteins characterized by seven transmembrane domains and a retinilydene chromophore bound in the protein core to a lysine via a Schiff base (Beja, O., et al. *Nature* 411, 786-789 (2001)). Over 5,000 microbial rhodopsins are known, and these proteins are found in all kingdoms of life. Microbial rhodopsins serve a variety of functions for their hosts: some are light-driven proton pumps (bacteriorhodopsin, proteorhodopsins), others are light-driven ion channels (channelrhodopsins), chloride pumps (halorhodopsins), or serve in a purely photosensory capacity (sensory rhodopsins).

[00113] The retinilydene chromophore imbues microbial rhodopsins with unusual optical properties. The linear and nonlinear responses of the retinal are highly sensitive to interactions with the protein host: small changes in the electrostatic environment can lead to large changes in absorption spectrum. These electro-optical couplings provide the basis for voltage sensitivity in microbial rhodopsins.

[00114] Some of the optical sensors described herein are natural proteins without modifications and are used in cells that do not normally express the microbial rhodopsin transfected to the cell, such

as eukaryotic cells. For example, as shown in the examples, the wild type Arch3 can be used in neural cells to specifically detect membrane potential and changes thereto.

[00115] Some of the microbial rhodopsins are derived from a microbial rhodopsin protein by modification of the protein to reduce or inhibit light-induced ion pumping of the rhodopsin protein. Such modifications permit the modified microbial rhodopsin proteins to sense voltage without altering the membrane potential of the cell with its native ion pumping activity and thus altering the voltage of the system. Other mutations impart other advantageous properties to microbial rhodopsin voltage sensors, including increased fluorescence brightness, improved photostability, tuning of the sensitivity and dynamic range of the voltage response, increased response speed, and tuning of the absorption and emission spectra.

[00116] Mutations that eliminate pumping in microbial rhodopsins in the present invention generally comprise mutations to the Schiff base counterion; a carboxylic amino acid (Asp or Glu) conserved on the third transmembrane helix (helix C) of the rhodopsin proteins. The amino acid sequence is RYX(DE) where X is a non-conserved amino acid. Mutations to the carboxylic residue directly affect the proton conduction pathway, eliminating proton pumping. Most typically the mutation is to Asn or Gln, although other mutations are possible, and based on the description provided herein, one skilled in the art can make different mutants which also result in reduced or absent ion pumping by the microbial rhodopsin protein. In one embodiment, the modified microbial rhodopsin proteins of the invention and the methods of the invention comprises the Asp to Asn or Gln mutation, or Glu to Asn or Gln mutation. In some embodiments, the protein consist essentially of Asp to Asn or Gln mutation, or Glu to Asn or Gln mutation. In some embodiments, the protein consist of Asp to Asn or Gln mutation, or Glu to Asn or Gln mutation.

[00117] Provided herein are illustrative exemplary optical voltage sensors and directions for making and using such sensors. Other sensors that work in a similar manner as optical sensors can be prepared and used based on the description and the examples provided herein.

[00118] Table 1a includes exemplary microbial rhodopsins useful according to the present invention. For example, mutations that eliminate pumping in microbial rhodopsins in the present invention generally comprise mutations to the Schiff base counterion; a carboxylic amino acid (Asp or Glu) conserved on the third transmembrane helix (helix C) of the rhodopsin proteins. Table 1a refers to the amino acid position in the sequence provided as the exemplary Genbank number. However, the position may be numbered slightly differently based on the variations in the available amino acid sequences. Based on the description of the motif described herein, a skilled artisan will easily be able to make similar mutations into other microbial rhodopsin genes to achieve the same functional feature, i.e. reduction in the pumping activity of the microbial rhodopsin in question.

Table 1a: Exemplary microbial rhodopsins useful according to the present invention.				
Microbial Rhodopsin	**Abbreviation**	**Genbank number**	**Amino acid mutation**	
Green-absorbing proteorhodopsin: a light-driven proton pump found in marine bacteria	GPR	AF349983; wild-type, (Nucleotide and protein disclosed as SEQ ID NOS 74-75, respectively)	D99N (SEQ ID NO: 76) in the specification, this mutation is also referred to as D97N	
Blue-absorbing proteorhodopsin: a light-driven proton pump found in marine bacteria.	BPR	AF349981; wild-type; (Nucleotide and protein disclosed as SEQ ID NOS 77-78, respectively)	D99N (SEQ ID NO: 79)	
Natronomonas pharaonis sensory rhodopsin II: a light-activated signaling protein found in the halophilic bacterium N. pharaonis. In the wild the sensory domain is paired with a transducer domain	NpSRII	Z35086.1; In one embodiment only the sensory domain, given by nucleotides 2112 – 2831 of sequence Z35086, is used (Nucleotide and "sensory domain" protein disclosed as SEQ ID NOS 80-81, respectively)	D75N (SEQ ID NO: 82)	
Bacteriorhodopsin: a light-driven proton pump found in Halobacterium salinarum	BR	NC_010364.1, nucleotides 1082241 to 1083029, or GenBank sequence M11720.1; ("M11720.1" nucleotide and protein disclosed as SEQ ID NOS 83-84, respectively	D98N (SEQ ID NO: 85)	
Archaerhodopsin Arch 3: a light-driven proton pump found in Halobacterium sodomense	Arch 3 (or Ar 3)	Chow B.Y. et al., Nature 463:98-102; ("Arch 3" wild-type protein disclosed as SEQ ID NO: 86)	D95N (SEQ ID NO: 87)	

[00119] The following Table 1b includes exemplary additional rhodopsins that can be mutated as indicated in the methods of the invention:

Table 1b				
Microbial Rhodopsin	**Abbreviation**	**Genbank number**	**Nucleic acid mutation**	**Amino Acid mutation**
Fungal Opsin Related Protein	Mac	AAG01180 (SEQ ID NO: 60)	G415 to A	D139N (SEQ ID NO: 61)
Cruxrhodopsin	Crux	BAA06678 (SEQ ID NO: 62)	G247 to A	D83N (SEQ ID NO: 63)

Table 1b				
Microbial Rhodopsin	**Abbreviation**	**Genbank number**	**Nucleic acid mutation**	**Amino Acid mutation**
Algal Bacteriorhodopsin	Ace	AAY82897 (SEQ ID NO: 64)	G265 to A	D89N (SEQ ID NO: 65)
Archaerhodopsin 1	Ar1	P69051 (SEQ ID NO: 66)	G289 to A	D97N (SEQ ID NO: 67)
Archaerhodopsin 2	Ar2	P29563 (SEQ ID NO: 68)	G286 to A	D96N (SEQ ID NO: 69)
Archaerhodopsin 3	Ar3	P96787 (SEQ ID NO: 70)	G283 to A	D95N (SEQ ID NO: 71)
Archaerhodopsin 4	Ar4	AAG42454 (SEQ ID NO: 72)	G292 to A	D98N (SEQ ID NO: 73)

Voltage Indicating Proteins (VIP)

[00120] We have developed a family of fluorescent voltage-indicating proteins (VIPs) based on Achaerhodopsins that function in mammalian cells, including neurons and human stem cell-derived cardiomyocytes. These proteins indicate electrical dynamics with sub-millisecond temporal resolution and sub-micron spatial resolution. We have demonstrated non-contact, high-throughput, and high-content studies of electrical dynamics in mammalian cells and tissues using optical measurement of membrane potential. These VIPs are broadly useful, particularly in eukaryotic, such as mammalian, including human cells.

[00121] We have developed VIPs based on Archaerhodopsin 3 (Arch 3) and its homologues. Arch 3 is Archaerhodopsin from *H. sodomense* and it is known as a genetically-encoded reagent for high-performance yellow/green-light neural silencing. Gene sequence at GenBank: GU045593.1 (synthetic construct Arch 3 gene, complete cds. Submitted 9/28/2009). We have shown that these proteins localize to the plasma membrane in eukaryotic cells and show voltage-dependent fluorescence.

[00122] We have also shown further improved membrane localization, with comparable voltage sensitivity, in ArchT, gene sequence at GenBank: HM367071.1 (synthetic construct ArchT gene, complete cds. Submitted 5/27/2010). ArchT is Archaerhodopsin from *Halorubrum sp.* TP009: genetically-encoded reagent for high-performance yellow/green-light neural silencing, 3.5x more light sensitive than Arch 3.

[00123] Table 1c summarizes exemplary sequences that can be used to create viral constructs that express the voltage indicators based on Archaerhodopsin.

Table 1c: Exemplary sequences that can be used to generate virus constructs with Arch 3 and ArchT		
Virus backbone	Lentivirus	SEQ ID NO: 24
Promoter	CamKII (neuron specific)	SEQ ID NO: 25
	CAG enhancer (pan cellular)	SEQ ID NO: 26
	CMV (pan cellular)	SEQ ID NO: 27
	Ubiquitin (pan cellular)	SEQ ID NO: 28
Voltage-sensing domain	Arch D95N	SEQ ID NO: 29
	ArchT D95N	SEQ ID NO: 30

[00124] Figure 10 shows existing genetically encoded fluorescent voltage indicators classified according to their sensitivity and speed—the two key parameters that determine the performance of an indicator. The proteins we developed are the Proteorhodopsin Optical Proton Sensor (PROPS), Arch 3 WT, and Arch 3 D95N, shown on the upper right. PROPS only functions in bacteria, while Arch 3 WT and Arch 3 D95N function in mammalian cells. We showed that microbial rhodopsin-based voltage indicators are faster and far more sensitive than other indicators.

[00125] Table 2 shows exemplary approximate characteristics of fluorescent voltage indicating proteins and contains representative members of all families of fluorescent indicators. Although the list on Table 2 is not comprehensive, one skilled in the art can readily see the characteristics of the types of proteins useful in the present invention.

Table 2: Representative members of all families of fluorescent indicators.			
Molecule	**Approx ΔF/F per 100 mV**	**Approx response time**	**Comments**
VSFP 2.3, Knopfel, T. et al. J. Neurosci. 30, 14998-15004 (2010)	9.5%	78 ms	Ratiometric (ΔR/R)
VSFP 2.4 Knopfel, T. et al. J. Neurosci. 30, 14998-15004 (2010)	8.9%	72 ms	Ratiometric (ΔR/R)
VSFP 3.1, Lundby, A., et al., PLoS One 3, 2514 (2008)	3%	1-20 ms	Protein
Mermaid, Perron, A. et al. Front Mol Neurosci. 2, 1-8 (2009)	9.2%	76	Ratiometric (ΔR/R)
SPARC, Ataka, K. &	0.5%	0.8 ms	Protein

Table 2: Representative members of all families of fluorescent indicators.			
Molecule	**Approx ΔF/F per 100 mV**	**Approx response time**	**Comments**
Pieribone, V. A. Biophys. J. 82, 509-516 (2002)			
Flash, Siegel, M. S. & Isacoff, E. Y. Neuron 19, 735-741 (1997)	5.1%	2.8 – 85 ms	Protein
PROPS, described herein; SEQ ID NO:	150%	5 ms	Protein
Arch 3 WT, described herein	66%	< 0.5 ms	Protein
Arch D95N	100%	41 ms	Protein

[00126] Figure 9 shows an optical recording of action potentials in a single rat hippocampal neuron. The data represents a single trial, in which spiking was induced by injection of a current pulse. The fluorescence shows clear bursts accompanying individual action potentials. This experiment is the first robust measurement of action potentials in a single mammalian neuron using a genetically encoded voltage indicator.

[00127] Figure 11 shows optical recordings of action potentials in a single HL-1 mouse cardiomyocyte expressing Arch 3 D95N - eGFP. Action potentials were recorded for up to 1000 s, with no signs of phototoxicity. This experiment is the first quantitative measurement of cardiac action potentials with a genetically encoded voltage indicator.

[00128] Figure 12 shows optical recordings of action potentials in human induced pluripotent stem cell (iPS)-derived cardiomyocytes, expressing Arch 3 D95N – eGFP. Human induced pluripotent stem cells (hiPSC) were provided by Cellular Dynamics Inc. Cells were plated into MatTek dishes coated in 0.1 % gelatin at a density of 20, 50, or 75 thousand cells per square centimeter. These conditions showed cells that were sparse and did not beat spontaneously (20K), a confluent monolayer that did beat spontaneously (50K), and a dense monolayer (75K). iPS cells were plated and maintained for 48 hours in plating medium, and thereafter fed every 48 hours with maintenance medium (both from Cellular Dynamics). iPS cells were transfected using Mirus LT-1 according to the manufacturer directions. To a tube containing 20 uL of optimem, we added 200 ng of DNA and 1.2 uL of the LT-1 transfection reagent. The DNA mixture was incubated at room temperature for 20 minutes. Fresh maintenence medium was added to the iPS cells during the

incubation and the DNA mixture was added dropwise over the plate. Cells were imaged 48 to 96 hours after transfection.

[00129] We observed synchronized beating of adjoining cells, indicating that VIPs can probe intercellular conduction. We recorded for more than 10 minutes continuously, with little phototoxicity. Within the population of cells we observed cells with action potentials matching those of ventricular, atrial, and nodal cells, as expected for this population. Addition of drugs led to changes in the action potential waveform that matched changes reported by conventional patch clamp.

Generation of fusions between microbial rhodopsins and GFP homologues with additional or improved properties

[00130] We fused VIPs with GFP-homologue proteins to develop a series of improved voltage indicators. Figure 16 illustrates these exemplary constructs and Figure 16 legend provides the sequences for these constructs. The new capabilities of these newly developed sensors include, for example, a spectral shift FRET (ssFRET) for enhanced brightness and 2-photon imaging, ratiometric voltage imaging, and multimodal sensors for simultaneous measurement of voltage and concentration.

Spectral shift FRET (ssFRET) for enhanced brightness and 2-photon imaging

[00131] A key limitation of the first generation of VIPs was that the endogenous fluorescence of the retinal was dim. Imaging required a specialized system comprising an intense red laser, a high numerical aperture objective, and an electron-multiplying CCD (EMCCD) camera. Ideally one would like an indicator bright enough to image on a conventional wide-field or confocal fluorescence microscope, or a 2-photon confocal microscope for in vivo applications.

[00132] ssFRET provides a path to brighter VIPs as shown in Figure 10. A GFP-homologue (generically referred to as GFP) is fused to the microbial rhodopsin (see, e.g., Figure 13). Voltage-dependent changes in the absorption spectrum of the retinal lead to voltage-dependent rates of nonradiative fluorescence resonance energy transfer (FRET) between the GFP and the retinal. Retinal in its absorbing, fluorescent state quenches the GFP, while retinal in the non-absorbing, non-fluorescent state does not quench the GFP. Thus one obtains anti-correlated fluorescence emission of the GFP and the retinal.

[00133] Thus in one embodiment, the invention provides a fusion protein comprising a GFP that is fused to a microbial rhodopsin or a modified microbial rhodopsin, such as a proteorhdopsin or archaerhodopsin. Such fusion proteins can be used in any and all of the methods of the present invention.

[00134] To maximize the degree of ssFRET between the GFP and the retinal we selected a GFP-homologue, mOrange2, whose emission overlaps maximally with the absorption of Arch 3 in its protonated state. The rate of FRET falls off very quickly with increasing distance between chromophores, so we constructed a series of truncated constructs in which the linker and non-essential

elements of the Arch 3 and mOrange2 were removed. Figure 15 shows that as the distance between the mOrange2 and the Arch 3 decreased, the ssFRET signal increased. This same strategy can be applied to generate ssFRET signals from other microbial rhodopsins and GFP homologues.

[00135] We have shown that the time response of fluorescence from mOrange2 to a step in Vm matches the time response of fluorescence from Arch D95N. This observation is consistent with ssFRET. Similar results can be seen for Figures 9A and 11 for fusions to Arch 3 WT.

Ratiometric voltage imaging

[00136] A key challenge in application of VIPs is to extract accurate values of the membrane potential, without systematic artifacts from photobleaching, variation in illumination intensity, cell movement, or variations in protein expression level. In cells that are accessible to patch clamp, one can calibrate the fluorescence as a function of membrane potential by varying the membrane potential under external control. However, a benefit of VIPs is that they function in systems that are inaccessible to patch clamp. In these cases direct calibration is not possible.

[00137] The Arch 3 (WT or D95N) fusion with eGFP enables ratiometric determination of membrane potential. Similar ratiometric determinations may be made using other rhodopsins such as those described in this application using the identical concept. The eGFP fluorescence is independent of membrane potential, The ratio of Arch 3 fluorescence to eGFP fluorescence provides a measure of membrane potential that is independent of variations in expression level, illumination, or movement. This construct does not undergo ssFRET due to the long linker between the eGFP and the Arch 3, and because the emission of eGFP has little spectral overlap with the absorption of Arch 3.

Multimodal sensors for simultaneous measurement of voltage and concentration

[00138] Membrane potential is only one of several mechanisms of signaling within cells. One often wishes to correlate changes in membrane potential with changes in concentration of other species, such as Ca++, H+ (i.e. pH), Na+, ATP, cAMP. We constructed fusions of Arch with pHluorin (a fluorescent pH indicator) and GCaMP3 (a fluorescent Ca++ indicator). One can also use fusions with other protein-based fluorescent indicators to enable other forms of multimodal imaging using the concept as taught herein. Concentration of ions such as sodium, potassium, chloride, and calcium can be simultaneously measured when the nucleic acid encoding the microbial rhodopsin is operably linked to or fused with an additional fluorescent ion sensitive indicator.

Additional fluorescent proteins

[00139] The term "additional fluorescent molecule" refers to fluorescent proteins other then microbial rhodopsins. Such molecules may include, e.g., green fluorescent proteins and their homologs.

[00140] Fluorescent proteins that are not microbial rhodopsins are well known and commonly used, and examples can be found, e.g., in a review The Family of GFP-Like Proteins: Structure, Function, Photophysics and Biosensor Applications. Introduction and Perspective, by Rebekka M. Wachter (Photochemistry and Photobiology Volume 82, Issue 2, pages 339–344, March 2006). Also, a review by Nathan C Shaner, Paul A Steinbach, & Roger Y Tsien, entitled A guide to choosing fluorescent proteins (Nature Methods - 2, 905 - 909 (2005)) provides examples of additional useful fluorescent proteins.

Targeting VIPs to intracellular organelles

[00141] We have shown targeting VIPs to intracellular organelles, including mitochondria, the endoplasmic reticulum, the sarcoplasmic reticulum, synaptic vesicles, and phagosomes. Accordingly, in one embodiment, the invention provides constructs, such as expression constructs, such as viral constructs comprising a microbial rhodopsin operably linked to a sequence targeting the protein to an intracellular organelle, including a mitochondrium, an endoplasmic reticulum, a sarcoplasmic reticulum, a synaptic vesicle, and a phagosome.

[00142] The invention further provides cells expressing the constructs, and further methods of measuring membrane potential changes in the cells expressing such constructs as well as methods of screening for agents that affect the membrane potential of one or more of the intracellular membranes.

The key advantages of Voltage Indicating Proteins (VIPs)

[00143] The newly developed VIPs show high sensitivity. In mammalian cells VIPs show about 3-fold increase in fluorescence between -150 mV and +150 mV. The response is linear over most of this range. We can measure membrane voltage with a precision of < 1 mV in a 1 s interval.

[00144] The newly developed VIPs show high speed. Arch 3 WT shows 90% of its step response in < 0.5 ms. A neuronal action potential lasts 1 ms, so this speed meets the benchmark for imaging electrical activity of neurons. However, Arch 3 WT retains the photoinduced proton-pumping, so illumination slightly hyperpolarizes the cell.

[00145] The modified microbial rhodopsin, Arch 3 D95N, has a 40 ms response time and lacks photoinduced proton pumping. Although the slower response time of this construct hampers detection of membrane potential and changes thereto in neurons, the Arch 3 D95N is fast enough to indicate membrane potential and action potentials in other types of cells, for example, in cardiomyocytes and does not perturb membrane potential in the cells wherein it is used.

[00146] The newly developed VIPs also show high photostability. VIPs are comparable to GFP in the number of fluorescence photons produced prior to photobleaching. We routinely watch VIPs in mammalian cells for many minutes, without signs of photobleaching or phototoxicity. VIPs have no homology to GFP, nor to any other known fluorescent protein.

[00147] The newly developed VIPs also show far red spectrum. VIPs are excited with a 633 nm laser, and the emission is in the near infrared, peaked at 710 nm. The emission is farther to the red than any existing fluorescent protein. These wavelengths coincide with low cellular autofluorescence and good transmission through tissue. This feature makes these proteins particularly useful in optical measurements of action potential as the spectrum facilitates imaging with high signal-to-noise, as well as multi-spectral imaging in combination with other fluorescent probes.

[00148] The newly developed VIPs further show high targetability. We have imaged VIPs in primary neuronal cultures, cardiomyocytes (HL-1 and human iPSC-derived), HEK cells, and Gram positive and Gram negative bacteria. We targeted a VIP to the endoplasmic reticulum, and to mitochondria. The ocnstructs are useful also for *in vivo* imaging in *C. elegans*, zebrafish, and mice.

[00149] With the microbial rhodopsin constructs of the invention further comprising a cell type- and/or a time-specific promotors, one can image membrane potential in any optically accessible cell type or organelle in a living organism.

[00150] In one embodiment, the design of a voltage sensor comprises, consists of, or consists essentially of selecting at least three elements: a promoter, a microbial rhodopsin voltage sensor, one or more targeting motifs, and an optional accessory fluorescent protein. Some non-limiting examples for each of these elements are listed in Tables 1a and 1b, and Table 3 below. In one embodiment, at least one element from each column is selected to create an optical voltage sensor with the desired properties. In some embodiments, methods and compositions for voltage sensing as described herein involves selecting: 1)A microbial rhodopsin protein, 2) one or more mutations to imbue the protein with sensitivity to voltage or to other quantities of interest and to eliminate light-driven charge pumping, 3) codon usage appropriate to the host species, 4) a promoter and targeting sequences to express the protein in cell types of interest and to target the protein to the sub-cellular structure of interest, 5) an optional fusion with a conventional fluorescent protein to provide ratiometric imaging, 6) a chromophore to insert into the microbial rhodopsin, and 7) an optical imaging scheme.

Table 3: Exemplary optical sensor combinations			
Promoter	**Voltage sensor**	**Targeting motif**	**Accessory fluorescent protein**
CMV (SEQ ID NO: 31)	hGPR (D97N)(SEQ ID NO: 35)	SS(β2nAChR)(SEQ ID NO: 39)	Venus(SEQ ID NO: 43)
14x UAS-E1b (SEQ ID NO: 32)	hGPR (D97N, ±E108Q, ±E142Q, ±L217D) (SEQ ID NO: 36)	SS(PPL)(SEQ ID NO: 40)	EYFP (SEQ ID NO: 44)
HuC(SEQ ID NO: 33)	hBPR (D99N)(SEQ ID NO: 37)	ER export motif(SEQ ID NO: 41)	TagRFP(SEQ ID NO: 45)
ara(SEQ ID NO: 34)	hNpSRII (D75N)(SEQ ID NO: 38)	TS from Kir2.1 (SEQ ID NO: 42)	
lac		MS	

[00151] In one embodiment, the optical sensor gene is encoded by a delivery vector. Such vectors include but are not limited to: plasmids (e.g. pBADTOPO, pCI-Neo, pcDNA3.0), cosmids, and viruses (such as a lentivirus, an adeno-associated virus, or a baculovirus).

[00152] In one embodiment, the green-absorbing proteorhodopsin (GPR) is used as the starting molecule. This molecule is selected for its relatively red-shifted absorption spectrum and its ease of expression in heterologous hosts such as *E. coli*. In another embodiment, the blue-absorbing proteorhodopsin (BPR) is used as an optical sensor of voltage. It is contemplated herein that a significant number of the microbial rhodopsins found in the wild can be engineered as described herein to serve as optical voltage sensors.

[00153] Microbial rhodopsins are sensitive to quantities other than voltage. Mutants of GPR and BPR, as described herein, are also sensitive to intracellular pH. It is also contemplated that mutants of halorhodopsin may be sensitive to local chloride concentration.

[00154] In one embodiment, the voltage sensor is selected from a microbial rhodopsin protein (wild-type or mutant) that provides a voltage-induced shift in its absorption or fluorescence. The starting sequences from which these constructs can be engineered include, but are not limited to, sequences listed in Tables 1a-1b, that list the rhodopsin and an exemplary mutation that can be made to the gene to enhance the performance of the protein product.

[00155] *Mutations to minimize the light-induced charge-pumping capacity.* The retinal chromophore is linked to a lysine by a Schiff base. A conserved aspartic acid serves as the proton acceptor adjacent to the Schiff base. Mutating this aspartic acid to asparagine suppresses proton pumping. Thus, in some embodiments, the mutations are selected from the group consisting of: D97N (green-absorbing proteorhodopsin), D99N (blue-absorbing proteorhodopsin), D75N (sensory

rhodopsin II), and D85N (bacteriorhodopsin). In other embodiments, residues that can be mutated to inhibit pumping include (using bacteriorhodopsin numbering) D96, Y199, and R82, and their homologues in other microbial rhodopsins. In another embodiment, residue D95 can be mutated in archaerhodopsin to inhibit proton pumping (e.g., D95N).

[00156] *Mutations are introduced to shift the absorption and emission spectra into a desirable range.* Residues near the binding pocket can be mutated singly or in combination to tune the spectra to a desired absorption and emission wavelength. In bacteriorhodopsin these residues include, but are not limited to, L92, W86, W182, D212, I119, and M145. Homologous residues may be mutated in other microbial rhodopsins. Thus, in some embodiments, the mutation to modify the microbial rhodopsin protein is performed at a residue selected from the group consisting of L92, W86, W182, D212, I119, M145.

[00157] *Mutations are introduced to shift the dynamic range of voltage sensitivity into a desired band.* Such mutations function by shifting the distribution of charge in the vicinity of the Schiff base, and thereby changing the voltage needed to add or remove a proton from this group. Voltage-shifting mutations in green-absorbing proteorhodopsin include, but are not limited to, E108Q, E142Q, L217D, either singly or in combination using green-absorbing proteorhodopsin locations as an example, or a homologous residue in another rhodopsin. In one embodiment, a D95N mutation is introduced into archaerhodopsin 3 to adjust the pKa of the Schiff base towards a neutral pH.

[00158] *Optionally mutations are introduced to enhance the brightness and photostability of the fluorescence.* Residues which when mutated may restrict the binding pocket to increase fluorescence include (using bacteriorhodopsin numbering), but are not limited to, Y199, Y57, P49, V213, and V48.

Codon usage

[00159] A large number of mammalian genes, including, for example, murine and human genes, have been successfully expressed in various host cells, including bacterial, yeast, insect, plant and mammalian host cells. Nevertheless, despite the burgeoning knowledge of expression systems and recombinant DNA technology, significant obstacles remain when one attempts to express a foreign or synthetic gene in a selected host cell. For example, translation of a synthetic gene, even when coupled with a strong promoter, often proceeds much more slowly than would be expected. The same is frequently true of exogenous genes that are foreign to the host cell. This lower than expected translation efficiency is often due to the protein coding regions of the gene having a codon usage pattern that does not resemble those of highly expressed genes in the host cell. It is known in this regard that codon utilization is highly biased and varies considerably in different organisms and that biases in codon usage can alter peptide elongation rates. It is also known that codon usage patterns are related to the relative abundance of tRNA isoacceptors, and that genes encoding proteins of high versus low abundance show differences in their codon preferences.

[00160] Codon-optimization techniques have been developed for improving the translational kinetics of translationally inefficient protein coding regions. These techniques are based on the

replacement of codons that are rarely or infrequently used in the host cell with those that are host-preferred. Codon frequencies can be derived from literature sources for the highly expressed genes of many organisms (see, for example, Nakamura et al., 1996, Nucleic Acids Res 24: 214-215). These frequencies are generally expressed on an 'organism-wide average basis' as the percentage of occasions that a synonymous codon is used to encode a corresponding amino acid across a collection of protein-encoding genes of that organism, which are preferably highly expressed. In one embodiment, the codons of a microbial rhodopsin protein are optimized for expression in a eukaryotic cell. In one embodiment, the eukaryotic cell is a human cell.

[00161] It is preferable but not necessary to replace all the codons of the microbial polynucleotide with synonymous codons having higher translational efficiencies in eukaryotic (e.g., human) cells than the first codons. Increased expression can be accomplished even with partial replacement. Typically, the replacement step affects at least about 5%, 10%, 15%, 20%, 25%, 30%, more preferably at least about 35%, 40%, 50%, 60%, 70% or more of the first codons of the parent polynucleotide. Suitably, the number of, and difference in translational efficiency between, the first codons and the synonymous codons are selected such that the protein of interest is produced from the synthetic polynucleotide in the eukaryotic cell at a level which is at least about 110%, suitably at least about 150%, preferably at least about 200%, more preferably at least about 250%, even more preferably at least about 300%, even more preferably at least about 350%, even more preferably at least about 400%, even more preferably at least about 450%, even more preferably at least about 500%, and still even more preferably at least about 1000%, of the level at which the protein is produced from the parent polynucleotide in the eukaryotic cell.

[00162] Generally, if a parent polynucleotide has a choice of low and intermediate translationally efficient codons, it is preferable in the first instance to replace some, or more preferably all, of the low translationally efficient codons with synonymous codons having intermediate, or preferably high, translational efficiencies. Typically, replacement of low with intermediate or high translationally efficient codons results in a substantial increase in production of the polypeptide from the synthetic polynucleotide so constructed. However, it is also preferable to replace some, or preferably all, of the intermediate translationally efficient codons with high translationally efficient codons for optimized production of the polypeptide.

[00163] Replacement of one codon for another can be achieved using standard methods known in the art. For example codon modification of a parent polynucleotide can be effected using several known mutagenesis techniques including, for example, oligonucleotide-directed mutagenesis, mutagenesis with degenerate oligonucleotides, and region-specific mutagenesis. Exemplary in vitro mutagenesis techniques are described for example in U.S. Pat. Nos. 4,184,917, 4,321,365 and 4,351,901 or in the relevant sections of Ausubel, et al. (CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, Inc. 1997) and of Sambrook, et al., (MOLECULAR CLONING. A LABORATORY MANUAL, Cold Spring Harbor Press, 1989). Instead of in vitro mutagenesis, the

synthetic polynucleotide can be synthesized de novo using readily available machinery as described, for example, in U.S. Pat. No.4,293,652. However, it should be noted that the present invention is not dependent on, and not directed to, any one particular technique for constructing the synthetic polynucleotide.

[00164] The genes for microbial rhodopsins (e.g., GPR) express well in *E. coli*, but less well in eukaryotic hosts. In one embodiment, to enable expression in eukaryotes a version of the gene with codon usage appropriate to eukaryotic (e.g., human) cells is designed and synthesized. This procedure can be implemented for any gene using publicly available software, such as e.g., the Gene Designer 2.0 package (available on the world wide web at dna20.com/genedesigner2/). Some of the "humanized" genes are referred to herein by placing the letter "h" in front of the name, e.g. hGPR. The Arch 3 rhodopsins and mutants thereof described herein and in the examples are all optimized for human codon usage.

Applications for VIPs in screens for drugs that target the following tissues or processes

[00165] The constructs disclosed in the present application can be used in methods for drug screening, e.g., for drugs targeting the nervous system. In a culture of cells expressing specific ion channels, one can screen for agonists or antagonists without the labor of applying patch clamp to cells one at a time. In neuronal cultures one can probe the effects of drugs on action potential initiation, propagation, and synaptic transmission. Application in human iPSC-derived neurons will enable studies on genetically determined neurological diseases, as well as studies on the response to environmental stresses (e.g. anoxia).

[00166] Similarly, the optical voltage sensing using the constructs provided herein provides a new and much improved methods to screen for drugs that modulate the cardiac action potential and its intercellular propagation. These screens will be useful both for determining safety of candidate drugs and to identify new cardiac drug leads. Identifying drugs that interact with the hERG channel is a particularly promising direction because inhibition of hERG is associated with ventricular fibrillation in patients with long QT syndrome. Application in human iPSC-derived cardiomyocytes will enable studies on genetically determined cardiac conditions, as well as studies on the response to environmental stresses (e.g. anoxia).

[00167] Additionally, the constructs of the present invention can be used in methods to study of development and wound healing. The role of electrical signaling in normal and abnormal development, as well as tissue repair, is poorly understood. VIPs enable studies of voltage dynamics over long times in developing or healing tissues, organs, and organisms, and lead to drugs that modulate these dynamics.

[00168] In yet another embodiment, the invention provides methods to screen for drugs that affect membrane potential of mitochondria. Mitochondria play an essential role in ageing, cancer, and

neurodegenerative diseases. Currently there is no good probe for mitochondrial membrane potential. VIPs provide such a probe, enabling searches for drugs that modulate mitochondrial activity.

[00169] The invention further provides methods to screen for drugs that modulate the electrophysiology of a wide range of medically, industrially, and environmentally significant microorganisms.

[00170] Prior to our discovery of VIPs, no measurement of membrane potential had been made in any intact prokaryote. We discovered that bacteria have complex electrical dynamics. VIPs enable screens for drugs that modulate the electrophysiology of a wide range of medically, industrially, and environmentally significant microorganisms. For instance, we found that electrical activity is correlated with efflux pumping in *E. coli*.

[00171] Changes in membrane potential are also associated with activation of macrophages. However, this process is poorly understood due to the difficulty in applying patch clamp to motile cells. VIPs enable studies of the electrophysiology of macrophages and other motile cells, including sperm cells for fertility studies. Thus the VIPs of the invention can be used in methods to screen for drugs or agents that affect, for example, immunity and immune diseases, as well as fertility.

[00172] The examples describe expression of VIPs in rat hippocampal neurons, mouse HL-1 cardiomyocytes, and human iPS-derived cardiomyocytes. In all cell types, single action potentials (APs) were readily observed. We tested the effects of drugs on the AP waveform.

[00173] For example, in one embodiment, the invention provides a method wherein the cell expressing a microbial rhodopsin is further exposed to a stimulus capable of or suspected to be capable of changing membrane potential.

[00174] Stimuli that can be used include candidate agents, such as drug candidates, small organic and inorganic molecules, larger organic molecules and libraries of molecules and any combinations thereof. One can also use a combination of a known drug, such as an antibiotic with a candidate agent to screen for agents that may increase the effectiveness of the one or more of the existing drugs, such as antibiotics.

[00175] The methods of the invention are also useful for vitro toxicity screening and drug development. For example, using the methods described herein one can make a human cardiomyocyte from induced pluripotent cells that stably express a modified archaerhodopsin wherein the proton pumping activity is substantially reduced or abolished. Such cells are particularly useful for in vitro toxicity screening in drug development.

PROPS: an exemplary optogenetic voltage sensor derived from GPR

[00176] GPR has seven spectroscopically distinguishable states that it passes through in its photocycle. In principle the transition between any pair of states is sensitive to membrane potential. In one embodiment, the acid-base equilibrium of the Schiff base was chosen as the wavelength-shifting transition, hence the name of the sensor: Proteorhodopsin Optical Proton Sensor (PROPS).

Characterization of the properties of PROPS and its uses are described herein in the Examples section. A brief discussion of PROPS is provided herein below.

[00177] The absorption spectrum of wild-type GPR is known to depend sensitively on the state of protonation of the Schiff base. When protonated, the absorption maximum is at 545 nm, and when deprotonated the maximum is at 412 nm. When GPR absorbs a photon, the retinal undergoes a 13-trans to cis isomerization, which causes a proton to hop from the Schiff base to nearby Asp97, leading to a shift from absorption at 545 nm to 412 nm. The PROPS design described herein seeks to recapitulate this shift in response to a change in membrane potential.

[00178] Two aspects of wild-type GPR can be changed for it to serve as an optimal voltage sensor. First, the pKa of the Schiff base can be be shifted from its wild-type value of ~12 to a value close to the ambient pH. When pKa ~ pH, the state of protonation becomes maximally sensitive to the membrane potential. Second, the endogenous charge-pumping capability can be be eliminated, because optimally, a voltage probe should not perturb the quantity under study. However, in some situations, a wild type microbial rhodopsin can be used, such as Arch 3 WT, which functions in neurons to measure membrane potential as shown in our examples.

[00179] In one embodiment, a single point mutation induces both changes in GPR. Mutating Asp97 to Asn eliminates a negative charge near the Schiff base, and destabilizes the proton on the Schiff base. The pKa shifts from ~12 to 9.8. In wild-type GPR, Asp97 also serves as the proton acceptor in the first step of the photocycle, so removing this amino acid eliminates proton pumping. This mutant of GPR is referred to herein as PROPS.

[00180] Similarly, in an analogous voltage sensor derived from BPR, the homologous mutation Asp99 to Asn lowers the pKa of the Schiff base and eliminates the proton-pumping photocycle. Thus, in one embodiment the optical sensor is derived from BPR in which the amino acid residue Asp99 is mutated to Asn.

[00181] In GPR, additional mutations shift the pKa closer to the physiological value of 7.4. In particular, mutations Glu108 to Gln and Glu142 to Gln individually or in combination lead to decreases in the pKa and to further increases in the sensitivity to voltage. Many mutations other than those discussed herein may lead to additional changes in the pKa and improvements in the optical properties of PROPS and are contemplated herein.

Expression Vectors and targeting sequences

[00182] Optical sensors can be expressed in a cell using an expression vector. The term " vector " refers to a carrier DNA molecule into which a nucleic acid sequence can be inserted for introduction into a host cell. An "expression vector" is a specialized vector that contains the necessary regulatory regions needed for expression of a gene of interest in a host cell. In some embodiments the gene of interest is operably linked to another sequence in the vector. In some embodiments, it is preferred that the viral vectors are replication defective, which can be achieved for example by removing all viral

nucleic acids that encode for replication. A replication defective viral vector will still retain its infective properties and enters the cells in a similar manner as a replicating vector, however once admitted to the cell a replication defective viral vector does not reproduce or multiply. The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in an expression vector.

[00183] Many viral vectors or virus-associated vectors are known in the art. Such vectors can be used as carriers of a nucleic acid construct into the cell. Constructs may be integrated and packaged into non-replicating, defective viral genomes like Adenovirus, Adeno-associated virus (AAV), or Herpes simplex virus (HSV) or others, including retroviral and lentiviral vectors, for infection or transduction into cells. The vector may or may not be incorporated into the cell's genome. The constructs may include viral sequences for transfection, if desired. Alternatively, the construct may be incorporated into vectors capable of episomal replication, e.g EPV and EBV vectors. The inserted material of the vectors described herein may be operatively linked to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that polynucleotide sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the polynucleotide sequence to be expressed, and maintaining the correct reading frame to permit expression of the polynucleotide sequence under the control of the expression control sequence, and production of the desired polypeptide encoded by the polynucleotide sequence. In some examples, transcription of an inserted material is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell- type in which expression is intended. It will also be understood that the inserted material can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of a protein. In some instances the promoter sequence is recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required for initiating transcription of a specific gene.

[00184] An "inducible promoter" is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to a "regulatory agent" (e.g., doxycycline), or a "stimulus" (e.g., heat). In the absence of a "regulatory agent" or "stimulus", the DNA sequences or genes will not be substantially transcribed. The term "not substantially transcribed" or "not substantially expressed" means that the level of transcription is at least 100-fold lower than the level of transcription observed in the presence of an appropriate stimulus or regulatory agent; preferably at least 200-fold, 300-fold, 400-fold, 500-fold or more. As used herein, the terms "stimulus" and/or "regulatory agent" refers to a chemical agent, such as a metabolite, a small molecule, or a physiological stress directly imposed upon the organism such as cold, heat, toxins, or

through the action of a pathogen or disease agent. A recombinant cell containing an inducible promoter may be exposed to a regulatory agent or stimulus by externally applying the agent or stimulus to the cell or organism by exposure to the appropriate environmental condition or the operative pathogen. Inducible promoters initiate transcription only in the presence of a regulatory agent or stimulus. Examples of inducible promoters include the tetracycline response element and promoters derived from the ß-interferon gene, heat shock gene, metallothionein gene or any obtainable from steroid hormone-responsive genes. Inducible promoters which may be used in performing the methods of the present invention include those regulated by hormones and hormone analogs such as progesterone, ecdysone and glucocorticoids as well as promoters which are regulated by tetracycline, heat shock, heavy metal ions, interferon, and lactose operon activating compounds. For review of these systems see Gingrich and Roder, 1998, Annu Rev Neurosci 21, 377-405. Tissue specific expression has been well characterized in the field of gene expression and tissue specific and inducible promoters are well known in the art. These promoters are used to regulate the expression of the foreign gene after it has been introduced into the target cell.

[00185] The promoter sequence may be a "cell- type specific promoter" or a "tissue-specific promoter" which means a nucleic acid sequence that serves as a promoter, i.e., regulates expression of a selected nucleic acid sequence operably linked to the promoter, and which affects expression of the selected nucleic acid sequence in specific cells or tissues where membrane potential is desired to be measured. In some embodiments, the cell-type specific promoter is a leaky cell-type specific promoter. The term "leaky" promoter refers to a promoter which regulates expression of a selected nucleic acid primarily in one cell type, but cause expression in other cells as well. For expression of an exogenous gene specifically in neuronal cells, a neuron-specific enolase promoter can be used (see Forss-Petter et al., 1990, Neuron 5: 187-197). For expression of an exogenous gene in dopaminergic neurons, a tyrosine hydroxylase promoter can be used. For expression in pituitary cells, a pituitary-specific promoter such as POMC may be used (Hammer et al., 1990, Mol. Endocrinol. 4:1689-97). Examples of muscle specific promoters include, for example α-myosin heavy chain promoter, and the MCK promoter. Other cell specific promoters active in mammalian cells are also contemplated herein. Such promoters provide a convenient means for controlling expression of the exogenous gene in a cell of a cell culture or within a mammal.

[00186] In some embodiments, the expression vector is a lentiviral vector. Lentiviral vectors useful for the methods and compositions described herein can comprise a eukaryotic promoter. The promoter can be any inducible promoter, including synthetic promoters, that can function as a promoter in a eukaryotic cell. For example, the eukaryotic promoter can be, but is not limited to, ecdysone inducible promoters, E1a inducible promoters, tetracycline inducible promoters etc., as are well known in the art. In addition, the lentiviral vectors used herein can further comprise a selectable marker, which can comprise a promoter and a coding sequence for a selectable trait. Nucleotide sequences encoding selectable markers are well known in the art, and include those that encode gene

products conferring resistance to antibiotics or anti-metabolites, or that supply an auxotrophic requirement. Examples of such sequences include, but are not limited to, those that encode thymidine kinase activity, or resistance to methotrexate, ampicillin, kanamycin, chloramphenicol, puromycinor zeocin, among many others.

[00187] In some embodiments the viral vector is an adeno-asscoated virus (AAV) vector. AAV can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell.

[00188] The type of vector one selects will also depend on whether the expression is intended to be stable or transient.

[00189] The invention also provides cells that are genetically engineered to express the microbial rhodopsin, such as VIPs or PROPS. The cell may be engineered to express the VIP or PROPS transiently or stably.

[00190] The invention provides methods of making both transiently expressing cells and cells and cell lines that express the microbial rhodopsins stably.

[00191] *Transient expression.* One of ordinary skill in the art is well equipped to engineer cells that are transiently transfected to express the VIPs or PROPS as described herein. Transduction and transformation methods for transient expression of nucleic acids are well known to one skilled in the art.

[00192] Transient transfection can be carried out, e.g., using calcium phosphate, by electroporation, or by mixing a cationic lipid with the material to produce liposomes, cationic polymers or highly branched organic compounds. All these are in routine use in genetic engineering.

[00193] *Stable expression of VIP or PROP in an eukaryotic cell.* One of ordinary skill in the art is well equipped to engineer cells that stably express the VIPs or PROPS as described herein. These methods are also in routine use in genetic engineering. Exemplary protocols can be found, e.g., in Essential Stem Cell Methods, edited by Lanza and Klimanskaya, published in 2008, Academic Press. For example, one can generate a virus that integrates into the genome and comprises a selectable marker, and infect the cells with the virus and screen for cells that express the marker, which cells are the ones that have incorporated the virus into their genome. For example, one can generate a VSV-g psuedotyped lenti virus with a puromycin selectable marker in HEK cells according to established procedures. Generally, one can use a stem cell specific promoter to encode a GFP if FACS sorting is necessary. The hiPS cultures are cultivated on embryonic fibroblast (EF) feeder layers or on Matrigel® cell culture substrate in fibroblast growth factor supplemented EF conditioned medium. The cells are dissociated by trypsinization to a single cell suspension The cells can be plated, e.g., 1 x 10^5 cells on a tissue culture 6-well plate pretreated with, e.g., Matrigel® cell culture substrate. To maintain the cells in an undifferentiated state, one can use, e.g., EF conditioned medium. About 6 hours after plating, one can add virus supernatant to adhered cells (use 5 x 10^6 IU virus per 1 x 10^5 cells). Add 6 μg/mL protamine sulfate to enhance virus infection. Cells are cultured with the virus

for 24 hours; washed, typically with PBS, and fresh media is added with a selection marker, such as 1 μg/mL puromycin. The medium is replaced about every 2 days with additional puromycin. Cells surviving after 1 week are re-plated, e.g., using the hanging drop method to form EBs with stable incorporation of gene.

[00194] In some embodiments, it is advantageous to express an optical voltage sensor (e.g., Arch 3 D94N) in only a single cell-type within an organism, and further, if desired, to direct the sensor to a particular subcellular structure within the cell. Upstream promoters control when and where the gene is expressed. Constructs are made that optimize expression in all eukaryotic cells. In one embodiment, the optical voltage sensor is under the control of a neuron-specific promoter.

[00195] The promoter sequence can be selected to restrict expression of the protein to a specific class of cells and environmental conditions. Common promoter sequences include, but are not limited to, CMV (cytomegalovirus promoter; a universal promoter for mammalian cells), 14x UAS-E1b (in combination with the transactivator Gal4, this promoter allows combinatorial control of transgene expression in a wide array of eukaryotes. Tissue-specific expression can be achieved by placing Gal4 under an appropriate promoter, and then using Gal4 to drive the UAS-controlled transgene), HuC (drives pan-neuronal expression in zebrafish and other teleosts), ara (allows regulation of expression with arabinose in bacteria) and lac (allows regulation of expression with IPTG in bacteria).

[00196] In some embodiments, the optical voltage sensor further comprises a localization or targeting sequence to direct or sort the sensor to a particular face of a biological membrane or subcellular organelle. Preferred localization sequences provide for highly specific localization of the protein, with minimal accumulation in other subcellular compartments. Example localization sequences that direct proteins to specific subcellular structures are shown below in Table 4.

Table 4: Exemplary protein localization sequences

Subcellular compartment	*Sequence*	*SEQ ID NO.*
Nuclear (import signal)	PPKKKRKV	1
Endoplasmic reticulum (import signal)	MSFVSLLLVGILFWATGAENLTKCEVFN	2
Endoplasmic reticulum (retention signal)	KDEL	3
Peroxisome (import signal)	SKL	4
Peroxisome (import signal)	(R/K)-(L/V/I)-XXXXX-(H/Q)-(L/A/F) (where X can be any amino acid).	5
Mitochondrial inner membrane	MLSLRNSIRFFKPATRTLCSSRYLL	6

Table 4: Exemplary protein localization sequences		
Subcellular compartment	*Sequence*	*SEQ ID NO.*
Mitochondrial outer membrane	MLRTSSLFTRRVQPSLFRNILRLQST	7
plasma membrane (cytosolic face)	MGCIKSKRKDNLNDDGVDMKT	8
plasma membrane (cytosolic face)	KKKKKKKSKTKCVIM	9
mitochondrial targeting sequence: human PINK1	MAVRQALGRGLQLGRALLLR FTGKPGRAYGLGRPGPAAGC VRGERPGWAAGPGAEPRRVG LGLPNRLRFFRQSVAGL	10
mitochondrial targeting sequence: human serine protease HTRA2	MAAPRAGRGAGWSLRAWRAL GGIRWGRRPRL	11
mitochondrial targeting sequence: human cytochrome oxidase 1	MFADRWLFST NHKDIGTLY	12
mitochondrial targeting sequence: human cytochrome oxidase 2	MAHAAQVGLQ DATSPIMEEL ITFHDH	13
mitochondrial targeting sequence: human protein phospatase 1K	MSTAALITLVRSGGNQVRRR VLLSSRLLQ	14
mitochondrial targeting sequence: human ATP synthase alpha	MLSVRVAAAVVRALPRRAGL VSRNALGSSFIAARNFHASNTHL	15
mitochondrial targeting sequence: human frataxin	MWTLGRRAVAGLLASPSPAQ AQTLTRVPRPAELAPLCGRRG	16

[00197] Other examples of localization signals are described in, e.g., "Protein Targeting", chapter 35 of Stryer, L., Biochemistry (4th ed.). W. H. Freeman, 1995 and Chapter 12 (pages 551-598) of Molecular Biology of the Cell, Alberts et al. third edition, (1994) Garland Publishing Inc. In some

embodiments, more than one discrete localization motif is used to provide for correct sorting by the cellular machinery. For example, correct sorting of proteins to the extracellular face of the plasma membrane can be achieved using an N-terminal signal sequence and a C-terminal GPI anchor or transmembrane domain.

[00198] Typically, localization sequences can be located almost anywhere in the amino acid sequence of the protein. In some cases the localization sequence can be split into two blocks separated from each other by a variable number of amino acids. The creation of such constructs via standard recombinant DNA approaches is well known in the art, as for example described in Maniatis, et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y, 1989).

[00199] **Targeting to the plasma membrane:** In some embodiments, constructs are designed to include signaling sequences to optimize localization of the protein to the plasma membrane. These can include e.g., a C-terminal signaling sequence from the β2 nicotinic acetylcholine receptor (MRGTPLLLVVSLFSLLQD (SEQ ID NO: 17), indicated with the one-letter amino acid code), and/or an endoplasmic reticulum export motif from Kir2.1, (FCYENEV) (SEQ ID NO: 18).

[00200] Additional improvements in plasma localization can be obtained by adding Golgi export sequences (e.g. from Kir2.1: RSRFVKKDGHCNVQFINV (SEQ ID NO: 19)) and membrane localization sequences (e.g. from Kir2.1: KSRITSEGEYIPLDQIDINV (SEQ ID NO: 20)) (Gradinaru, V. et al. *Cell* (2010)). In some embodiments, the targeting sequence is selected to regulate intracellular transport of the protein to the desired subcellular structure. In one embodiment the protein is targeted to the plasma membrane of a eukaryotic cell. In this case the targeting sequence can be designed following the strategy outlined in e.g., Gradinaru et al., "Molecular and Cellular Approaches for Diversifying and Extending Optogenetics," Cell 141, 154-165 (2010). The term "signal sequence" refers to N-terminal domains that target proteins into a subcellular locale e.g., the endoplasmic reticulum (ER), and thus are on their way to the plasma membrane. Signal sequences used in optogenetic voltage sensors can be derived from the proteins β2-n-acetylcholine receptor (SS B2nAChR) and PPL. In addition, there is an endogenous signaling sequence on microbial rhodopsin proteins that can be harnessed for appropriate subcellular targeting. A trafficking signal (TS) can optionally be inserted into the genome C-terminal to the microbial rhodopsin and N-terminal to the accessory fluorescent protein. In one embodiment, the trafficking signal is derived from the Kir2.1 protein as specified in Gradinaru et al. In another embodiment, an ER export motif is inserted at the C-terminus of the accessory fluorescent protein.

[00201] **Targeting mitochondria**: For measuring mitochondrial membrane potential or for studying mitochondria, one may wish to localize PROPS to the mitochondrial inner membrane or mitochondrial outer membrane, in which case appropriate signaling sequences can be added to the rhodopsin protein.

[00202] Optogenetic voltage sensors can be targeted to the inner mitochondrial membrane, following a procedure such as that described in e.g., A. Hoffmann, V. Hildebrandt, J. Heberle, and G.

Büldt, "Photoactive mitochondria: in vivo transfer of a light-driven proton pump into the inner mitochondrial membrane of Schizosaccharomyces pombe," Proc. Natl. Acad. Sci. USA 91, PNAS 9367-9371 (1994).

Cells

[00203] Cells that are useful according to the invention include eukaryotic and prokaryotic cells. Eukaryotic cells include cells of non-mammalian invertebrates, such as yeast, plants, and nematodes, as well as non-mammalian vertebrates, such as fish and birds. The cells also include mammalian cells, including human cells. The cells also include immortalized cell lines such as HEK, HeLa, CHO, 3T3, which may be particularly useful in applications of the methods for drug screens. The cells also include stem cells, pluripotent cells, progenotir cells, and induced pluripotent cells. Differentiated cells including cells differentiated from the stem cells, pluripotent cells and progenotir cells are included as well.

[00204] In some embodiments, the cells are cultured in vitro or ex vivo. In some embodiments, the cells are part of an organ or an organism.

[00205] In some embodiment, the cell is an "artificial cell" or a "synthetic cell" created by bioengineering (see, e.g., Creation of a Bacterial Cell Controlled by a Chemically Synthesized Genome, Daniel G. Gibson et al., Science 2 July 2010: Vol. 329 no. 5987 pp. 52-56; Cans, Ann-Sofie, Andes-Koback, Meghan, and Keating, Christine D. Positioning Lipid Membrane Domains in Giant Vesicles by Micro-organization of Aqueous Cytoplasm Mimic. J. Am. Chem. Soc., 2008).

[00206] The methods can also be applied to any other membrane-bound structure, which may not necessarily be classified as a cell. Such membrane bound structures can be made to carry the microbial rhodopsin proteins of the invention by, e.g., fusing the membranes with cell membrane fragments that carry the microbial rhodopsin proteins of the invention.

[00207] Cells include also zebrafish cardiomyocytes; immune cells (primary murine and human cultures and iPS-derived lines for all, in addition to the specific lines noted below), including B cells (e.g., human Raji cell line, and the DT40 chicken cell line), T cells (e.g., human Jurkat cell line), Macrophages, Dendritic cells, and Neutrophils (e.g., HL-60 line). Additionally, one can use glial cells: astrocytes and oligodendrocytes; pancreatic beta cells; hepatocytes; non-cardiac muscle cells; endocrine cells such as parafollicular and chromaffin; and yeast cells. Cells further include neuronal cells, such as neurons.

[00208] The cell can also be a Gram positive or a Gram negative bacteria, as well as pathogenic bacteria of either Gram type. The pathogenic cells are useful for applications of the method to, e.g., screening of novel antibiotics that affect membrane potential to assist in destruction of the bacterial cell or that affect membrane potential to assist destruction of the bacterial cell in combination with the membrane potential affecting agent; or in the search for compounds that suppress efflux of antibiotics.

[00209] The membrane potential of essentially any cell, or any phospholipid bilayer enclosed structure, can be measured using the methods and compositions described herein. Examples of the cells that can be assayed are a primary cell e.g., a primary hepatocyte, a primary neuronal cell, a primary myoblast, a primary mesenchymal stem cell, primary progenitor cell, or it may be a cell of an established cell line. It is not necessary that the cell be capable of undergoing cell division; a terminally differentiated cell can be used in the methods described herein. In this context, the cell can be of any cell type including, but not limited to, epithelial, endothelial, neuronal, adipose, cardiac, skeletal muscle, fibroblast, immune cells, hepatic, splenic, lung, circulating blood cells, reproductive cells, gastrointestinal, renal, bone marrow, and pancreatic cells. The cell can be a cell line, a stem cell, or a primary cell isolated from any tissue including, but not limited to brain, liver, lung, gut, stomach, fat, muscle, testes, uterus, ovary, skin, spleen, endocrine organ and bone, etc. Where the cell is maintained under in vitro conditions, conventional tissue culture conditions and methods can be used, and are known to those of skill in the art. Isolation and culture methods for various cells are well within the knowledge of one skilled in the art. The cell can be a prokaryotic cell, a eukaryotic cell, a mammalian cell or a human cell. In one embodiment, the cell is a neuron or other cell of the brain. In some embodiment, the cell is a cardiomyocyte. In some embodiments, the cell is cardiomyocyte that has been differentiated from an induced pluripotent cell.

Reference value

[00210] The invention provides method for measuring membrane potential in a cell expressing a nucleic acid encoding a microbial rhodopsin protein, the method comprising the steps of (a) exciting at least one cell comprising a nucleic acid encoding a microbial rhodopsin protein with light of at least one wave length; and (b) detecting at least one optical signal from the at least one cell, wherein the level of fluorescence emitted by the at least one cell compared to a reference is indicative of the membrane potential of the cell.

[00211] The term "reference" as used herein refers to a baseline value of any kind that one skilled in the art can use in the methods. In some embodiments, the reference is a cell that has not been exposed to a stimulus capable of or suspected to be capable of changing membrane potential. In one embodiment, the reference is the same cell transfected with the microbial rhodopsin but observed at a different time point. In another embodiment, the reference is the fluorescence of a homologue of Green Fluorescent Protein (GFP) operably fused to the microbial rhodopsin.

Detecting fluorescence from a modified microbial rhodopsin

[00212] In the methods of the invention, the cells are excited with a light source so that the emitted fluorescence can be detected. The wavelength of the excitation light depends on the fluorescent molecule. For example, the archerhodopsin constructs in the examples are all excitable using light with wavelengths varying between λ=594nm and λ=645nm. Alternatively, the range may

be between λ=630-645nm. For example a commonly used Helium Neon laser emits at λ=632.8nm and can be used in excitation of the fluorescent emission of these molecules.

[00213] In some embodiments a second light is used. For example, if the cell expresses a reference fluorescent molecule or a fluorescent molecule that is used to detect another feature of the cell, such a pH or Calcium concentration. In such case, the second wavelength differs from the first wavelength. Examples of useful wavelengths include wavelengths in the range of λ=447-594nm, for example, λ=473nm, λ=488nm, λ=514nm, λ=532nm, and λ=561nm.

[00214] The hardware and software needed to take maximal advantage of VIPs depends on the type of assay, and can be easily optimized and selected by a skilled artisan based on the information provided herein. Existing instrumentation can be easily used or adapted for the detection of VIPs and PROPs. The factors that determine the type of instrumentation include, precision and accuracy, speed, depth penetration, multiplexing and throughput.

[00215] *Precision and accuracy*: In determining the detection system, one should evaluate whether one needs absolute or relative measurement of voltage. Absolute measurements of membrane potential are typically done ratiometrically, with either two excitation wavelengths or two emission wavelengths. Relative voltage changes in a single cell can be performed with single-band excitation and detection.

[00216] *Speed*: To measure action potentials in a neuron one needs sub-millisecond temporal resolution. This requires either high-speed CCDs, or high-speed confocal microscopes which can scan custom trajectories. Slower dynamics and quasi steady state voltages can be measured with conventional cameras. These measurements can be used, for example, in methods and assays that are directed to screening of agents in cardiac cells, such as cardiomyocytes.

[00217] *Depth penetration*: Imaging in deep tissue may require confocal microscopy or lateral sheet illumination microscopy. Alternatively, deep imaging may require the development of nonlinear microscopies, including two-photon fluorescence or second harmonic generation. Conventional epifluorescence imaging works well for cells in culture, and total internal reflection fluorescence (TIRF) provides particularly high signal-to-noise ratios in images of adherent cells.

[00218] *Multiplexing with other optical imaging and control*: One can combine imaging of VIPs with other structural and functional imaging, of e.g. pH, calcium, or ATP. One may also combine imaging of VIPs with optogenetic control of membrane potential using e.g. channelrhodopsin, halorhodopsin, and archaerhodopsin. If optical measurement and control are combined in a feedback loop, one can perform all-optical patch clamp to probe the dynamic electrical response of any membrane.

[00219] *Throughput*: One can also integrate robotics and custom software for screening large libraries or large numbers of conditions which are typically encountered in high throughput drug screening methods.

Spectroscopic readouts of voltage-induced shifts in microbial rhodopsins

[00220] The spectroscopic states of microbial rhodopsins are typically classified by their absorption spectrum. However, in some cases there is insufficient protein in a single cell to detect spectral shifts via absorbance alone. Any of the following several optical imaging techniques can be used to probe other state-dependent spectroscopic properties.

a) Fluorescence

[00221] It was found that many microbial rhodopsin proteins and their mutants produce measurable fluorescence. For example, PROPS fluorescence is excited by light with a wavelength between wavelength of 500 and 650 nm, and emission is peaked at 710 nm. The rate of photobleaching of PROPS decreases at longer excitation wavelengths, so one preferable excitation wavelength is in the red portion of the spectrum, near 633 nm. These wavelengths are further to the red than the excitation and emission wavelengths of any other fluorescent protein, a highly desirable property for *in vivo* imaging. Furthermore, the fluorescence of PROPS shows negligible photobleaching, in stark contrast to all other known fluorophores. When excited at 633 nm, PROPS and GFP emit a comparable numbers of photons prior to photobleaching. Thus microbial rhodopsins constitute a new class of highly photostable, membrane-bound fluorescent markers.

[00222] It was further found that the fluorescence of PROPS is exquisitely sensitive to the state of protonation of the Schiff base in that only the protonated form fluoresces. Thus voltage-induced changes in protonation lead to changes in fluorescence.'

[00223] In some embodiments, the fluorescence of PROPS is detected using e.g., a fluorescent microscope, a fluorescent plate reader, FACS sorting of fluorescent cells, etc.

b) Spectral Shift Fluorescence Resonance Energy Transfer (FRET)

[00224] FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein-protein interactions, protein-DNA interactions, and protein conformational changes. For monitoring the complex formation between two molecules (e.g., retinal and microbial rhodopsin), one of them is labeled with a donor and the other with an acceptor, and these fluorophore-labeled molecules are mixed. When they are dissociated, the donor emission is detected upon the donor excitation. On the other hand, when the donor and acceptor are in proximity (1-10 nm) due to the interaction of the two molecules, the acceptor emission is predominantly observed because of the intermolecular FRET from the donor to the acceptor.

[00225] A fluorescent molecule appended to a microbial rhodopsin can transfer its excitation energy to the retinal, but only if the absorption spectrum of the retinal overlaps with the emission spectrum of the fluorophore. Changes in the absorption spectrum of the retinal lead to changes in the fluorescence brightness of the fluorophore. To perform spectral shift FRET, a fluorescent protein is

fused with the microbial rhodopsin voltage sensor, and the fluorescence of the protein is monitored. This approach has the advantage over direct fluorescence that the emission of fluorescent proteins is far brighter than that of retinal, but the disadvantage of being an indirect readout, with smaller fractional changes in fluorescence.

[00226] In some embodiments, voltage-induced changes in the absorption spectrum of microbial rhodopsins are detected using spectral shift FRET

c) Rhodopsin Optical Lock-in Imaging (ROLI)

[00227] The absorption spectrum of many of the states of retinal is temporarily changed by a brief pulse of light. In ROLI, periodic pulses of a "pump" beam are delivered to the sample. A second "probe" beam measures the absorbance of the sample at a wavelength at which the pump beam induces a large change in absorbance. Thus the pump beam imprints a periodic modulation on the transmitted intensity of the probe beam. These periodic intensity changes are detected by a lock-in imaging system. In contrast to conventional absorption imaging, ROLI provides retinal-specific contrast. Modulation of the pump at a high frequency allows detection of very small changes in absorbance.

[00228] In some embodiments, the fluorescence of PROPS is detected using rhodopsin optical lock-in imaging.

d) Raman

[00229] Raman spectroscopy is a technique that can detect vibrational, rotational, and other low-frequency modes in a system. The technique relies on inelastic scattering of monochromatic light (e.g., a visible laser, a near infrared laser or a near ultraviolet laser). The monochromatic light interacts with molecular vibrations, phonons or other excitations in the system, resulting in an energy shift of the laser photons. The shift in energy provides information about the phonon modes in the system.

[00230] Retinal in microbial rhodopsin molecules is known to have a strong resonant Raman signal. This signal is dependent on the electrostatic environment around the chromophore, and therefore is sensitive to voltage.

[00231] In some embodiments, voltage-induced changes in the Raman spectrum of microbial rhodopsins are detected using Raman microscopy.

e) Second Harmonic Generation (SHG)

[00232] Second harmonic generation, also known in the art as "frequency doubling" is a nonlinear optical process, in which photons interacting with a nonlinear material are effectively "combined" to form new photons with twice the energy, and therefore twice the frequency and half the wavelength of the initial photons.

[00233] SHG signals have been observed from oriented films of bacteriorhodopsin in cell membranes. SHG is an effective probe of the electrostatic environment around the retinal in optical voltage sensors. Furthermore, SHG imaging involves excitation with infrared light which penetrates deep into tissue. Thus SHG imaging can be used for three-dimensional optical voltage sensing using the optical sensors described herein.

[00234] In some embodiments, voltage-induced changes in the second harmonic spectrum of microbial rhodopsins are detected using SHG imaging.

Fusion protein with a moiety that produces an optical signal

[00235] Although microbial rhodopsin proteins are themselves fluorescent in response to changes in voltage, in some applications it may desired or necessary to enhance the level of fluorescence or provide another optical signal (e.g., a colorimetric signal) to permit detection of voltage changes. Further, a moiety that produces an optical signal can be attached to the microbial rhodopsin to monitor the subcellular localization of the rhodopsin protein. Thus, in some embodiments, the modified microbial rhodopsin proteins further comprise a moiety that produces an optical signal, thereby enhancing the optical signal measured from the modified microbial rhodopsin protein or permitting localization studies to be performed for the rhodopsin protein.

[00236] For example, a gene for a fluorescent protein of the GFP family or a homolog thereof can optionally be appended or as referred to in the claims "operably linked" to the nucleic acid encoding the microbial rhodopsin. In one embodiment, the identity of the fluorescent protein, its linker to the voltage-sensing complex, and the location of this linker in the overall protein sequence are selected for one of two functions: either to serve as an indicator of the level and distribution of gene expression products; or to serve as an alternative readout of voltage, independent of the endogenous fluorescence of the retinal.

[00237] For example, when the fluorescent protein serves as an indicator of protein localization, it enables quantitative optical voltage measurements that are not confounded by cell-to-cell variation in expression levels. The fluorescence of the fluorescent protein and the microbial rhodopsin can be measured simultaneously and the ratio of these two signals provides a concentration-independent measure of membrane potential.

[00238] In one embodiment, the microbial rhodopsin protein is a PROPS fusion protein comprising a fluorescent protein. For example, an N-terminal fusion of PROPS with the fluorescent protein Venus. This protein provides a stable reference indicating localization of PROPS within the cell and permitting ratiometric imaging of Venus and PROPS fluorescence. Ratiometric imaging permits quantitative measurements of membrane potential because this technique is insensitive to the total quantity of protein within the cell. Other fluorescent proteins may be used in lieu of Venus with similar effects. In some embodiments, the fluorescent polypeptide is selected from the group consisting of GFP, YFP, EGFP, EYFP, EBFB, DsRed, RFP and fluorescent variants thereof.

[00239] In one embodiment, the microbial rhodopsin is an archaerhodopsin fused with or operably linked to an additional fluorescent protein, such as a GFP or a homolog therof.

Chromophore

[00240] In the wild, microbial rhodopsins contain a bound molecule of retinal which serves as the optically active element. These proteins will also bind and fold around many other chromophores with similar structure, and possibly preferable optical properties. Analogues of retinal with locked rings cannot undergo trans-cis isomerization, and therefore have higher fluorescence quantum yields (Brack, T. et al. *Biophys. J.* 65, 964-972 (1993)). Analogues of retinal with electron-withdrawing substituents have a Schiff base with a lower pKa than natural retinal and therefore may be more sensitive to voltage (Sheves, M., et al. *Proc. Nat. Acad. Sci. U. S. A.* 83, 3262-3266 (1986); Rousso, I., et al. *Biochemistry* 34, 12059-12065 (1995)). Covalent modifications to the retinal molecule may lead to optical voltage sensors with significantly improved optical properties and sensitivity to voltage.

Advantages of the methods and compositions described herein

[00241] The key figures of merit for an optical voltage sensor are its response speed and its sensitivity (fractional change in fluorescence per 100 mV change in membrane potential). Figure 10 compares these attributes for previous protein-based fluorescent voltage indicators and those contemplated herein. Additional important attributes include the ability to target the indicator to a particular cell type or sub-cellular structure, photostability, and low phototoxicity.

[00242] Previous protein-based efforts focused on fusing one or more fluorescent proteins to transmembrane voltage sensing domains. A change in voltage induces a conformational change in the voltage sensing domain, which moves the fluorescent proteins, and changes their fluorescence. The reliance on conformational motion of multiple large protein domains makes these approaches unavoidably slow. Furthermore, the conformational shifts of most voltage sensing domains are small, leading to small changes in fluorescence.

[00243] The most sensitive indicators from the VSFP 2.x family have a change in fluorescence of $\Delta F/F = 10\%$ per 100 mV. VSFP 2.x proteins respond in approximately 100 milliseconds, far too slow to detect a 1 ms action potential in a neuron (Perron, A. et al. *Front Mol Neurosci.* 2 (2009); Mutoh, H. et al. *PLoS One* 4, e4555 (2009)). The SPARC family of voltage sensors has a 1 ms response time, but shows a fluorescence change of < 1% per 100 mV (Baker, B. J. et al. *J. Neurosci. Methods* 161, 32-38 (2007); Ataka, K. & Pieribone, V. A. *Biophys. J.* 82, 509-516 (2002)). Prior to the present study described herein, two decades of research on fluorescent voltage sensors had not yet yielded a protein that could signal individual neuronal action potentials *in vivo*.

[00244] Scientists have also developed small organic dyes that show voltage-sensitive fluorescence. These lipophilic molecules incorporate into the cell membrane where voltage leads to shifts in conformation or electronic energy levels and thereby to changes in optical properties. These

molecules respond quickly (less than 1 ms, typically), and have sensitivities as large as 34% per 100 mV, but cannot be targeted, are often difficult to deliver, and are highly toxic (Krauthamer, V., et al. *J. Fluoresc.* 1, 207-213 (1991); Fromherz, P., et al. *Eur. Biophys. J.* 37, 509-514 (2008); Sjulson, L. & Miesenbock, G. *J. Neurosci.* 28, 5582 (2008)), see e.g. US Patent Nos. 7,867,282, 6,107,066, and 5,661,035). None of these optical voltage sensors employs a microbial rhodopsin protein that is configured to run "backwards" to convert changes in membrane potential into changes in an optically detectable signal.

[00245] The approach to optical voltage sensing described herein is different from previous efforts. As described herein a protein is used that has a strong electro-optical coupling in the wild. Microbial rhodopsins in the wild serve to transduce sunlight into a membrane potential. The optical voltage sensors described herein use this function in reverse, transducing a membrane potential into a readily detectable optical signal. As Figure 12 shows, the exemplary microbial rhodopsin voltage sensors (PROPS, Arch 3 WT, Arch 3 D95N) exceed other protein-based indicators on the key figures of merit.

Membrane Fusion mediated delivery of an optical sensor

[00246] Membrane fusion reactions are common in eukaryotic cells. Membranes are fused intracellularly in processes including endocytosis, organelle formation, inter-organelle traffic, and constitutive and regulated exocytosis. Intercellularly, membrane fusion occurs during sperm-egg fusion and myoblast fusion.

[00247] Membrane fusion has been induced artificially by the use of liposomes, in which the cell membrane is fused with the liposomal membrane, and by various chemicals or lipids, which induce cell-cell fusion to produce heterokaryons. Naturally occurring proteins shown to induce fusion of biological membranes are mainly fusion proteins of enveloped viruses. Thus, in some embodiments, the optical sensor is administered using a liposome comprising a fusogenic protein.

[00248] It is generally believed that membrane fusion under physiological conditions is protein-mediated. This has led to the development of liposomes that contain fusion-promoting proteins (proteoliposomes), with decreased cytotoxicity (see, for example, Cheng, Hum. Gene Ther. 7:275-282 (1996); Hara et al., Gene 159:167-174 (1995); and Findeis et al., Trends Biotechnol., 11:202-205 (1993)).

[00249] The only proteins conclusively shown to induce intercellular fusion of biological membranes are those of enveloped viruses and two proteins from nonenveloped viruses. All enveloped viruses encode proteins responsible for fusion of the viral envelope with the cell membrane. These viral fusion proteins are essential for infection of susceptible cells. The mechanism of action of fusion proteins from enveloped viruses have served as a paradigm for protein-mediated membrane fusion (see, for example, White, Ann. Rev. Physiol., 52:675-697 (1990); and White, Science, 258:917-924 (1992)).

[00250] Most enveloped virus fusion proteins are relatively large, multimeric, type I membrane proteins, as typified by the influenza virus HA protein, a low pH-activated fusion protein, and the Sendai virus F protein, which functions at neutral pH. These are structural proteins of the virus with the majority of the fusion protein oriented on the external surface of the virion to facilitate interactions between the virus particle and the cell membrane.

[00251] According to the mechanism of action of fusion proteins from enveloped viruses, fusion of the viral envelope with the cell membrane is mediated by an amphipathic alpha-helical region, referred to as a fusion peptide motif, that is present in the viral fusion protein. This type of fusion peptide motif is typically 17 to 28 residues long, hydrophobic (average hydrophobicity of about 0.6±0.1), and contains a high content of glycine and alanine, typically 36%±7% (White, Annu. Rev. Physiol., 52:675-697 (1990).

[00252] All of the enveloped virus fusion proteins are believed to function via extensive conformational changes that, by supplying the energy to overcome the thermodynamic barrier, promote membrane fusion. These conformational changes are frequently mediated by heptad repeat regions that form coiled coil structures (see Skehel and Wiley, Cell, 95:871-874 (1998)). Recognition of the importance of fusion peptide motifs in triggering membrane fusion has resulted in the use of small peptides containing fusion peptide motifs to enhance liposome-cell fusion (see, for example, Muga et al., Biochemistry 33:4444-4448 (1994)).

[00253] Enveloped virus fusion proteins also trigger cell-cell fusion, resulting in the formation of polykaryons (syncytia). Synthesis of the viral fusion protein inside the infected cell results in transport of the fusion protein through the endoplasmic reticulum and Golgi transport system to the cell membrane, an essential step in the assembly and budding of infectious progeny virus particles from the infected cell (Petterson, Curr. Top. Micro. Immunol., 170:67-106 (1991)). The synthesis, transport, and folding of the fusion protein is facilitated by a variety of components, including signal peptides to target the protein to the intracellular transport pathway, glycosylation signals for N-linked carbohydrate addition to the protein, and a transmembrane domain to anchor the protein in the cell membrane. These proteins have been used in reconstituted proteoliposomes ('virosomes') for enhanced, protein-mediated liposome-cell fusion in both cell culture and *in vivo* (see, for example, Ramani et al., FEBS Lett., 404:164-168 (1997); Scheule et al., Am. J. Respir. Cell Mol. Biol., 13:330-343 (1995); and Grimaldi, Res. Virol., 146:289-293 (1995)).

[00254] Thus, in some embodiments of the methods and compositions described herein, a micelle, liposome or other artificial membrane comprising the modified microbial rhodopsin and a fusion protein is administered to a cell or a subject to mediate delivery of the optical sensor protein to the cell by membrane fusion. In a preferred embodiment, the composition further comprises a targeting sequence to target the delivery system to a particular cell-type. If desired, the exogenous lipid of an artificial membrane composition can further comprise a targeting moiety (e.g., ligand) that binds to mammalian cells to facilitate entry. For example, the composition can include as a ligand an

asialoglycoprotein that binds to mammalian lectins (e.g., the hepatic asialoglycoprotein receptor), facilitating entry into mammalian cells. Single chain antibodies, which can target particular cell surface markers, are also contemplated herein for use as targeting moieties. Targeting moieties can include, for example, a drug, a receptor, an antibody, an antibody fragment, an aptamer, a peptide, a vitamin, a carbohydrate, a protein, an adhesion molecule, a glycoprotein, a sugar residue or a glycosaminoglycan, a therapeutic agent, a drug, or a combination of these. A skilled artisan can readily design various targeting moieties for modifying an artificial membrane based on the intended target cell to be assessed using an optical sensor as described herein.

[00255] For methods using membrane fusion mediated delivery, it is contemplated that the optical sensor to be used is expressed and produced in a heterologous expression system. Different expression vectors comprising a nucleic acid that encodes an optical sensor or derivative as described herein for the expression of the optical sensor can be made for use with a variety of cell types or species. The expression vector should have the necessary 5' upstream and 3' downstream regulatory elements such as promoter sequences, ribosome recognition and binding TATA box, and 3' UTR AAUAAA transcription termination sequence for efficient gene transcription and translation in the desired cell. In some embodiments, the optical sensors are made in a heterologous protein expression system and then purified for production of lipid-mediated delivery agents for fusion with a desired cell type. In such embodiments, the expression vector can have additional sequences such as 6X-histidine (SEQ ID NO: 21), V5, thioredoxin, glutathione-S-transferase, c-Myc, VSV-G, HSV, FLAG, maltose binding peptide, metal-binding peptide, HA and "secretion" signals (e.g., Honeybee melittin Pho, BiP), which are incorporated into the expressed recombinant optical sensor for ease of purification. In addition, there can be enzyme digestion sites incorporated after these sequences to facilitate enzymatic removal of additional sequence after they are not needed. These additional sequences are useful for the detection of optical sensor expression, for protein purification by affinity chromatography, enhanced solubility of the recombinant protein in the host cytoplasm, for better protein expression especially for small peptides and/or for secreting the expressed recombinant protein out into the culture media, into the periplasm of the prokaryote bacteria, or to the spheroplast of yeast cells. The expression of recombinant optical sensors can be constitutive in the host cells or it can be induced, e.g., with copper sulfate, sugars such as galactose, methanol, methylamine, thiamine, tetracycline, infection with baculovirus, and (isopropyl-beta-D-thiogalactopyranoside) IPTG, a stable synthetic analog of lactose, depending on the host and vector system chosen.

[00256] Examples of other expression vectors and host cells are the pET vectors (Novagen), pGEX vectors (Amersham Pharmacia), and pMAL vectors (New England labs. Inc.) for protein expression in *E. coli* host cells such as BL21, BL21(DE3) and AD494(DE3)pLysS, Rosetta (DE3), and Origami(DE3) (Novagen); the strong CMV promoter-based pcDNA3.1 (Invitrogen) and pCIneo vectors (Promega) for expression in mammalian cell lines such as CHO, COS, HEK-293, Jurkat, and MCF-7; replication incompetent adenoviral vector vectors pAdeno X, pAd5F35, pLP-Adeno-X-CMV

(Clontech), pAd/CMV/V5-DEST, pAd-DEST vector (Invitrogen) for adenovirus-mediated gene transfer and expression in mammalian cells; pLNCX2, pLXSN, and pLAPSN retrovirus vectors for use with the Retro-X™ system from Clontech for retroviral-mediated gene transfer and expression in mammalian cells; pLenti4/V5-DEST™, pLenti6/V5-DEST™, and pLenti6.2/V5-GW/lacZ (Invitrogen) for lentivirus-mediated gene transfer and expression in mammalian cells; adenovirus-associated virus expression vectors such as pAAV-MCS, pAAV-IRES-hrGFP, and pAAV-RC vector (Stratagene) for adeno-associated virus-mediated gene transfer and expression in mammalian cells; BACpak6 baculovirus (Clontech) and pFastBac™ HT (Invitrogen) for the expression in Spodopera frugiperda 9 (Sf9) and Sf11 insect cell lines; pMT/BiP/V5-His (Invitrogen) for the expression in Drosophila Schneider S2 cells; Pichia expression vectors pPICZα, pPICZ, pFLDα and pFLD (Invitrogen) for expression in Pichia pastoris and vectors pMETα and pMET for expression in P. methanolica ; pYES2/GS and pYD1 (Invitrogen) vectors for expression in yeast Saccharomyces cerevisiae . Recent advances in the large scale expression heterologous proteins in Chlamydomonas reinhardtii are described by Griesbeck C. et. al. 2006 Mol. Biotechnol. 34:213-33 and Fuhrmann M. 2004, Methods Mol Med. 94:191-5. Foreign heterologous coding sequences are inserted into the genome of the nucleus, chloroplast and mitochondria by homologous recombination. The chloroplast expression vector p64 carrying the versatile chloroplast selectable marker aminoglycoside adenyl transferase (aadA), which confers resistance to spectinomycin or streptomycin, can be used to express foreign protein in the chloroplast. The biolistic gene gun method can be used to introduce the vector in the algae. Upon its entry into chloroplasts, the foreign DNA is released from the gene gun particles and integrates into the chloroplast genome through homologous recombination.

[00257] Cell-free expression systems are also contemplated. Cell-free expression systems offer several advantages over traditional cell-based expression methods, including the easy modification of reaction conditions to favor protein folding, decreased sensitivity to product toxicity and suitability for high-throughput strategies such as rapid expression screening or large amount protein production because of reduced reaction volumes and process time. The cell-free expression system can use plasmid or linear DNA. Moreover, improvements in translation efficiency have resulted in yields that exceed a milligram of protein per milliliter of reaction mix. An example of a cell-free translation system capable of producing proteins in high yield is described by Spirin AS. et. al., Science 242:1162 (1988). The method uses a continuous flow design of the feeding buffer which contains amino acids, adenosine triphosphate (ATP), and guanosine triphosphate (GTP) throughout the reaction mixture and a continuous removal of the translated polypeptide product. The system uses *E. coli* lysate to provide the cell-free continuous feeding buffer. This continuous flow system is compatible with both prokaryotic and eukaryotic expression vectors. As an example, large scale cell-free production of the integral membrane protein EmrE multidrug transporter is described by Chang G. el. al., Science 310:1950-3 (2005). Other commercially available cell-free expression systems include the Expressway™ Cell-Free Expression Systems (Invitrogen) which utilize an *E. coli*-based

in-vitro system for efficient, coupled transcription and translation reactions to produce up to milligram quantities of active recombinant protein in a tube reaction format; the Rapid Translation System (RTS) (Roche Applied Science) which also uses an E. coli-based in-vitro system; and the TNT Coupled Reticulocyte Lysate Systems (Promega) which uses a rabbit reticulocyte-based in-vitro system.

Applications of optical voltage sensors

[00258] Provided herein are areas in which an improved optical voltage indicator can be applied both in commercial and scientific endeavors.

Drug screens

[00259] A recent article reported that "Among the 100 top-selling drugs, 15 are ion-channel modulators with a total market value of more than $15 billion." (Molokanova, E. & Savchenko, A. *Drug Discov. Today* 13, 14-22 (2008)). However, searches for new ion-channel modulators are limited by the absence of good indicators of membrane potential (Przybylo, M., et al. *J. Fluoresc.*, 1-19 (2010)). In some embodiments, the optical sensors described herein are used to measure or monitor membrane potential changes in response to a candidate ion channel modulator. Such screening methods can be performed in a high throughput manner by simultaneously screening multiple candidate ion channel modulators in cells.

Stem cells

[00260] Many genetically determined diseases of the nervous system and heart lack good animal models. In some embodiments, the optical sensors described herein are expressed in stem cells, either induced pluripotent or stem cells isolated from cord blood or amniotic fluid, or embryonic stem cells derived from humans or fetuses known to carry or be affected with a genetic defect. In some embodiments, the embryonal stem cells are of non-human origin. Alternatively the optical sensors are expressed in progeny of the stem cells, either progenitor cells or differentiated cell types, such as cardiac or neuronal cells. Expression of voltage indicators in these cell types provides information on the electrophysiology of these cells and the response of membrane potential to candidate agents or to changes in ambient conditions (e.g. anoxia). Additionally, expression of voltage indicators in stem cells enables studies of the differentiation and development of stem cells into electrically active cell types and tissues.

[00261] The genetic defect may be a single gene alteration, or a deletion, insertion, duplication or a rearrangement or one or more nucleic acids, including large chromosomal alterations.

[00262] Stem cells may be isolated and manipulated according to methods known to one skilled in the art. Patents describing methods of making and using, e.g., primate embryonic stem cells are described in, e.g., U.S. patent Nos. 7,582,479; 6,887,706; 6,613,568; 6,280,718; 6,200,806; and

5,843,780. Additionally, for example, human cord blood derived unrestricted somatic stem cells are described in U.S. Patent No. 7,560,280 and progenitor cells from wharton's jelly of human umbilical cord in U.S. Patent No. 7,547,546.

[00263] Induced pluripotent stem cells may be produced by methods described, for example, in U.S. Patent Application Publication No. 20110200568, European Patent Application Publication No. 01970446, and U.S. Paten Application Publication No. US2008/0233610. Additional methods for making and using induced pluripotent stem cells are also described in application U.S. Serial Nos. 10/032,191, titled "Methods for cloning mammals using reprogrammed donor chromatin or donor cells," and 10/910,156, "Methods for altering cell fate." These patent applications relate to technology to alter the state of a cell, such as a human skin cell, by exposing the cell's DNA to the cytoplasm of another reprogramming cell with differing properties. Detailed description of the reprogramming factors used in making induced pluripotent stem cells, including expression of genes OCT4, SOX2, NANOG, cMYC, LIN28 can also be found, for example, in PCT/US2006/030632.

[00264] Methods for differentiating stem cells or pluripotent cells into differentiated cells are also well known to one skilled in the art.

Brain imaging

[00265] The human brain functions by sending electrical impulses along its ~10^{11} neurons. These patterns of firing are the origin of every human thought and action. Yet there is currently no good way to observe large-scale patterns of electrical activity in an intact brain (Baker, B. J. et al. *J. Neurosci. Methods* 161, 32-38 (2007); Baker, B. J. et al. *Brain Cell Biology* 36, 53-67 (2008)).

[00266] An improved optical voltage sensor can lead to unprecedented insights in neuroscience. The device can allow mapping of brain activity in patients and/or cells of patients with psychiatric and neurological diseases, and in victims of traumatic injuries or animal models modeling such diseases and injuries.

[00267] Optical imaging of neuronal activity can also form the basis for improved brain-machine interfaces for people with disabilities. For imaging in the brain, the optical sensor is administered by direct injection into the site to be analyzed (with or without accompanying electroporation) or the optical sensor is delivered using a viral vector. Alternatively the optical sensor may be administered through the formation of a transgenic organism, or through application of the Cre-Lox recombination system.

Microbiology

[00268] Bacteria are host to dozens of ion channels of unknown function (Martinac, B., et al. *Physiol. Rev.* 88, 1449 (2008)). Most bacteria are too small for direct electrophysiological measurements, so their electrical properties are almost entirely unknown.

[00269] Upon expressing PROPS in *E. coli*, it was found that *E. coli* undergo a previously unknown electrical spiking behavior. The data described herein in the Examples section is the first report of spontaneous electrical spiking in any bacterium. This result establishes the usefulness of voltage sensors in microbes.

[00270] Furthermore, we found that electrical spiking in *E. coli* is coupled to efflux of a cationic membrane permeable dye. It is thus plausible that electrical spiking is correlated to efflux of other cationic compounds, including antibiotics. Optical voltage indicators may prove useful in screens for inhibitors of antibiotic efflux.

[00271] Optical voltage sensors will unlock the electrophysiology of the millions of species of microorganisms which have proven too small to probe via conventional electrophysiology. This information will be useful for understanding the physiology of bacteria with medical, industrial, and ecological applications.

Mitochondria and metabolic diseases

[00272] Mitochondria are membrane-bound organelles which act as the ATP factories in eukaryotic cells. A membrane voltage powers the mitochondrial ATP synthase. Dysfunction of mitochondria has been implicated in a variety of neurodegenerative diseases, diabetes, cancer, cardiovascular disease, and aging. Thus there is tremendous interest in measuring mitochondrial membrane potential *in vivo*, although currently available techniques are severely limited (Verburg, J. & Hollenbeck, P. J. *J. Neurosci.* 28, 8306 (2008); Ichas, F., et al. *Cell* 89, 1145-1154 (1997); Johnson, L. V., et al. *Proc. Natl. Acad. Sci. U. S. A.* 77, 990 (1980)).

[00273] The exemplary optical voltage sensor described herein (PROPS) can be tagged with peptide sequences that direct it to the mitochondrial inner membrane (Hoffmann, A., et al. *Proc. Nat. Acad. Sci. U. S. A.* 91, 9367 (1994)) or the mitochondrial outer membrane, where it serves as an optical indicator of mitochondrial membrane potential.

Imaging in human cells and vertebrate models (e.g., rat, mouse, zebrafish)

[00274] As described in Example 2, we also expressed Arch 3 in HEK293T cells. Fluorescence of Arch 3 in HEK 293T cells was readily imaged in an inverted fluorescence microscope with red illumination (λ = 640 nm, I = 540 W/cm2), a high numerical aperture objective, a Cy5 filter set, and an EMCCD camera. The cells exhibited fluorescence predominantly localized to the plasma membrane (Fig. 6C). Cells not expressing Arch 3 were not fluorescent. Cells showed 17% photobleaching over a continuous 10-minute exposure, and retained normal morphology during this interval.

[00275] The fluorescence of HEK cells expressing Arch 3 was highly sensitive to membrane potential, as determined via whole-cell voltage clamp. We developed an algorithm to combine pixel intensities in a weighted sum such that the output, was a nearly optimal estimate of membrane

potential V determined by conventional electrophysiology. Fig. 6C shows an example of a pixel-weight matrix, indicating that the voltage-sensitive protein was localized to the cell membrane; intracellular Arch 3 contributed fluorescence but no voltage-dependent signal. The fluorescence increased by a factor of 2 between -150 mV and +150 mV, with a nearly linear response throughout this range (Fig. 6D). The response of fluorescence to a step in membrane potential occurred within the 500 μs time resolution of our imaging system on both the rising and falling edge (Fig. 6E). Application of a sinusoidally varying membrane potential led to sinusoidally varying fluorescence; at f = 1 kHz, the fluorescence oscillations retained 55% of their low-frequency amplitude (Fig. 18). Arch 3 reported voltage steps as small as 10 mV, with an accuracy of 625 $\mu V/(Hz)^{1/2}$ over timescales < 12 s (Fig. 19).

[00276] We tested Arch 3 as a voltage indicator in cultured rat hippocampal neurons, using viral delivery. Neurons expressing Arch 3 showed voltage-dependent changes in fluorescence localized to the cell membrane. Under whole cell current clamp, cells exhibited spiking upon injection of current pulses of 200 pA. Individual spikes were accompanied by clearly identifiable increases of fluorescence (Fig. 7A). At a 2 kHz image acquisition rate, the signal-to-noise ratio in the fluorescence (spike amplitude:baseline noise) was 10.5. A spike-finding algorithm correctly identified 99.6% of the spikes (based on comparison to simultaneously recorded membrane potential), with a false-positive rate of 0.7% (n = 269 spikes) (Fig. 7B). Single cells were observed for up to 4 minutes of cumulative exposure, with no detectable change in resting potential or spike frequency.

[00277] We imaged the dynamics of action potentials with sub-cellular resolution by averaging multiple temporally registered movies of single spikes (Fig. 7C). In and near the soma, the optically determined waveform of the action potential was uniform and matched the electrically recorded waveform. However in very thin processes the peak of the action potential lagged by up to 1 ms (Fig. 7D). These observations are consistent with multiple-patch recordings on single neurons (Stuart, G. J. & Sakmann, B. Active propagation of somatic action potentials into neocortical pyramidal cell dendrites. Nature 367, 69-72 (1994)); but such recordings are technically demanding and only probe the variation in membrane potential at a small number of points. We show that Arch 3 may be used to map intracellular dynamics of action potentials. Similarly, other archaerhodopsins can be expected to work in a eukaryotic cell as membrane potential indicators.

[00278] Thus, using a model system of Arch 3, we showed that the membrane potential of a mammalian cell can be detected using archaerhodopsins and modified archaerhodopsins.

Gene Delivery Methods

[00279] The nucleic acids encoding the microbial rhodopsin proteins of the invention are introduced to the cell or organ or organism of interest uding routine gene delivery methods. are administered to a subject for the purpose of imaging membrane potential changes in cells of a subject. In one embodiment, the optical sensors are introduced to the cell via expression vectors.

[00280] The various gene delivery methods currently being applied to stem cell engineering include viral and non viral vectors, as well as biological or chemical methods of transfection. The methods can yield either stable or transient gene expression in the system used.

Viral Gene Delivery Systems

[00281] Because of their high efficiency of transfection, genetically modified viruses have been widely applied for the delivery of genes into stem cells.

DNA Virus Vectors

(i) Adenovirus

[00282] Adenoviruses are double stranded, nonenveloped and icosahedral viruses containing a 36 kb viral genome (Kojaoghlanian et al., 2003). Their genes are divided into early (E1A, E1B, E2, E3, E4), delayed (IX, IVa2) and major late (L1, L2, L3, L4, L5) genes depending on whether their expression occurs before or after DNA replication. More than51 human adenovirus serotypes have been described which can infect and replicate in a wide range of organs. The viruses are classified into the following subgroups: A--induces tumor with high frequency and short latency, B--are weakly oncogenic, and C--are non-oncogenic (Cao et al., 2004; Kojaoghlanian et al., 2003).

[00283] These viruses have been used to generate a series of vectors for gene transfer cellular engineering. The initial generation of adenovirus vectors were produced by deleting the E1 gene (required for viral replication) generating a vector with a 4 kb cloning capacity. An additional deletion of E3 (responsible for host immune response) allowed an 8 kb cloning capacity (Bett et al., 1994; Danthinne and Imperiale, 2000; Danthinne and Werth, 2000). The second generation of vectors was produced by deleting the E2 region (required for viral replication) and/or the E4 region (participating in inhibition of host cell apoptosis) in conjunction with E1 or E3 deletions. The resultant vectors have a cloning capacity of 10-13 kb (Armentano et al., 1995). The third "gutted" generation of vectors was produced by deletion of the entire viral sequence with the exception of the inverted terminal repeats (ITRs) and the cis acting packaging signals. These vectors have a cloning capacity of 25 kb (Kochanek et al., 2001) and have retained their high transfection efficiency both in quiescent and dividing cells.

[00284] Importantly, the adenovirus vectors do not normally integrate into the genome of the host cell, but they have shown efficacy for transient gene delivery into adult stem cells. These vectors have a series of advantages and disadvantages. An important advantage is that they can be amplified at high titers and can infect a wide range of cells (Benihoud et al., 1999; Kanerva and Hemminki, 2005). The vectors are generally easy to handle due to their stability in various storing conditions. Adenovirus type 5 (Ad5) has been successfully used in delivering genes in human and mouse stem cells (Smith-Arica et al., 2003). The lack of adenovirus integration into host cell genetic material can in many instances be seen as a disadvantage, as its use allows only transient expression of the therapeutic gene.

[00285] The following provides examples to show that a skilled artisan can readily transducer cells with contructs expressing microbial rhodopsins of the present invention to eukaryotic, such as mammalian cells. For example in a study evaluating the capacity of mesenchymal stem cells to undergo chondrogenesis when TGF-beta1 and bone morphogencic protein-2 (BMP-2) were delivered by adenoviral-mediated expression, the chondrogenesis was found to closely correlated with the level and duration of the transiently expressed proteins. Transgene expression in all aggregates was highly transient, showing a marked decrease after 7 days. Chondrogenesis was inhibited in aggregates modified to express >100 ng/ml TGF-beta1 or BMP-2; however, this was partly due to the inhibitory effect of exposure to high adenoviral loads (Mol. Ther. 2005 August; 12 (2):219-28. Gene-induced chondrogenesis of primary mesenchymal stem cells in vitro. Palmer G D, Steinert A, Pascher A, Gouze E, Gouze J N, Betz O, Johnstone B, Evans C H, Ghivizzani S C). In a second model using rat adipose derived stem cells transduced with adenovirus carrying the recombinant human bone morphogenic protein-7 (BMP-7) gene showed promising results for an autologous source of stem cells for BMP gene therapy. However, activity assessed by measuring alkaline phosphatase in vitro was transient and peaked on day 8. Thus the results were similar to those found in the chondrogenesis model (Cytotherapy. 2005; 7 (3):273-81).

[00286] Thus for experiments that do not require stable gene expression adenovirus vectors is a good option.

[00287] Adenovirus vectors based on Ad type 5 have been shown to efficiently and transiently introduce an exogenous gene via the primary receptor, coxsackievirus, and adenovirus receptor (CAR). However, some kinds of stem cells, such as MSC and hematopoietic stem cells, cannot be efficiently transduced with conventional adenovirus vectors based on Ad serotype 5 (Ad5), because of the lack of CAR expression. To overcome this problem, fiber-modified adenovirus vectors and an adenovirus vector based on another serotype of adenovirus have been developed. (Mol. Pharm. 2006 March-April; 3 (2):95-103. Adenovirus vector-mediated gene transfer into stem cells. Kawabata K, Sakurai F, Koizumi N, Hayakawa T, Mizuguchi H. Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka 567-0085, Japan).

[00288] Such modifications can be readily applied to the use of the microbial rhodopsin constructs described herein, particularly in the applications relating to stem cells.

(ii) Adeno-Associated Virus

[00289] Adeno-Associated viruses (AAV) are ubiquitous, noncytopathic, replication-incompetent members of ssDNA animal virus of parvoviridae family (G. Gao et al., New recombinant serotypes of AAV vectors. Curr Gene Ther. 2005 June; 5 (3):285-97). AAV is a small icosahedral virus with a 4.7 kb genome. These viruses have a characteristic termini consisting of palindromic repeats that fold into a hairpin. They replicate with the help of helper virus, which are usually one of the many serotypes of adenovirus. In the absence of helper virus they integrate into the human genome at a specific locus

(AAVS1) on chromosome 19 and persist in latent form until helper virus infection occurs (Atchison et al., 1965, 1966). AAV can transduce cell types from different species including mouse, rat and monkey. Among the serotypes, AAV2 is the most studied and widely applied as a gene delivery vector. Its genome encodes two large opening reading frames (ORFs) rep and cap. The rep gene encodes four proteins Rep 78, Rep 68, Rep 52 and Rep 40 which play important roles in various stages of the viral life cycle (e.g. DNA replication, transcriptional control, site specific integration, accumulation of single stranded genome used for viral packaging). The cap gene encodes three viral capsid proteins VP1, VP2, VP3 (Becerra et al., 1988; Buning et al., 2003). The genomic 3' end serves as the primer for the second strand synthesis and has terminal resolution sites (TRS) which serve as the integration sequence for the virus as the sequence is identical to the sequence on chromosome 19 (Young and Samulski, 2001; Young et al., 2000).

[00290] These viruses are similar to adenoviruses in that they are able to infect a wide range of dividing and non-dividing cells. Unlike adenovirus, they have the ability to integrate into the host genome at a specific site in the human genome. Unfortunately, due to their rather bulky genome, the AAV vectors have a limited capacity for the transfer of foreign gene inserts (Wu and Ataai, 2000).

RNA Virus Vectors

(i) Retroviruses

[00291] Retroviral genomes consist of two identical copies of single stranded positive sense RNAs, 7-10 kb in length coding for three genes; gag, pol and env, flanked by long terminal repeats (LTR) (Yu and Schaffer, 2005). The gag gene encodes the core protein capsid containing matrix and nucleocapsid elements that are cleavage products of the gag precursor protein. The pol gene codes for the viral protease, reverse transcriptase and integrase enzymes derived from gag-pol precursor gene. The env gene encodes the envelop glycoprotein which mediates viral entry. An important feature of the retroviral genome is the presence of LTRs at each end of the genome. These sequences facilitate the initiation of viral DNA synthesis, moderate integration of the proviral DNA into the host genome, and act as promoters in regulation of viral gene transcription. Retroviruses are subdivided into three general groups: the oncoretroviruses (Maloney Murine Leukenmia Virus, MoMLV), the lentiviruses (HIV), and the spumaviruses (foamy virus) (Trowbridge et al., 2002).

[00292] Retroviral based vectors are the most commonly used integrating vectors for gene therapy. These vectors generally have a cloning capacity of approximately 8 kb and are generated by a complete deletion of the viral sequence with the exception of the LTRs and the cis acting packaging signals.

[00293] The retroviral vectors integrate at random sites in the genome. The problems associated with this include potential insertional mutagenesis, and potential oncogenic activity driven from the LTR. The U3 region of the LTR harbors promoter and enhancer elements, hence this region when

deleted from the vector leads to a self-inactivating vector where LTR driven transcription is prevented. An internal promoter can then be used to drive expression of the transgene.

[00294] The initial studies of stem cell gene transfer in mice raised the hope that gene transfer into humans would be equally as efficient (O'Connor and Crystal, 2006). Gene transfer using available retroviral vector systems to transfect multi-lineage long-term repopulating stem cells is still significantly more efficient in the mouse.

(ii) Lentivirus

[00295] Lentiviruses are members of Retroviridae family of viruses (M. Scherr et al., Gene transfer into hematopoietic stem cells using lentiviral vectors. Curr Gene Ther. 2002 February; 2 (1):45-55). They have a more complex genome and replication cycle as compared to the oncoretroviruses (Beyer et al., 2002). They differ from simpler retroviruses in that they possess additional regulatory genes and elements, such as the tat gene, which mediates the transactivation of viral transcription (Sodroski et al., 1996) and rev, which mediates nuclear export of unspliced viral RNA (Cochrane et al., 1990; Emerman and Temin, 1986).

[00296] Lentivirus vectors are derived from the human immunodeficiency virus (HIV-1) by removing the genes necessary for viral replication rendering the virus inert. Although they are devoid of replication genes, the vector can still efficiently integrate into the host genome allowing stable expression of the transgene. These vectors have the additional advantage of a low cytotoxicity and an ability to infect diverse cell types. Lentiviral vectors have also been developed from Simian, Equine and Feline origin but the vectors derived from Human Immunodeficiency Virus (HIV) are the most common (Young et al., 2006).

[00297] Lentivirus vectors are generated by deletion of the entire viral sequence with the exception of the LTRs and cis acting packaging signals. The resultant vectors have a cloning capacity of about 8 kb. One distinguishing feature of these vectors from retroviral vectors is their ability to transduce dividing and non-dividing cells as well as terminally differentiated cells (Kosaka et al., 2004). The lentiviral delivery system is capable of high infection rates in human mesenchymal and embryonic stem cells. In a study by Clements et al., the lentiviral backbone was modified to express mono- and bi-cistronic transgenes and was also used to deliver short hairpin ribonucleic acid for specific silencing of gene expression in human stem cells. (Tissue Eng. 2006 July; 12 (7):1741-51. Lentiviral manipulation of gene expression in human adult and embryonic stem cells. Clements M O, Godfrey A, Crossley J, Wilson S J, Takeuchi Y, Boshoff C).

[00298] Table below summarizes some of the qualities of the viral vectors.

Vector	Insert capacity (kb)	Tropism	Vector genome form	Expression	Efficiency
Enveloped					
Retrovirus	8	Dividing cells only	Integrated	Stable	High
Lentivirus	8	Dividing and non-dividing	Integrated	Stable	High
Non-enveloped					
Adeno-associated virus	<5	Dividing and non-dividing	Episomal and integrated	Stable	High
Adenovirus	2-24	Dividing and non-dividing	Episomal	Transient	High

Non-Viral Gene Delivery Systems

(i) Methods for the Facilitated Integration of Genes

[00299] In addition to the viral based vectors discussed above, other vector systems that lack viral sequence can be used. The alternative strategies include conventional plasmid transfer and the application of targeted gene integration through the use of integrase or transposase technologies. These represent important new approaches for vector integration and have the advantage of being both efficient, and often site specific in their integration. Currently three recombinase systems are available for genetic engineering: cre recombinase from phage P1 (Lakso et al., 1992; Orban et al., 1992), FLP (flippase) from yeast 2 micron plasmid (Dymecki, 1996; Rodriguez et al., 2000), and an integrase isolated from streptomyses phage I C31 (Ginsburg and Calos, 2005). Each of these recombinases recognize specific target integration sites. Cre and FLP recombinase catalyze integration at a 34 bp palindromic sequence called lox P (locus for crossover) and FRT (FLP recombinase target) respectively. Phage integrase catalyzes site-specific, unidirectional recombination between two short att recognition sites in mammalian genomes. Recombination results in integration when the att sites are present on two different DNA molecules and deletion or inversion when the att sites are on the same molecule. It has been found to function in tissue culture cells (in vitro) as well as in mice (in vivo).

[00300] The Sleeping Beauty (SB) transposon is comprised of two inverted terminal repeats of 340 base pairs each (Izsvak et al., 2000). This system directs the precise transfer of specific constructs from a donor plasmid into a mammalian chromosome. The excision and integration of the transposon from a plasmid vector into a chromosomal site is mediated by the SB transposase, which can be delivered to cells as either in a cis or trans manner (Kaminski et al., 2002). A gene in a

chromosomally integrated transposon can be expressed over the lifetime of a cell. SB transposons integrate randomly at TA-dinucleotide base pairs although the flanking sequences can influence integration.

Physical Methods to Introduce Vectors into Cells

(i) Electroporation

[00301] Electroporation relies on the use of brief, high voltage electric pulses which create transient pores in the membrane by overcoming its capacitance. One advantage of this method is that it can be utilized for both stable and transient gene expression in most cell types. The technology relies on the relatively weak nature of the hydrophobic and hydrophilic interactions in the phospholipid membrane and its ability to recover its original state after the disturbance. Once the membrane is permeabilized, polar molecules can be delivered into the cell with high efficiency. Large charged molecules like DNA and RNA move into the cell through a process driven by their electrophoretic gradient. The amplitude of the pulse governs the total area that would be permeabilized on the cell surface and the duration of the pulse determines the extent of permeabilization (Gabriel and Teissie, 1997). The permeabilized state of the cell depends on the strength of the pulses. Strong pulses can lead to irreversible permeabilization, irreparable damage to the cell and ultimately cell death. For this reason electroporation is probably the harshest of gene delivery methods and it generally requires greater quantities of DNA and cells. The effectiveness of this method depends on many crucial factors like the size of the cell, replication and temperature during the application of pulse (Rols and Teissie, 1990).

[00302] The most advantageous feature of this technique is that DNA can be transferred directly into the nucleus increasing its likelihood of being integrated into the host genome. Even cells difficult to transfect can be stably transfected using this method (Aluigi et al., 2005; Zernecke et al., 2003). Modification of the transfection procedure used during electroporation has led to the development of an efficient gene transfer method called nucleofection. The Nucleofector.TM. technology, is a non-viral electroporation-based gene transfer technique that has been proven to be an efficient tool for transfecting hard-to-transfect cell lines and primary cells including MSC (Michela Aluigi, Stem Cells Vol. 24, No. 2, February 2006, pp. 454-461).

Biomolecule-Based Methods

(i) Protein Transduction Domains (PTD)

[00303] PTD are short peptides that are transported into the cell without the use of the endocytotic pathway or protein channels. The mechanism involved in their entry is not well understood, but it can occur even at low temperature (Derossi et al. 1996). The two most commonly used naturally occurring PTDs are the trans-activating activator of transcription domain (TAT) of human immunodeficiency virus and the homeodomain of Antennapedia transcription factor. In addition to these naturally

occurring PTDs, there are a number of artificial peptides that have the ability to spontaneously cross the cell membrane (Joliot and Prochiantz, 2004). These peptides can be covalently linked to the pseudo-peptide backbone of PNA (peptide nucleic acids) to help deliver them into the cell.

(ii) Liposomes

[00304] Liposomes are synthetic vesicles that resemble the cell membrane. When lipid molecules are agitated with water they spontaneously form spherical double membrane compartments surrounding an aqueous center forming liposomes. They can fuse with cells and allow the transfer of "packaged" material into the cell. Liposomes have been successfully used to deliver genes, drugs, reporter proteins and other biomolecules into cells (Felnerova et al., 2004). The advantage of liposomes is that they are made of natural biomolecules (lipids) and are nonimmunogenic.

[00305] Diverse hydrophilic molecules can be incorporated into them during formation. For example, when lipids with positively charged head group are mixed with recombinant DNA they can form lipoplexes in which the negatively charged DNA is complexed with the positive head groups of lipid molecules. These complexes can then enter the cell through the endocytotic pathway and deliver the DNA into lysosomal compartments. The DNA molecules can escape this compartment with the help of dioleoylethanolamine (DOPE) and are transported into the nucleus where they can be transcribed (Tranchant et al., 2004).

[00306] Despite their simplicity, liposomes suffer from low efficiency of transfection because they are rapidly cleared by the reticuloendothelial system due to adsorption of plasma proteins. Many methods of stabilizing liposomes have been used including modification of the liposomal surface with oligosaccharides, thereby sterically stabilizing the liposomes (Xu et al., 2002).

(iii) Immunoliposomes

[00307] Immunoliposomes are liposomes with specific antibodies inserted into their membranes. The antibodies bind selectively to specific surface molecules on the target cell to facilitate uptake. The surface molecules targeted by the antibodies are those that are preferably internalized by the cells so that upon binding, the whole complex is taken up. This approach increases the efficiency of transfection by enhancing the intracellular release of liposomal components. These antibodies can be inserted in the liposomal surface through various lipid anchors or attached at the terminus of polyethylene glycol grafted onto the liposomal surface. In addition to providing specificity to gene delivery, the antibodies can also provide a protective covering to the liposomes that helps to limit their degradation after uptake by endogenous RNAses or proteinases (Bendas, 2001). To further prevent degradation of liposomes and their contents in the lysosomal compartment, pH sensitive immunoliposomes can be employed (Torchilin, 2006). These liposomes enhance the release of liposomal content into the cytosol by fusing with the endosomal membrane within the organelle as they become destabilized and prone to fusion at acidic pH.

[00308] In general non-viral gene delivery systems have not been as widely applied as a means of gene delivery into stem cells as viral gene delivery systems. However, promising results were demonstrated in a study looking at the transfection viability, proliferation and differentiation of adult neural stem/progenitor cells into the three neural lineages neurons. Non-viral, non-liposomal gene delivery systems (ExGen500 and FuGene6) had a transfection efficiency of between 16% (ExGen500) and 11% (FuGene6) of cells. FuGene6-treated cells did not differ from untransfected cells in their viability or rate of proliferation, whereas these characteristics were significantly reduced following ExGen500 transfection. Importantly, neither agent affected the pattern of differentiation following transfection. Both agents could be used to genetically label cells, and track their differentiation into the three neural lineages, after grafting onto ex vivo organotypic hippocampal slice cultures (J Gene Med. 2006 January; 8 (1):72-81. Efficient non-viral transfection of adult neural stem/progenitor cells, without affecting viability, proliferation or differentiation. Tinsley R B, Faijerson J, Eriksson P S).

(iv) Polymer-Based Methods

[00309] The protonated .epsilon.-amino groups of poly L-lysine (PLL) interact with the negatively charged DNA molecules to form complexes that can be used for gene delivery. These complexes can be rather unstable and showed a tendency to aggregate (Kwoh et al., 1999). The conjugation of polyethylene glycol (PEG) was found to lead to an increased stability of the complexes (Lee et al., 2005, Harada-Shiba et al., 2002). To confer a degree of tissue-specificity, targeting molecules such as tissue-specific antibodies have also been employed (Trubetskoy et al., 1992, Suh et al., 2001).

[00310] An additional gene carrier that has been used for transfecting cells is polyethylenimine (PEI) which also forms complexes with DNA. Due to the presence of amines with different pKa values, it has the ability to escape the endosomal compartment (Boussif et al., 1995). PEG grafted onto PEI complexes was found to reduce the cytotoxicity and aggregation of these complexes. This can also be used in combination with conjugated antibodies to confer tissue-specificity (Mishra et al., 2004, Shi et al., 2003, Chiu et al., 2004, Merdan et al., 2003).

Targeted gene delivery – site-specific recombinations

[00311] In certain embodiments, a non-human, transgenic animal comprising a targeting vector that further comprises recombination sites (e.g., Lox sites, FRT sites) can be crossed with a non-human, transgenic animal comprising a recombinase (e.g., Cre recombinase, FLP recombinase) under control of a particular promoter. It has been shown that these site-specific recombination systems, although of microbial origin for the majority, function in higher eukaryotes, such as plants, insects and mice. Among the site-specific recombination systems commonly used, there may be mentioned the Cre/Lox and FLP/FRT systems. The strategy normally used consists of inserting the loxP (or FRT) sites into the chromosomes of ES cells by homologous recombination, or by conventional transgenesis, and then of delivering Cre (or FLP) for the latter to catalyze the recombination reaction.

The recombination between the two loxP (or FRT) sites may be obtained in ES cells or in fertilized eggs by transient expression of Cre or using a Cre transgenic mouse. Such a strategy of somatic mutagenesis allows a spatial control of the recombination because the expression of the recombinase is controlled by a promoter specific for a given tissue or for a given cell.

[00312] A detailed description of the FRT system can be found, e.g., in U.S. Patent No. 7,736,897.

[00313] The P1 bacteriophage uses Cre-lox recombination to circularize and facilitate replication of its genomic DNA when reproducing. Since being discovered, the bacteriophage's recombination strategy has been developed as a technology for genome manipulation. Because the cre gene and loxP sites are not native to the mouse genome, they are introduced by transgenic technology into the mouse genomes (Nagy A. 2000. Cre recombinase: the universal reagent for genome tailoring. Genesis 26:99-109). The orientation and location of the loxP sites determine whether Cre recombination induces a deletion, inversion, or chromosomal translocation (Nagy A. 2000. Cre recombinase: the universal reagent for genome tailoring. Genesis 26:99-109). The cre/lox system has been successfully applied in mammalian cell cultures, yeasts, plants, mice, and other organisms (Araki K, Imaizumi T, Okuyama K, Oike Y, Yamamura K. 1997. Efficiency of recombination by Cre transient expression in embryonic stem cells: comparison of various promoters. J Biochem (Tokyo) 122:977-82). Much of the success of Cre-lox is due to its simplicity. It requires only two components: (a) Cre recombinase: an enzyme that catalyzes recombination between two loxP sites; and (b) LoxP sites: a specific 34-base pair bp) sequences consisting of an 8-bp core sequence, where recombination takes place, and two flanking 13-bp inverted repeats.

Cell-mediated delivery

[00314] In one embodiment, the optical sensors of the present invention are delivered using e.g., a cell expressing the optical sensor. A variety of means for administering cells to subjects are known to those of skill in the art. Such methods can include systemic injection, for example i.v. injection or implantation of cells into a target site in a subject. Cells may be inserted into a delivery device which facilitates introduction by injection or implantation into the subjects. Such delivery devices may include tubes, e.g., catheters, for injecting cells and fluids into the body of a recipient subject. In one preferred embodiment, the tubes additionally have a needle, e.g., a syringe, through which the cells of the invention can be introduced into the subject at a desired location. The cells may be prepared for delivery in a variety of different forms. For example, the cells may be suspended in a solution or gel or embedded in a support matrix when contained in such a delivery device. Cells may be mixed with a pharmaceutically acceptable carrier or diluent in which the cells of the invention remain viable. Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. The solution is preferably sterile and fluid. Preferably, the solution is stable under the conditions of manufacture and

storage and preserved against the contaminating action of microorganisms such as bacteria and fungi through the use of, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. Solutions of the invention may be prepared by incorporating cells as described herein in a pharmaceutically acceptable carrier or diluent and, as required, other ingredients enumerated above, followed by filtered sterilization. It is preferred that the mode of cell administration is relatively non-invasive, for example by intravenous injection, pulmonary delivery through inhalation, oral delivery, buccal, rectal, vaginal, topical, or intranasal administration.

[00315] However, the route of cell administration will depend on the tissue to be treated and may include implantation or direct injection. Methods for cell delivery are known to those of skill in the art and can be extrapolated by one skilled in the art of medicine for use with the methods and compositions described herein. Direct injection techniques for cell administration can also be used to stimulate transmigration through the entire vasculature, or to the vasculature of a particular organ, such as for example liver, or kidney or any other organ. This includes non-specific targeting of the vasculature. One can target any organ by selecting a specific injection site, such as e.g., a liver portal vein. Alternatively, the injection can be performed systemically into any vein in the body. This method is useful for enhancing stem cell numbers in aging patients. In addition, the cells can function to populate vacant stem cell niches or create new stem cells to replenish the organ, thus improving organ function. For example, cells may take up pericyte locations within the vasculature. Delivery of cells may also be used to target sites of active angiogenesis. If so desired, a mammal or subject can be pre-treated with an agent, for example an agent is administered to enhance cell targeting to a tissue (e.g., a homing factor) and can be placed at that site to encourage cells to target the desired tissue. For example, direct injection of homing factors into a tissue can be performed prior to systemic delivery of ligand-targeted cells.

[00316] Method of using stem cells, such as neural stem cells to deliver agents through systemic administration and via intracranial administration to home in on a tumor or to an injured parts of brain have been described (see, e.g., U.S. Patent Nos. 7,655,224; and 7,393,526). Accordingly, one can also modify such cells to express the desired voltage sensor for delivery into the organs, such as the brain.

[00317] As used in this specification and the appended claims, the singular forms “a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[00318] It is understood that the foregoing detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those skilled in the art, may be made without departing from the spirit and scope of the present invention. These

publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

Some definitions

[00319] As used herein the term "optical sensor" refers to a microbial rhodopsin protein employed to yield an optical signal indicative of the voltage drop across the membrane in which it is embedded

[00320] As used herein the phrase "reduced ion pumping activity" means a decrease in the endogenous ion pumping activity of a modified microbial rhodopsin protein of at least 10% compared to the endogenous pumping activity of the natural microbial rhodopsin protein from which the modified rhodopsin is derived. The ions most commonly pumped by microbial rhodopsins are H^+ and Cl^-. In some embodiments, the ion pumping activity of a modified rhodopsin protein is at least 20% lower, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% lower than the endogenous ion pumping activity of the corresponding native microbial rhodopsin protein.

[00321] In some embodiments, the modified microbial rhodopsin has no detectable ion pumping activity.

[00322] As used herein, the term "endogenous ion pumping activity" refers to the movement of ions through a native microbial rhodopsin protein that occurs in response to light stimuli.

[00323] As used herein, the term "native microbial rhodopsin protein" or "natural microbial rhodopsin protein" refers to a rhodopsin protein prepared or isolated from a microbial (e.g., bacterial, archaeal, or eukaryotic) source. Such natural microbial rhodopsin proteins, when isolated, retain characteristics (e.g., pKa, ion pumping activity etc) that are substantially similar to the microbial rhodopsin protein in its native environment (e.g., in a microbial cell). Some non-limiting examples of microbial rhodopsin proteins useful with the methods described herein include green-absorbing proteorhodopsin (GPR; GenBank accession number AF349983), blue-absorbing proteorhodopsin (BPR, GenBank accession number AF349981), *Natromonas pharaonis* sensory rhodopsin II (NpSRII; GenBank accession number Z35086.1), and bacteriorhodopsin (BR; the protein encoded by GenBank sequence NC_010364.1, nucleotides 1082241-1083029, wherein 1082241 is designated as 1 herein, GenBank accession number M11720.1, or as described by e.g., Beja O, et al., (2000). *Science 289* (5486): 1902–1904), and archaerhodopsin (see e.g., Chow B.Y. et al., Nature 463:98-102 (2010) and the Examples in this application).

[00324] As used herein, the term "modified microbial rhodopsin protein" refers to a native microbial rhodopsin protein comprising at least one mutation. Mutations can be in the nucleic acid

sequence (e.g., genomic or mRNA sequence), or alternatively can comprise an amino acid substitution. Such amino acid substitutions can be conserved mutations or non-conserved mutations. As well-known in the art, a "conservative substitution" of an amino acid or a "conservative substitution variant" of a polypeptide refers to an amino acid substitution which maintains: 1) the structure of the backbone of the polypeptide (e.g. a beta sheet or alpha-helical structure); 2) the charge or hydrophobicity of the amino acid; or 3) the bulkiness of the side chain. More specifically, the well-known terminologies "hydrophilic residues" relate to serine or threonine. "Hydrophobic residues" refer to leucine, isoleucine, phenylalanine, valine or alanine. "Positively charged residues" relate to lysine, arginine or histidine. "Negatively charged residues" refer to aspartic acid or glutamic acid. Residues having "bulky side chains" refer to phenylalanine, tryptophan or tyrosine. To avoid doubt as to nomenclature, the term "D97N" or similar terms specifying other specific amino acid substitutions means that the Asp (D) at position 97 of the protein sequence is substituted with Asn (N). A "conservative substitution variant" of D97N would substitute a conservative amino acid variant of Asn (N) that is not D.

[00325] The terminology "conservative amino acid substitutions" is well known in the art, which relates to substitution of a particular amino acid by one having a similar characteristic (e.g., similar charge or hydrophobicity, similar bulkiness). Examples include aspartic acid for glutamic acid, or isoleucine for leucine. A list of exemplary conservative amino acid substitutions is given in the Table 5 below. A conservative substitution mutant or variant will 1) have only conservative amino acid substitutions relative to the parent sequence, 2) will have at least 90% sequence identity with respect to the parent sequence, preferably at least 95% identity, 96% identity, 97% identity, 98% identity or 99%; and 3) will retain voltage sensing activity as that term is defined herein.

Table 5: Conservative Amino Acid Substitutions

For Amino Acid	Code	Replace With
Alanine	A	D-ala, Gly, Aib, β-Ala, Acp, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, Aib, β-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met

Table 5: Conservative Amino Acid Substitutions		
For Amino Acid	**Code**	**Replace With**
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4 or 5-phenylproline, AdaA, AdaG, cis-3,4 or 5-phenylproline, Bpa, D-Bpa
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or-L-1-oxazolidine-4-carboxylic acid (Kauer, U.S. Pat. No. (4,511,390)
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met (O), D-Met (O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met (O), D-Met (O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG

[00326] A non-conservative mutation is any other amino acid substitution other than the conservative substitutions noted in the above table.

[00327] Methods of making conservative amino acid substitutions are also well known to one skilled in the art and include but are not limited to site-specific mutagenesis using oligonucleotide primers and polymerase chain reactions. Optical sensor variants can be expressed and assayed for voltage sensing activity, pKa, and fluorescence detection by methods known in the art and/or described herein to verify that the desired activities of the optical sensor are retained or augmented by the amino acid substitutions. It is contemplated that conservative amino acid substitution variants of the optical sensors described herein can have enhanced activity or superior characteristics for sensing voltage relative to the parent optical sensor. Certain silent or neutral missense mutations can also be made in the nucleic acid encoding an optical sensor by a mutation that does not change the encoded amino acid sequence of the encoded optical sensor. These types of mutations are useful to optimize codon usage which improve recombinant protein expression and production in the desired cell type. Specific site-directed mutagenesis of a nucleic acid encoding an optical sensor in a vector can be used to create specific amino acid mutations and substitutions. Site-directed mutagenesis can be carried out using, e. g. the QUICKCHANGE® site-directed mutagenesis kit from STRATAGENE® according to manufacture's instructions, or by any method known in the art.

[00328] As used herein, the term "membrane potential" refers to a calculated difference in voltage between the interior and exterior of a cell. In one embodiment membrane potential, ΔV, is determined by the equation $\Delta V = V_{interior} - V_{exterior}$. For example, if the outside voltage is 100 mV, and the inside voltage is 30 mV, then the difference is –70 mV. Under resting conditions, the membrane potential is predominantly determined by the ion having the greatest conductance across the membrane. In many cells, the membrane potential is determined by potassium, which yields a resting membrane potential of approximately -70mV. Thus by convention, a cell under resting conditions has a negative membrane potential. In some cells when a membrane potential is reached that is equal to or greater than a threshold potential, an action potential is triggered and the cell undergoes depolarization (i.e., a large increase in the membrane potential). Often, when a cell undergoes depolarization, the membrane potential reverses and reaches positive values (e.g., 55mV). During resolution of the membrane potential following depolarization towards the resting membrane potential, a cell can "hyperpolarize." The term "hyperpolarize" refers to membrane potentials that are more negative than the resting membrane potential, while the term "depolarize" refers to membrane potentials that are less negative (or even positive) compared to the resting membrane potential. Membrane potential changes can arise by movement of ions through ion channels or ion pumps embedded in the membrane. Membrane potential can be measured across any cellular membrane that comprises ion channels or ion pumps that can maintain an ionic gradient across the membrane (e.g., plasma membrane, mitochondrial inner and outer membranes etc.).

[00329] As used herein, the term "change in the membrane potential" refers to an increase (or decrease) in ΔV of at least 1mV that is either spontaneous or in response to e.g., environmental or chemical stimuli (e.g., cell-to-cell communication, ion channel modulation, contact with a candidate agent etc.) compared to the resting membrane potential measured under control conditions (e.g., absence of an agent, impaired cellular communication, etc.). In some embodiments, the membrane potential ΔV is increased by at least 10 mV, at least 15 mV, at least 20 mV, at least 25 mV, at least 30 mV, at least 35 mV, at least 40 mV, at least 45 mV, at least 50 mV, at least 55 mV, at least 60 mV, at least 65 mV, at least 70 mV, at least 75 mV, at least 80 mV, at least 85 mV, at least 90 mV, at least 95 mV, at least 100 mV, at least 105 mV, at least 110 mV, at least 115 mV, at least 120 mV, at least 125 mV, at least 130 mV, at least 135 mV, at least 140 mV, at least 145 mV, at least 150 mV, at least 155 mV, at least 160 mV, at least 165 V, at least 170 mV, at least 180 mV, at least 190 mV, at least 200 mV or more compared to the membrane potential of a similar cell under control conditions. In other embodiments, the membrane potential is decreased by at least 3 mV, at least 5 mV, at least 10 mV, at least 15 mV, at least 20 mV, at least 25 mV, at least 30 mV, at least 35 mV, at least 40 mV, at least 45 mV, at least 50 mV, at least 55 mV, at least 60 mV, at least 65 mV, at least 70 mV, at least 75 mV, at least 80 mV, at least 85 mV, at least 90 mV, at least 95 mV, at least 100 mV, at least 105 mV, at least 110 mV, at least 115 mV, at least 120 mV, at least 125 mV, at least 130 mV, at least 135 mV, at

least 140 mV, at least 145 mV, at least 150 mV or more compared to the membrane potential of a similar cell under control conditions.

[00330] As used herein, the phrase "localizes to a membrane of the cell" refers to the preferential localization of the modified microbial rhodopsin protein to the membrane of a cell and can be achieved by e.g., modifying the microbial rhodopsin to comprise a signal sequence that directs the rhodopsin protein to a membrane of the cell (e.g., the plasma membrane, the mitochondrial outer membrane, the mitochondrial inner membrane etc.). In some embodiments, at least 40% of the modified microbial rhodopsin protein in the cell is localized to the desired cellular membrane compartment (e.g., plasma membrane, mitochondrial membrane etc); in other embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% of the modified microbial rhodopsin protein is localized to the desired cellular membrane compartment. Similarly, the phrase "localized to a subcellular compartment" refers to the preferential localization of the microbial rhodopsin protein to a particular subcellular compartment (e.g., mitochondria, endoplasmic reticulum, peroxisome etc.). In some embodiments, at least 40% of the modified microbial rhodopsin protein in the cell is localized to the desired subcellular compartment; in other embodiments, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% of the modified microbial rhodopsin protein is localized to the desired subcellular compartment.

[00331] In some embodiment, about 100% is localized to the desired cellular membrane or compartment.

[00332] As used herein, the term "introducing to a cell" refers to any method for introducing either an expression vector encoding an optical sensor or a recombinant optical sensor protein described herein into a host cell. Some non-limiting examples of introducing an expression vector into a cell include, for example, calcium phosphate transfection, electroporation, lipofection, or a method using a gene gun or the like. In one embodiment, a recombinant optical sensor protein is introduced to a cell by membrane fusion using a lipid mediated delivery system, such as micelles, liposomes, etc.

[00333] As used herein, the phrase "a moiety that produces an optical signal" refers to a molecule (e.g., retinal), or moiety of a molecule, capable of producing a detectable signal such as e.g., fluorescence, chemiluminescence, a colorimetric signal etc. In one embodiment, the modified microbial rhodopsin comprises a fusion molecule with a moiety that produces an optical signal.

[00334] As used herein, the phrases "change in the level of fluorescence" or "a change in the level of the optical signal" refer to an increase or decrease in the level of fluorescence from the modified microbial rhodopsin protein or an increase or decrease in the level of the optical signal induced by a change in voltage or membrane potential. In some embodiments, the level of fluorescence or level of optical signal in a cell is increased by at least at least 2%, at least 5%, at least 10%, 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 50-fold, at least 100-fold,

at least 500-fold, at least 600-fold, at least 700-fold, at least 800-fold, at least 900-fold, at least 1000-fold, at least 2000-fold, at least 5000-fold, at least 10000-fold or more compared to the same cell or a similar cell under control conditions. Alternatively, the level of fluorescence or level of optical signal in a cell is decreased by at least by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or even 100% (i.e., no detectable signal) compared to the same cell or a similar cell under control culture conditions.

[00335] As used herein, the phrase "modulates ion channel activity" refers to an increase or decrease in one or more properties of an ion channel that manifests as a change in the membrane potential of a cell. These properties include, e.g., open- or closed-state conductivity, threshold voltage, kinetics and/or ligand affinity. In some embodiments, the one or more properties of interest of an ion channel of a cell as measured by e.g., a change in membrane potential of the cell. In some embodiments, the activity of an ion channel is increased by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, at least 100-fold, at least 1000-fold or more in the presence of an agent compared to the activity of the ion channel in the absence of the agent. In other embodiments, the parameter of interest of an ion channel is decreased by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% in the presence of an agent compared to the activity of the ion channel in the absence of the agent. In some embodiments, the parameter of an ion channel is absent in the presence of an agent compared to the activity of the ion channel in the absence of the agent.

[00336] As used herein, the term "inhibitor of antibiotic efflux" refers to an agent that decreases the level of antibiotic efflux from a bacterial cell by at least 20% compared to the level of antibiotic efflux in the absence of the agent. In some embodiments, antibiotic efflux is decreased by at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or even 100% (i.e., absent) in the presence of an agent compared to the level of antibiotic efflux in the absence of the agent.

[00337] As used herein, the term "targeting sequence" refers to a moiety or sequence that homes to or preferentially associates or binds to a particular tissue, cell type, receptor, organelle, or other area of interest. The addition of a targeting sequence to an optical sensor composition will enhance the delivery of the composition to a desired cell type or subcellular location. The addition to, or expression of, a targeting sequence with the optical sensor in a cell enhances the localization of the optical sensor to a desired location within an animal or subject.

[00338] As used herein, the phrase "homologous mutation in another microbial rhodopsin that corresponds to the amino acid mutation in bacteriorhodopsin" refers to mutation of a residue in a desired microbial rhodopsin that is expected to have a similar effect to a substantially similar mutation in bacteriorhodopsin. One of skill in the art can easily locate a homologous residue in their desired

microbial rhodopsin by performing an alignment of conserved regions of the desired microbial rhodopsin with a bacteriorhodopsin sequence using a computer program such as ClustalW.

[00339] Examples of homologous mutations include the mutations made in the Examples set forth in this application.

[00340] As used herein the term "comprising " or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

[00341] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00342] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00343] Provided herein are optical sensors comprising a microbial rhodopsin or a modified microbial rhodopsin protein with a reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived. In one embodiment, the composition comprises a vector encoding or an engineered cell comprising the microbial rhodopsin protein or the modified microbial rhodopsin protein with a reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived. In some embodiments, the microbial rhodopsin is directed to a membrane, such as plasma membrane or mitochondrial membrane.

[00344] Accordingly, the invention provides a method for measuring membrane potential in a cell expressing a nucleic acid encoding a microbial rhodopsin protein, the method comprising the steps of: (a) exciting, in vitro, ex vivo or in vivo, at least one cell comprising a nucleic acid encoding a microbial rhodopsin protein with light of at least one wave length; and (b)detecting, in vitro, at least one optical signal from the at least one cell, wherein the level of fluorescence emitted by the at least one cell compared to a reference is indicative of the membrane potential of the cell.

[00345] In some or any embodiment or aspect of the invention, the he microbial rhodopsin protein is a modified microbial rhodopsin protein with reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived.

[00346] In some or any embodiment or aspect of the invention, the modified microbial rhodopsin comprises a mutated proton acceptor proximal to the Schiff Base.

[00347] In some or any embodiment or aspect of the invention, the microbial rhodopsin protein is a member of the proteorhodopsin family of proteins.

[00348] In some or any embodiment or aspect of the invention the microbial rhodopsin protein is a member of the archaerhodopsin family of proteins.

[00349] In some or any embodiment or aspect of the invention the at least one wave length is a wave length between λ = 594-645 nm.

[00350] In some or any embodiment or aspect of the invention the cell is a prokaryotic cell.

[00351] In some or any embodiment or aspect of the invention the cell is a eukaryotic cell.

[00352] In some or any embodiment or aspect of the invention the eukaryotic cell is a mammalian cell.

[00353] In some or any embodiment or aspect of the invention the eukaryotic cell is a stem cell or a pluripotent or a progenitor cell.

[00354] In some or any embodiment or aspect of the invention the eukaryotic cell is an induced pluripotent cell.

[00355] In some or any embodiment or aspect of the invention the eukaryotic cell is a neuron.

[00356] In some or any embodiment or aspect of the invention the eukaryotic cell is a cardiomyocyte.

[00357] In some or any embodiment or aspect of the invention the method further comprises a step of transfecting, in vitro, ex vivo or in vivo, the at least one cell with a vector comprising the nucleic acid encoding the microbial rhodopsin protein.

[00358] In some or any embodiment or aspect of the invention, the nucleic acid encoding the microbial rhodopsin protein is operably linked to a cell-type specific promoter.

[00359] In some or any embodiment or aspect of the invention, the nucleic acid encoding the microbial rhodopsin protein is operably linked to a membrane-targeting nucleic acid sequence.

[00360] In some or any embodiment or aspect of the invention, the membrane-targeting nucleic acid is a plasma membrane targeting nucleic acid sequence.

[00361] In some or any embodiment or aspect of the invention, the membrane-targeting nucleic acid sequence is a subcellular compartment-targeting nucleic acid sequence.

[00362] In some or any embodiment or aspect of the invention, the subcellular compartment is selected from a mitochondrial membrane, an endoplasmic reticulum, a sarcoplastic reticulum, a synaptic vesicle, an endosome and a phagosome.

[00363] In some or any embodiment or aspect of the invention, the nucleic acid encoding a microbial rhodopsin protein is operably linked to a nucleic acid encoding at least one additional fluorescent protein or a chromophore.

[00364] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is a fluorescent protein capable for indicating the ion concentration in the cell.

[00365] In some or any embodiment or aspect of the invention, the fluorescent protein capable for indicating ion concentration is a calcium indicator.

[00366] In some or any embodiment or aspect of the invention, the fluorescent protein capable for indicating ion concentration is a pH indicator.

[00367] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is capable of undergoing nonradiative fluorescence resonance energy transfer to the microbial rhodopsin, with a rate of energy transfer dependent on the membrane potential.

[00368] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

[00369] In some or any embodiment or aspect of the invention, brightness of the fluorescent protein is insensitive to membrane potential and local chemical environment.

[00370] In some or any embodiment or aspect of the invention, the method further comprises steps of exciting, in vitro, ex vivo or in vivo, the at least one cell with light of at least a first and a second wavelength; and detecting, in vitro, ex vivo or in vivo, the at least first and the second optical signal resulting from the excitation with the at least the first and the second wavelength from the at least one cell.

[00371] In some or any embodiment or aspect of the invention, the at least second wave length is between λ = 447-594 nm.

[00372] In some or any embodiment or aspect of the invention, the method further comprises a step of calculating the ratio of the fluorescence emission from the microbial rhodopsin to the fluorescence emission of the at least one additional fluorescent protein to obtain a measurement of membrane potential independent of variations in expression level.

[00373] In some or any embodiment or aspect of the invention, the method further comprises the step of exposing, in vitro, ex vivo or in vivo, the at least one cell to a stimulus capable of, or suspected to be capable of changing membrane potential.

[00374] In some or any embodiment or aspect of the invention, the stimulus a candidate agent.

[00375] In some or any embodiment or aspect of the invention, the stimulus is a change to the composition of the cell culture medium.

[00376] In some or any embodiment or aspect of the invention, the stimulus is an electrical current.

[00377] In some or any embodiment or aspect of the invention, further comprising the step of measuring, in vitro, ex vivo or in vivo the at least one optical signal at a first and at least at a second time point.

[00378] In some or any embodiment or aspect of the invention, the first time point is before exposing the at least one cell to a stimulus and the at least second time point is after exposing the at least one cell to the stimulus.

[00379] In some or any embodiment or aspect of the invention, wherein the method comprises a plurality of cells, for example in a highthroughput assay.

[00380] In another embodiment, the invention provides an isolated and purified nucleic acid encoding a modified member of the archaerhodopsin family of proteins with reduced ion pumping activity compared to a natural member of the archaerhodopsin family of proteins from which it is derived.

[00381] In some or any embodiment or aspect of the invention, the modified member of an archaerhodopsin family of proteins comprises a mutated proton acceptor proximal to the Schiff Base.

[00382] In some or any embodiment or aspect of the invention, the modified member of the archaerhodopsin family of proteins with reduced ion pumping activity compared to a natural member of the archaerhodopsin family of proteins from which it is derived is operably linked to a nucleic acid sequence encoding a membrane-targeting nucleic acid sequence.

[00383] In some or any embodiment or aspect of the invention, the membrane-targeting nucleic acid sequence is a plasma membrane targeting nucleic acid sequence.

[00384] In some or any embodiment or aspect of the invention, the membrane-targeting nucleic acid sequence is a subcellular membrane-targeting nucleic acid sequence.

[00385] In some or any embodiment or aspect of the invention, the subcellular membrane is a mitochondrial membrane, an endoplasmic reticulum, a sarcoplastic reticulum, a synaptic vesicle, an endosome or a phagosome.

[00386] In some or any embodiment or aspect of the invention, the modified microbial rhodopsin of the archaerhodopsin family is operably linked to a cell-type specific promoter.

[00387] In some or any embodiment or aspect of the invention, the modified microbial rhodopsin of the archaerhodopsin family is operably linked to a nucleic acid encoding at least one additional fluorescent protein or a chromophore.

[00388] In some or any embodiment or aspect of the invention, wherein the at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

[00389] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is a fluorescent protein capable for indicating ion concentration in the cell.

[00390] In some or any embodiment or aspect of the invention, the fluorescent protein capable for indicating ion concentration is a calcium indicator.

[00391] In some or any embodiment or aspect of the invention, the fluorescent protein capable for indicating ion concentration is a pH indicator.

[00392] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is capable of undergoing nonradiative fluorescence resonance energy transfer to the microbial rhodopsin, with a rate of energy transfer dependent on the membrane potential.

[00393] In some or any embodiment or aspect of the invention, the isolated and purified nucleic further comprises a vector.

[00394] In some or any embodiment or aspect of the invention, the vector is a viral vector, such as a lentiviral vector or an adeno-associated viral vector.

[00395] In another embodiment, the invention provides a kit comprising the isolated and purified nucleic acid encoding the modified microbial proteins of archaerhodopsin family as disclosed herein in a suitable container. The nucleic acids may be provided in dry form or in a suitable buffer.

[00396] Another embodiment of the invention provides an isolated cell comprising a nucleic acid encoding a microbial rhodopsin protein, wherein the microbial rhodopsin protein is a modified

microbial rhodopsin protein with reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived.

[00397] In some or any embodiment or aspect of the invention, the modified microbial rhodopsin protein comprises a mutated proton acceptor proximal to the Schiff Base.

[00398] In some or any embodiment or aspect of the invention, the microbial rhodopsin is a member of the proteorhodopsin family.

[00399] In some or any embodiment or aspect of the invention, the microbial rhodopsin is a member of the archaerhodopsin family.

[00400] In some or any embodiment or aspect of the invention, the cell is a eukaryotic cell.

[00401] In some or any embodiment or aspect of the invention, the cell is a prokaryotic cell.

[00402] In some or any embodiment or aspect of the invention, the modified microbial rhodopsin gene is operably linked to a promoter.

[00403] In some or any embodiment or aspect of the invention, the promoter is a cell-type specific promoter.

[00404] In some or any embodiment or aspect of the invention, the nucleic acid encoding the modified microbial rhodopsin protein is operably linked to a membrane-targeting nucleic acid sequence.

[00405] In some or any embodiment or aspect of the invention, the membrane-targeting nucleic acid is a plasma membrane targeting nucleic acid sequence.

[00406] In some or any embodiment or aspect of the invention, the membrane-targeting nucleic acid is a subcellular compartment-targeting nucleic acid sequence.

[00407] In some or any embodiment or aspect of the invention, the subcellular compartment is selected from a mitochondrial membrane, an endoplasmic reticulum, a sarcoplastic reticulum, a synaptic vesicle, an endosome and a phagosome.

[00408] In some or any embodiment or aspect of the invention, the nucleic acid encoding the modified microbial rhodopsin protein is operably linked to a nucleic acid encoding at least one additional fluorescent protein or chromophore.

[00409] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

[00410] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is capable of undergoing nonradiative fluorescence resonance energy transfer to the microbial rhodopsin, with a rate of energy transfer dependent on the membrane potential.

[00411] In some or any embodiment or aspect of the invention, the least one additional fluorescent protein is a fluorescent protein whose brightness is insensitive to membrane potential and local chemical environment.

[00412] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is capable of indicating the ion concentration in the cell.

[00413] In some or any embodiment or aspect of the invention,the fluorescent protein capable for indicating ion concentration is a calcium indicator.

[00414] In some or any embodiment or aspect of the invention, the fluorescent protein capable for indicating ion concentration is a pH indicator.

[00415] In some or any embodiment or aspect of the invention, the cell is a stem cell, a pluripotent cell, or an induced pluripotent cell, or differentiated or undifferentiated progeny thereof.

[00416] In some or any embodiment or aspect of the invention, the cell is a differentiated cell.

[00417] In some or any embodiment or aspect of the invention, the differentiated cell is a neuron.

[00418] In some or any embodiment or aspect of the invention, the differentiated cell is a cardiomyocyte.

[00419] The invention further provides, in one embodiment, an isolated cell comprising the isolated and purified nucleic acid of any of the isolated and purified nucleic acids or nucleic acid constructs comprising the modified microbial rhodopsin of archaerhodopsin family as described above.

[00420] In one embodiment, the invention provides a kit comprising a plurality of cells as described herein in a suitable cell culture medium and a container.

[00421] In yet another embodiment, the invention provides a method of making an engineered cell for optical measurement of membrane potential comprising the steps of transducing a cell with a nucleic acid encoding a microbial rhodopsin protein.

[00422] In some or any embodiment or aspect of the invention, the microbial rhodopsin protein is a modified microbial rhodopsin protein with reduced ion pumping activity compared to a natural microbial rhodopsin protein.

[00423] In some or any embodiment or aspect of the invention, the modified microbial rhodopsin comprises a mutated proton acceptor proximal to the Schiff Base.

[00424] In some or any embodiment or aspect of the invention, the nucleic acid encoding a microbial rhodopsin protein is operably linked to a membrane targeting nucleic acid sequence.

[00425] In some or any embodiment or aspect of the invention, the membrane targeting nucleic acid sequence is a plasma membrane targeting nucleic acid sequence.

[00426] In some or any embodiment or aspect of the invention, the membrane targeting nucleic acid sequence is a subcellular membrane targeting nucleic acid sequence.

[00427] In some or any embodiment or aspect of the invention, the subcellular membrane is a mitochondrial membrane, an endoplasmic reticulum, a sarcoplastic reticulum, a synaptic vesicle, an endosome or a phagosome.

[00428] In some or any embodiment or aspect of the invention, the nucleic acid encoding the microbial rhodopsin protein is operably linked to a cell-type specific promoter.

[00429] In some or any embodiment or aspect of the invention, the nucleic acid encoding the microbial rhodopsin protein is operably linked to an additional nucleic acid encoding at least one additional fluorescent protein or a chromophore.

[00430] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

[00431] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is capable of indicating ion concentration in the cell.

[00432] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is capable of indicating ion concentration in the cell is a calcium indicator.

[00433] In some or any embodiment or aspect of the invention, the at least one additional fluorescent protein is capable of indicating ion concentration in the cell is a pH indicator.

[00434] In some or any embodiment or aspect of the invention, the cell is a differentiated cell.

[00435] In some or any embodiment or aspect of the invention, the differentiated cell is a neuron.

[00436] In some or any embodiment or aspect of the invention, the differentiated cell is a cardiomyocyte.

[00437] In some or any embodiment or aspect of the invention, the cell is a pluripotent cell, a stem cell or an induced pluripotent stem cell.

[00438] In some or any embodiment or aspect of the invention, the method further comprises a step of differentiating the pluripotent cell, the stem cell or the induced pluripotent stem cell into a differentiated cell.

[00439] In some or any embodiment or aspect of the invention, the transducing is performed using a transient transfection.

[00440] In some or any embodiment or aspect of the invention, the transducing is performed using a stable transfection.

[00441] In some or any embodiment or aspect of the invention, the cell is transduced with the isolated and purified nucleic acid as described herein.

[00442] In some embodiments, the method is performed to a non-human cells.

[00443] In some embodiments and aspects, the invention provides a cell that has been genetically engineered to express a microbial rhodopsin that is not naturally present in the cell, and methods of using such cells.

[00444] Also provided herein are methods for making an optical sensor comprising: (a) modifying a microbial rhodopsin protein to reduce the ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived, and (b) introducing the modified microbial rhodopsin protein into a cell, thereby producing a genetically encoded optical sensor. In one embodiment, the step of introducing comprises introducing a gene for the modified microbial rhodopsin protein into the cell.

[00445] In another aspect, the invention provides for a method for measuring a change in a membrane potential of a cell, the method comprising: measuring an optical signal in a cell, wherein the cell comprises a natural microbial rhodopsin or a modified microbial rhodopsin protein with reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived, wherein the microbial rhodopsin or the modified microbial rhodopsin protein localizes to a membrane of the cell and, wherein a change in the level of fluorescence of the rhodopsin or the modified microbial rhodopsin is indicative of a change in the membrane potential of the cell.

[00446] Also described herein is a method of screening for an agent that modulates membrane potential, either directly or through an effect on ion channel activity, the method comprising: (a) contacting a cell with a candidate agent, wherein the cell comprises a microbial rhodopsin or a modified microbial rhodopsin protein with reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived, wherein the microbial rhodopsin or the modified microbial rhodopsin protein localizes to a membrane of the cell, and (b) measuring the optical signal, wherein a change in the level of fluorescence of the rhodopsin or the modified microbial rhodopsin in the presence of the candidate agent is indicative of a change in the membrane potential across the membrane of the cell, thereby indicating that the candidate agent modulates membrane potential directly or through an effect on ion channel activity.

[00447] In addition, provided herein is a method of screening for a modulator of antibiotic efflux in a bacterial cell, the method comprising: (a) contacting a bacterial cell with a candidate agent, wherein the cell comprises a microbial rhodopsin or a modified microbial rhodopsin protein having reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived, wherein the microbial rhodopsin or the modified microbial rhodopsin protein localizes to a membrane of the cell, and (b) measuring the time-dependent fluctuations in the level of fluorescence of the microbial rhodopsin or the modified microbial rhodopsin, wherein a change in the fluctuations in the presence of the candidate agent is indicative of a change in fluctuations in the membrane potential across the membrane of the cell, thereby indicating that the candidate agent is a modulator of antibiotic efflux in the bacterial cell. In one embodiment, the candidate agent is an inhibitor of antibiotic efflux. In an alternate embodiment, the candidate agent enhances antibiotic efflux.

[00448] Also described herein are nucleic acid constructs for expressing a microbial rhodopsin and a modified microbial rhodopsin protein with a reduced ion pumping activity compared to the natural microbial rhodopsin protein from which it is derived comprising at least one nucleic acid sequence encoding the modified microbial rhodopsin protein that is modified for codon usage appropriate for the eukaryotic cell.

[00449] In another embodiment, described herein are vectors comprising at least one nucleic acid encoding a voltage indicator protein operably linked to a promoter, e.g., a tissue-specific promoter, such as a neuron-specific promoter or a cardiac tissue, such as cardiomyocyte-specific promoter.

[00450] In some embodiments, the vector also comprises a membrane-targeting signal sequence to membranes such as the plasma membrane, mitochondria, the endoplasmic reticulum, the sarcoplasmic reticulum, synaptic vesicles, and phagosomes. In some embodiments, the vector comprises at least two nucleic acids each encoding a different voltage indicator protein. In some embodiments, the vector further comprises a nucleic acids encoding, e.g., a pH indicator (e.g., pHluorin) or a calcium indicator (e.g., GCaMP3). Use of such a combination enables simultaneous monitoring of voltage and ion concentration (pH or Ca^{2+}, respectively).

[00451] In another embodiment, the invention provides a cell expressing, either stably or transiently, at least one nucleic acid construct or vector encoding a microbial rhodopsin or a modified microbial rhodopsin described in the specification.

[00452] In another aspect, methods are provided for measuring a change in the membrane potential of at least one cell in a cellular circuit, the method comprising: measuring the level of fluorescence of the microbial rhodopsin or the modified microbial rhodopsin in at least one cell in a cellular circuit, wherein the at least one cell in a cellular circuit comprises a microbial rhodopsin or a modified microbial rhodopsin protein having reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived, wherein the microbial rhodopsin or the modified microbial rhodopsin protein localizes to a membrane of the cell, and wherein a change in the level of fluorescence is indicative of a change in the membrane potential across a membrane of the at least one cell in said cellular circuit. In some and in all aspects of the invention, the microbial rhodopsin targets the plasma membrane of the cell.

[00453] Another aspect provided herein is a high-throughput system for optically measuring membrane potential, the system comprising: (a) a plurality of engineered cells comprising on their membrane at least one modified microbial rhodopsin protein having reduced ion pumping activity compared to a natural microbial rhodopsin protein from which it is derived, and, (b) means for detecting fluorescence of the modified microbial rhodopsin protein.

[00454] In one embodiment of the cells or methods described above, the cell is a eukaryotic cell or a prokaryotic cell.

[00455] In one embodiment, the eukaryotic cell is a mammalian cell.

[00456] In another embodiment of the cell is a stem cell or a pluripotent cell, including an induced pluripotent cell or a stem cell.

[00457] In some embodiments, the cell is a progenitor cell, such as a neural progenitor or a cardiac progenitor cell.

[00458] In some embodiments, the cell is a differentiated cell, such as a neural cell or a cardiomyocyte.

[00459] In another embodiment of the cells or methods described above, the prokaryotic cell is a bacterial cell.

[00460] In one embodiment, the step of introducing comprises introducing a gene for the microbial rhodopsin or the modified rhodopsin protein into the cell.

[00461] In one and all aspects of the embodiments described herein, the cell is a eukaryotic cell. In one and all embodiments described herein, the cell does not naturally express a microbial rhodopsin.

[00462] In another embodiment of the cells or methods described above, the introducing is performed using a viral vector.

[00463] In another embodiment of the cells or methods described above, the introducing is performed using electroporation.

[00464] In other embodiments of the cells or methods described herein, the introducing is performed using lipofection, $CaPO_4$, lipid-mediated delivery (e.g., liposome, micelle etc.), transfection or transformation.

[00465] In another embodiment of the cells or methods described above, the introducing is performed in vivo into at least one cell in a subject.

[00466] In another embodiment of the cells or methods described above, the cell is genetically engineered to encode the modified microbial rhodopsin protein.

[00467] In another embodiment of the cells or methods described above, the cell is a stem cell.

[00468] In another embodiment of the cells or methods described above, the stem cell selected from the group consisting of an induced pluripotent cell, an embryonic stem cell, an adult stem cell, and a neuronal stem cell.

[00469] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein further comprises a moiety that produces an optical signal.

[00470] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein comprises an amino acid mutation compared to the natural microbial rhodopsin protein from which it is derived.

[00471] In some embodiments, the microbial rhodopsin is a proteorhodopsin.

[00472] In some embodiments, the microbial rhodopsin is Archaerhodopsin. In some embodiments, the Archaerhodopsin is Archaerhodopsin 3 (Arch3) or any of its homologues.

[00473] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein comprising a mutation is green-absorbing proteorhodopsin.

[00474] In another embodiment of the cells or methods described above, the mutation is selected from the group consisting of: D97N, E108Q, E142Q, and L217D.

[00475] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein comprising a mutation is blue-absorbing proteorhodopsin.

[00476] In another embodiment of the cells or methods described above, the mutation is D85N in bacteriorhodopsin or D99N in blue-absorbing proteorhodopsin.

[00477] In another embodiment of the cells or methods described above, the amino acid mutation is at residue V48, P49, Y57, L92, W86, I119, M145, W182, Y199, D212, and V213 in bacteriorhodopsin (e.g., a protein encoded by the nucleic acid sequence GenBank NC_010364.1, sequences 1082241-1083029, wherein the nucleotides 1082241is designated herein as nucleic acid residue 1 or GenBank accession sequence M11720.1) or the homologous mutations in another microbial rhodopsin.

[00478] In one embodiment, the microbial rhodopsin is archaerhodopsin, such as Arh-3 (see e.g., Chow, B.Y. et al., Nature 463:98-102 (2010)). In another embodiment, the archaerhodopsin Arh-3 comprises a D95N amino acid mutation or a mutation in a homologous location of another archaerhodopsin. Other achaerhodopsins include, but are not limited to archaerhodopsin-1 and -2 found in Halorubrum sp. (see, e.g., Enami et al. J Mol Biol. 2006 May 5;358(3):675-85. Epub 2006 Mar 3)

[00479] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein is localized to a subcellular compartment.

[00480] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein is localized to the plasma membrane, the mitochondrial inner membrane, or the mitochondrial outer membrane.

[00481] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein further comprises a targeting sequence.

[00482] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein comprises a Golgi export sequence, a membrane localization sequence, and/or an endoplasmic reticulum export sequence.

[00483] In another embodiment of the cells or methods described above, the membrane localization sequence comprises a C-terminal signaling sequence from the β2 nicotinic acetylcholine receptor.

[00484] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein further comprises a fluorescent moiety or a chromophore.

[00485] In another embodiment of the cells or methods described above, the fluorescence or optical signal is detected by fluorescence, spectral shift fluorescence resonance energy transfer (FRET), rhodopsin optical lock-in imaging (ROLI), Raman, or second harmonic generation (SHG).

[00486] In another embodiment of the cells or methods described above, the eukaryotic cell is a neuronal cell.

[00487] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein is expressed in the cell.

[00488] In another embodiment of the cells or methods described above, the modified microbial rhodopsin protein is expressed from a nucleic acid sequence encoding the modified microbial rhodopsin protein having a modified codon usage such that the codon usage is appropriate for the

eukaryotic cell but the amino acid sequence remains substantially similar to the modified microbial rhodopsin protein.

[00489] In another embodiment of the cells or methods described above, the bacterial cell is an antibiotic insensitive strain of bacteria.

[00490] In another embodiment of the cells or methods described above, the rhodopsin protein further comprises a moiety that produces an optical signal.

[00491] In another embodiment of the cells or methods described above, the moiety that produces an optical signal comprises a fluorescent moiety or a chromophore.

[00492] In another embodiment of the cells or methods described above, the construct further comprises a eukaryotic promoter.

[00493] In another embodiment of the cells or methods described above, the eukaryotic promoter is an inducible promoter, or a tissue-specific promoter.

[00494] In another embodiment of the cells or methods described above, the cellular circuit comprises at least two neuronal cells.

[00495] In another embodiment of the cells or methods described above, the ion pumping activity of the modified microbial rhodopsin protein is inhibited.

[00496] In another embodiment of the cells or methods described above, the modified microbial rhodopsin has no measurable ion pumping activity.

[00497] In another embodiment of the cells or methods described above, the candidate agent inhibits, increases, or reduces the ion channel activity.

EXAMPLES

[00498] Voltage is used by biological systems to convey information on both cellular and organismal scales. Traditional voltage probes rely on electrodes to make physical contact with the cell and can be used in limited numbers. Described herein is a new class of fluorescent proteins based on microbial rhodopsins, whose fluorescence is exquisitely sensitive to the membrane potential of a cell. The probes, have a far red fluorescent excitiation, a near infrared emission, a millisecond response time, and are extremely photostable.

EXAMPLE 1

Proteorhodopsin as a fluorescent sensor of protons

[00499] Proteorhodopsins are a large family of photoactive transmembrane proteins recently discovered in marine bacteria. Green-absorbing proteorhodopsin (GPR) is a light-driven proton pump that converts solar energy into a proton-motive force used by its host to power cellular machinery. The directional motion of a proton through GPR is accompanied by a series of dramatic color shifts in the protein. The approach of the study was to determine whether GPR could be run backward: could a

transmembrane potential drive a proton through the protein and thereby alter its color? Described herein is a protein-based colorimetric indicator of membrane potential using such an approach.

[00500] A covalently bound retinylidene is the visible chromophore of GPR. A Schiff base (SB) links the retinal to the ε-amino group of lysine 231. Early in the wild-type photocycle, a proton leaves the SB, causing a shift in the peak absorption from λ_{max} = 535 nm to λ_{max} = 421 nm. An increase in pH also induces this color shift and indicates that the SB has a $pK_a > 12$. It was reasoned that a change in membrane potential would alter the electrochemical potential of the proton on the SB, and thereby affect the pK_a. The Nernst equation indicates that a ΔV of 59 mV at the SB corresponds to a ΔpK_a of 1 pH unit. If the pK_a became lower than the ambient pH, the SB loses its proton and a color change would ensue. Electrochromic responses were shown to occur in bulk in the closely related protein bacteriorhodopsin when a point mutation was made to the SB counterion Asp85.

[00501] A mutation of the GPR counterion, Asp97Asn, was constructed. The mutant has been reported to not pump protons in response to light, and has a SB with pK_a = 9.9 which is much closer to physiological pH. The purified protein undergoes a visible color change between pH 8.4 and 10.4. Although the pK_a was still > 2 pH units above ambient, the protein was tested as a voltage sensor. This engineered protein is referred to herein as a Proteorhodopsin Optical Proton Sensor (PROPS).

[00502] Optical absorption is insufficiently sensitive to detect color changes in the small amount of PROPS in a single cell. Therefore, a different spectroscopic readout was sought. Surprisingly, PROPS expressed in *E. coli* showed clearly visible fluorescence (λ_{exc} = 633 nm, λ_{em} = 700 – 750 nm) localized to the membrane. Furthermore the fluorescence of purified PROPS vanished at high pH, indicating that only the protonated SB is fluorescent with a 633 nm excitation. *E. coli* – PROPS/+retinal, or +PROPS/-retinal exhibited 100-fold lower fluorescence than cells +PROPS/+retinal (SOM) confirming the emission is arising from the functional protein.

[00503] PROPS has several photophysical properties that are advantageous for imaging. The red excitation and near infrared emission fall in spectral bands of little background autofluorescence. At pH 7.4, purified PROPS has fluorescence quantum yield (QY) = 1.0×10^{-3}, while WT GPR has QY = 1.3×10^{-3}. The low QY of PROPS is partially offset by its remarkable photostability. At a laser intensity of 350 mW/cm^2, PROPS photobleaches to 50% of its initial intensity in > 30 minutes, while under the same measurement conditions the organic fluorophore Alexa™ 647 photobleaches to 50% in 24 s. PROPS constitutes a new class of far-red fluorescent protein with no homology to GFP.

PROPS is sensitive to the local concentration of protons

[00504] To test the sensitivity of PROPS to protons, the protein was simultaneously imaged with the pH sensitive dye, BCECF, inside intact *E. coli* treated with CCCP to equalize internal and external pH. A pH titration of the fluorescence of both fluorophores shows that PROPS is indeed sensitive to protons with the fluorescence decreasing at increasing pH.

[00505] To test the response of PROPS to voltage, *E. coli* membranes were rendered permeable to K^+ by treatment with EDTA and valinomycin and subjected to shocks of KCl using a homebuilt flow chamber. Intensity differences were calculated as a function of the imposed membrane voltage. PROPS shows a clear decrease in fluorescence upon a KCl up-shock consistent with the model that higher membrane voltage lowers the fluorescence from the protonated state. Using these measurements, a $\Delta F/F$ per 100 mV of 500% was calculated. These experiments confirm that PROPS is sensitive to a linear combination of the internal pH and voltage.

***E. coli* expressing PROPS have periodic flashes of fluorescence**

[00506] PROPS was expressed in *E. coli*, the cells were immobilized on a glass coverslip, and imaged in an inverted epifluorescence microscope while gently flowing minimal medium at pH 7 over the cells. Many cells exhibited cell-wide flashes in fluorescence in which the fluorescence increased by a factor of up to 8. The flashes occurred simultaneously (to within the 10 ms imaging resolution) and homogeneously over the extent of a cell. Flashes were uncorrelated between neighboring cells.

[00507] Within a nominally homogeneous population of cells in a single microscope field of view, a variety of temporal dynamics were observed. The most common flashes had a rise time of 350 ms, a duration of 1 s and a return to baseline over 1000 ms. Many cells flashed periodically, with a typical frequency of 0.2 Hz. Some cells occasionally had "slow flashes", lasting from 20 s to 40 s. The fast and slow flashes reached the same maximum intensity. A third motif was a "ringing pattern" in which the fluorescence oscillations became smaller in amplitude and higher in frequency until the cell settled at an intermediate intensity. Some cells were quiescent for many minutes, flashed once, and then returned to darkness; others had periods of quiescence punctuated by brief bursts of flashing. Some cells were permanently bright, and some were permanently dark. Four strains of *E. coli* showed blinks with two different plasmids encoding PROPS (arabinose or IPTG induction). Flashing occurred in cells immobilized via poly-L-lysine, cells left to settle on an uncoated coverslip, and cells immobilized on an agarose pad. Flashing was observed on two independent microscopic imaging systems.

[00508] It was sought to determine whether flashing was induced by the expression of PROPS and/or by the laser used in imaging. The same field of cells was monitored under increasing laser power over 2 orders of magnitude. The blinking was unchanged until a threshold intensity of 100 W/cm^2 at which point the blinking rose dramatically. The cause of the increased blinking is unclear. The sharp increase in blinking allows two regimes of PROPS (i) monitoring endogenous activity at low powers (< 50 W/cm^2) and (ii) enhancing activity at high powers (> 100 W/cm^2). To increase the fraction of cells undergoing blinking, the rest of the studies were conducted in the high power regime.

[00509] It was also observed that strain JY29 showed some "sub-threshold" flashes with rise and fall times as brief as 4 ms. This observation is significant because it establishes that PROPS responds to voltage fluctuations on a timescale comparable to the duration of a neuronal action potential (1ms).

At present it is not clear whether the observed 4ms timescale is set by the response speed of PROPS or by the intrinsic voltage dynamics in *E. coli.*

Flashes are due to swings in voltage, not pH

[00510] PROPS are sensitive to both voltage and pH, but the transient blinks in fluorescence could be a function of the internal pH (pH_i), the external pH (pH_o), and the membrane potential (ΔV). The study sought to determine this unknown response function, and furthermore to determine whether flashing was caused by changes in pH_i, ΔV, or both. Independent measures of these quantities were deemed necessary.

[00511] To measure pH_i, cells were incubated with EDTA and a fluorescent indicator of pH, BCECF-AM. This dye is taken up by the cells and converted to a fluorescent membrane-impermeable form. The fluorescence of the BCECF and of the PROPS was simultaneously measured. The BCECF fluorescence was largely constant as pH_o was varied from 6.5 – 9, consistent with earlier findings that *E. coli* maintain homeostasis of $pH_i \sim 7.8$ over this range of pH_o. The PROPS baseline (non-blinking) fluorescence was also largely constant over this range of pH_o, indicating that the SB in PROPS is not exposed to the extracellular medium. The ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was then added, which renders the cell membrane permeable to protons so that changes in pH_o induce corresponding changes in pH_i. Both the BCECF and the PROPS fluorescence changed much more in response to changed pH_o, indicating that PROPS is sensitive to pH_i. CCCP treated cells with BCECF responded to step changes in pH_o fast enough that if there were a change in pH_i during a flash, a change in BCECF fluorescence would be detectable.

[00512] A fresh sample of blinking cells was prepared with EDTA and BCECF and two-color movies were recorded showing simultaneous fluorescence of BCECF and PROPS (data not shown). These cells continued to flash in the PROPS channel, but the BCECF fluorescence remained constant to within measurement error. This indicates that there is little or no change in pH_i during a blink.

[00513] To correlate flashing with ΔV, PROPS fluorescence and flagellar rotation of *E. coli* strain JY29 were simultaneously observed. These cells have a sticky flagellum which binds to a glass coverslip, causing the cell body to rotate at an angular velocity proportional to the cellular PMF. Strain JY29 lacks CheY and thus its flagellar motor does not reverse direction. Flashes were associated with slowing or pauses of the rotation indicating that blinks occur as a lowering of the cellular PMF. Since pH_o and pH_i remain constant, the membrane potential must be reduced to account for the change in PMF.

Blinks are sensitive to pH, benzoate, and $[Na^+]$ and $[K^+]$.

[00514] Flashing cells were subjected to chemical perturbations in an effort to deduce the mechanism of the voltage changes. In unstirred medium in a sealed chamber, the flashing ceased over

~ 15 minutes; but re-started upon addition of freshly oxygenated medium. Removal of oxygen from the medium using Oxyrase™ also reversibly eliminated flashing. From these experiments it was concluded that flashing requires aerobic respiration. Under continuous gentle flow of aerated minimal medium, cells flashed continuously for > 1 hr. Cells stored in minimal media for several days at 4 °C started flashing when warmed to room temperature.

[00515] Flashing was highly sensitive to pH_o, occurring only between pH 7 and 9. Flashing frequencies increased and on-time decreased at lower pH. Flashing was more likely to be periodic at lower pH. Extremes of pH (< 6 or > 9.5) irreversibly eliminated flashing, causing cells to enter the bright state in an abrupt transition. Addition of CCCP (20 μg/mL) rapidly and irreversibly eliminated the blinking, causing all cells to become bright.

[00516] Ionic shocks were tested to determine the ions conducted during the voltage swing. Cells in buffer containing just Na^+, K^+, Cl^-, and PO_4 were able to blink for > 30 minutes at pH 7.5. Removal of just Na^+ or K^+ did not immediately stop blinking, but removal of both ions caused dramatic fluorescence changes. Likewise, a 2 M KCl shock induced dramatic effects, but a 2 M NaCl shock did not. The evidence does not point to specific ions, but rather a channel that is capable of transporting multiple types of ions. Further tests on the mechanisms of blinking and efflux are being conducted with genetic knockouts.

[00517] The development of PROPS represents an important step in molecular probes. With the advent of the optogenetic tools for controlling neural activity based on channel rhodopsin II and halorhodopsin, microbial rhodopsins have gained interest as tools for neuroscience. PROPS adds an important tool in neuroscience through its ability to probe electrical activity with light. The far red shifted excitation and emission spectra of PROPS can combine easily with current efforts of channel rhodopsin to provide fully optical control and read-out of membrane potentials.

[00518] Using the unique capabilities of PROPS spontaneous electrical activity in respirating *E. coli* was discovered. The discrete nature of the spikes is reminiscent of localization of the Crz1 protein to the nucleus of yeast during Ca^{++} adaption, although the localization on yeast occurs on a much slower timescale (minutes rather than seconds.) The biological significance of electrical spiking in *E. coli* is at present unclear.

[00519] PROPS can also be used to determine (i) the molecular components and mechanisms behind bacterial blinking , (ii) the biological role that each of the three types of blinks serve, (iii) other cellular behaviors, and (iv) blinking patterns of other bacterial strains. In one embodiment, GPR is engineered to have a higher fluorescent quantum yield. GPR, its mutants, and other microbial rhodopsins are a promising family of fluorescent indicators of membrane potential. In one embodiment, PROPS and its variants may be used to monitor changes in membrane potential in other types of electrically active cells, such as neurons.

EXAMPLE 2: Arch 3 can be run backward and thereby provide a voltage sensor

[00520] Archaerhodopsin 3 (Arch 3) from Halorubrum sodomense is a light-driven outward proton pump, capturing solar energy for its host (Ihara, K. et al. Evolution of the archaeal rhodopsins: evolution rate changes by gene duplication and functional differentiation. J. Mol. Biol. 285, 163-174 (1999)). Recently Arch 3 was expressed in mammalian neurons, wherein it enabled optical silencing of neural activity, and was shown to be minimally perturbative to endogenous function in the dark (Chow, B. Y. et al. High-performance genetically targetable optical neural silencing by light-driven proton pumps. Nature 463, 98-102 (2010)). We have now demonstrated that Arch 3 can be run backward: that a membrane potential can alter the optical properties of the protein, and thereby provide a voltage sensor that function through a mechanism similar to PROPS.

[00521] At neutral pH Arch 3 was pink, but at high pH the protein turned yellow (Fig. 8A), with a pKa for the transition of 10.1. Based on homology to other microbial rhodopsins (Lanyi, J. K. Bacteriorhodopsin. Annu. Rev. Physiol. 66, 665-688 (2004); Friedrich, T. et al. Proteorhodopsin is a light-driven proton pump with variable vectoriality. J. Mol. Biol. 321, 821-838 (2002)), we attributed the pH-induced color change to deprotonation of the Schiff Base (SB) which links the retinal chromophore to the protein core (Dioumaev, A. K. et al. Proton transfers in the photochemical reaction cycle of proteorhodopsin. Biochemistry 41, 5348-5358 (2002)). We reasoned that a change in membrane potential might change the local electrochemical potential of the proton at the SB, tipping the acid-base equilibrium and inducing a similar color shift (Fig. 6A). This mechanism of voltage-induced color shift has previously been reported in dried films of bacteriorhodopsin (Kolodner, P., Lukashev, E. P., Ching, Y. & Rousseau, D. L. Electric-field-induced Schiff-base deprotonation in D85N mutant bacteriorhodopsin. Proc. Nat. Acad. Sci. U. S. A. 93, 11618-11621 (1996)), and formed the basis of voltage sensitivity in PROPS (Kralj, J. M., Hochbaum, D. R., Douglass, A. D. & Cohen, A. E. Electrical spiking in Escherichia coli probed with a fluorescent voltage indicating protein. Science 333, 345-348 (2011)).

[00522] Changes in optical absorption would be challenging to detect in a single cell, due to the small quantity of protein available. However, most microbial rhodopsins are weakly fluorescent (Lenz, M. O. et al. First steps of retinal photoisomerization in proteorhodopsin. Biophys. J. 91, 255-262 (2006)), so we characterized purified Arch 3 as a prospective fluorescent indicator (Table 6). At neutral pH, Arch emitted far red fluorescence (λem = 687 nm), while at high pH Arch was not fluorescent (Fig. 6B, Fig. 17). The fluorescence quantum yield of Arch was low (9 × 10-4) but the photostability was comparable to members of the GFP family (Shaner, N. C., Steinbach, P. A. & Tsien, R. Y. A guide to choosing fluorescent proteins. Nat. Meth. 2, 905 (2005)), yielding approximately 25% as many photons prior to photobleaching as eGFP. The broad absorption peak enabled excitation at λ = 640 nm, a wavelength where few other cellular components absorb, and the far red emission occurred in a spectral region of little background autofluorescence.

[00523] Fluorescence of Arch 3 in HEK 293 cells was readily imaged in an inverted fluorescence microscope with red illumination (λ = 640 nm, I = 540 W/cm^2), a high numerical aperture objective, a Cy5 filter set, and an EMCCD camera. The cells exhibited fluorescence predominantly localized to the plasma membrane (Fig. 6C). Cells not expressing Arch were not fluorescent. Cells showed 17% photobleaching over a continuous 10-minute exposure, and retained normal morphology during this interval.

[00524] The fluorescence of HEK cells expressing Arch was highly sensitive to membrane potential, as determined via whole-cell voltage clamp. We developed an algorithm to combine pixel intensities in a weighted sum such that the output, was a nearly optimal estimate of membrane potential V determined by conventional electrophysiology. Fig. 6C shows an example of a pixel-weight matrix, indicating that the voltage-sensitive protein was localized to the cell membrane; intracellular Arch contributed fluorescence but no voltage-dependent signal. The fluorescence increased by a factor of 2 between -150 mV and +150 mV, with a nearly linear response throughout this range (Fig. 6D). The response of fluorescence to a step in membrane potential occurred within the 500 μs time resolution of our imaging system on both the rising and falling edge (Fig. 6E). Application of a sinusoidally varying membrane potential led to sinusoidally varying fluorescence; at f = 1 kHz, the fluorescence oscillations retained 55% of their low-frequency amplitude (Fig. 18). Arch reported voltage steps as small as 10 mV, with an accuracy of 625 μV/(Hz)$^{1/2}$ over timescales < 12 s (Fig. 19). Over longer timescales laser power fluctuations and cell motion degraded the accuracy.

[00525] We tested Arch 3 as a voltage indicator in cultured rat hippocampal neurons, using viral delivery. Neurons expressing Arch showed voltage-dependent changes in fluorescence localized to the cell membrane. Under whole cell current clamp, cells exhibited spiking upon injection of current pulses of 200 pA. Individual spikes were accompanied by clearly identifiable increases of fluorescence (Fig. 7A). At a 2 kHz image acquisition rate, the signal-to-noise ratio in the fluorescence (spike amplitude:baseline noise) was 10.5. A spike-finding algorithm correctly identified 99.6% of the spikes (based on comparison to simultaneously recorded membrane potential), with a false-positive rate of 0.7% (n = 269 spikes) (Fig. 7B). Single cells were observed for up to 4 minutes of cumulative exposure, with no detectable change in resting potential or spike frequency.

[00526] We imaged the dynamics of action potentials with sub-cellular resolution by averaging multiple temporally registered movies of single spikes (Fig. 7C). In and near the soma, the optically determined waveform of the action potential was uniform and matched the electrically recorded waveform. However in very thin processes the peak of the action potential lagged by up to 1 ms (Fig. 7D). These observations are consistent with multiple-patch recordings on single neurons (Stuart, G. J. & Sakmann, B. Active propagation of somatic action potentials into neocortical pyramidal cell dendrites. Nature 367, 69-72 (1994)); but such recordings are technically demanding and only probe

the variation in membrane potential at a small number of points. The present results suggest that Arch may be used to map intracellular dynamics of action potentials.

[00527] In the absence of added retinal, neurons expressing Arch showed clearly identifiable fluorescence flashes accompanying individual spikes (Fig. 20), indicating that neurons contained sufficient endogenous retinal to populate some of the protein. Experiments with Arch and other microbial rhodopsins in vivo have shown that endogenous retinal is sufficient for optogenetic control of neural activity 25. Thus Arch may function as a voltage indicator in vivo without exogenous retinal.

[00528] Illumination at 640 nm was far from the peak of the Arch absorption spectrum (λ = 558 nm), but the imaging laser nonetheless induced photocurrents of 10 – 20 pA in HEK cells expressing Arch 3 (Fig. 8A). We sought to develop a mutant which did not perturb the membrane potential, yet which maintained voltage sensitivity. The mutation D85N in bacteriorhodopsin eliminated proton pumping 26, so we introduced the homologous mutation, D95N, into Arch 3. This mutation eliminated the photocurrent (Fig. 8A) and shifted several other photophysical properties of importance to voltage sensing (Table 6, Fig. 8, Fig. 21). Movies of the fluorescence response to changes in membrane potential were also taken and showed the result visually. Arch D95N was more sensitive and brighter than Arch 3 WT, but had a slower response (Fig. 8A-8C).

[00529] Under illumination conditions typically used for imaging neural activity (I = 1800 W/cm2 in total internal reflection (TIR) mode), the light-induced outward photocurrent was typically 10 pA in neurons expressing Arch 3 WT. Under current-clamp conditions this photocurrent shifted the resting potential of the neurons by up to -20 mV. For neurons near their activation threshold, this photocurrent could suppress firing (Fig. 9A), so we explored the non-pumping variant D95N as a voltage indicator in neurons. Illumination of Arch D95N did not perturb membrane potential in neurons (Fig. 9B).

[00530] Arch 3 D95N reported neuronal action potentials on a single-trial basis (Fig. 9C). The response to a depolarizing current pulse was dominated by the slow component of the step response; yet the fast component of the response was sufficient to indicate action potentials.

Protein purification

[00531] A lentiviral backbone plasmid encoding Arch 3-EGFP (FCK:Arch 3-EGFP) was a generous gift from Dr. Edward Boyden (MIT). The gene for the rhodopsin was cloned into pet28b vector using the restriction sites EcoRI and NcoI. The D95N mutation was created using the QuikChangeII kit™ (Agilent) using the forward primer (5'-TTATGCCAGGTACGCCAACTGGCTGTTTACCAC) (SEQ ID NO: 22) and the reverse primer (5'-GTGGTAAACAGCCAGTTGGCGTACCTGGCATAA) (SEQ ID NO: 23).

[00532] Arch and its D95N mutant were expressed and purified from *E. coli*, following Bergo, V., et al. (Conformational changes detected in a sensory rhodopsin II-transducer complex. J. Biol. Chem.

278, 36556-36562 (2003)). Briefly, *E. coli* (strain BL21, pet28b plasmid) was grown in 1 L of LB with 100 μg/mL kanamycin, to an O.D 600 of 0.4 at 37 °C. All-*trans* retinal (5 μM) and inducer (IPTG 0.5 mM) were added and cells were grown for an additional 3.5 hours in the dark. Cells were harvested by centrifugation and resuspended in 50 mM Tris, 2 mM $MgCl_2$ at pH 7.3 and lysed with a tip sonicator for 5 minutes. The lysate was centrifuged and the pellet was resuspended in PBS supplemented with 1.5% dodecyl maltoside (DM). The mixture was homogenized with a glass/teflon Potter Elvehjem™ homogenizer and centrifuged again. The supernatant was concentrated and washed using a centricon 30k MWCO filter to a final volume of 3 mL.

Spectroscopic characterization of Arch WT and D95N

[00533] The absorption spectra of purified Arch 3 WT and D95N were determined using an Ocean Optics™ USB4000 spectrometer with a DT-MINI-2-GS light source. Absorption spectra, for Arch 3 WT and D95N, were measured as a function of pH between pH 6 and 11. To determine the fluorescence emission spectra, proteins in a quartz cuvette were illuminated with the uncollimated beam of a 100 mW, 532 nm laser (Dragon Lasers, 532GLM100) or a 25 mW, 633 nm HeNe laser (Spectra-Physics). Scattered laser light was blocked with a 532 nm Raman notch filter (Omega Optical, XR03) or a 710/100 emission filter (Chroma), and fluorescence was collected perpendicular to the illumination with a 1000 micron fiber, which passed the light to an Ocean Optics QE65000 spectrometer. Spectra were integrated for 2 seconds.

[00534] The fluorescence quantum yields of Arch 3 WT and D95N were determined by comparing the integrated emission intensity to emission of a sample of the dye ALEXA FLUOR® 647 Conjugate. Briefly, the concentrations of micromolar solutions of dye and protein were determined using a visible absorption spectrum. We used the extinction coefficients of 270,000 $M^{-1}cm^{-1}$ for ALEXA FLUOR® 647 and 63,000 $M^{-1}cm^{-1}$ for Arch 3 WT and D95N, assuming that these microbial rhodopsins have the same extinction coefficient as bacteriorhodopsin. The dye solution was then diluted 1:1000 to yield a solution with comparable fluorescence emission to the Arch 3. The fluorescence emission spectra of dye and protein samples were measured with 633 nm excitation. The quantum yield was then determined by the formula

[00535] $$QY_{Arch} = \frac{Fl_{Arch}}{Fl_{Alexa}} * \frac{\varepsilon_{Alexa}}{\varepsilon_{Arch}} * \frac{c_{Alexa}}{c_{Arch}} * QY_{Alexa}$$

[00536] where *Fl* is the integrated fluorescence from 660 to 760 nm, ε is the extinction coefficient at 633 nm and *c* is the concentration.

Relative photostability of Arch 3 and eGFP

[00537] To perform a direct comparison of photostability of Arch 3 and eGFP we studied the photobleaching of the Arch 3-eGFP fusion. This strategy guaranteed a 1:1 stoichiometry of the two

fluorophores, simplifying the analysis. The experiments were performed on permeabilized cells, in the microscope, with video recording as the cells photobleached. We first recorded a movie of photobleaching of Arch 3 under 640 nm illumination; then on the same field of view we recorded photobleaching of eGFP under 488 nm illumination, with illumination intensity adjusted to yield approximately the same initial count rate as for Arch 3. Fluorescence background levels were obtained from nearby protein-free regions of each movie and were subtracted from the intensity of the protein-containing regions. The area under each photobleaching timetrace was calculated, yielding an estimate of the total number of detected photons from each fluorophore. The eGFP emission (λ_{max} = 509 nm) and the Arch emission (λ_{max} = 687 nm) were collected through different emission filters, so the raw counts were corrected for the transmission spectra of the filters and the wavelength-dependent quantum yield of the EMCCD camera. The result was that the relative number of photons emitted prior to photobleaching for eGFP:Arch 3 WT was 3.9:1, and for eGFP:Arch D95N this ratio was 10:1.

HEK cell culture

[00538] HEK-293 cells were grown at 37 °C, 5% CO_2, in DMEM supplemented with 10% FBS and penicillin-streptomycin. Plasmids were transfected using LIPOFECTAMINE™ and PLUS reagent™ (Invitrogen) following the manufacturer's instructions, and assayed between 48-72 hours later. The day before recording, cells were re-plated onto glass-bottom dishes (MatTek) at a density of ~5000 cells/cm^2.

[00539] The concentration of endogenous retinal in the HEK cells was not known, so the cells were supplemented with retinal by diluting stock retinal solutions (40 mM, DMSO) in growth medium to a final concentration of 5 μM, and then placing the cells back in the incubator for 1 – 3 hours. All imaging and electrophysiology were performed in Tyrode's buffer (containing, in mM: 125 NaCl, 2 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 30 glucose pH 7.3, and adjusted to 305-310 mOsm with sucrose). Only HEK cells having reversal potentials between -10 and -40 mV were included in the analysis.

Microscopy

[00540] Simultaneous fluorescence and whole-cell patch clamp recordings were acquired on a home-built, inverted epifluorescence microscope at room temperature. A diode laser operating at 100 mW, 640 nm (CrystaLaser, DL638-100-O) provided wide-field illumination at an intensity typically between 500 – 2000 W/cm^2. To minimize background signal from out-of-focus debris, illumination was often performed in through-the-objective total internal reflection fluorescence (TIRF) mode. Fluorescence emission was collected using a 60x, 1.45 NA oil immersion objective (Olympus), and separated from scattered excitation using a 660 – 760 nm bandpass emission filter (Chroma). For fast imaging of dynamic fluorescence responses and action potentials, images were acquired on an Andor

iXon$^+$ 860 camera™ operating at up to 2,000 frames/s (using a small region of interest and pixel binning). Slower images with higher spatial resolution were acquired on an Andor iXon$^+$ 897 EMCCD. Custom software written in LabView (National Instruments) was used to synchronize illumination, collection of images, recording of membrane potential and cell current, and application of electrical stimuli to the cell.

Electrophysiology

[00541] Filamented glass micropipettes (WPI) were pulled to a tip resistance of 3-10 MΩ, fire polished, and filled with internal solution (containing, in mM: 125 Potassium gluconate, 8 NaCl, 0.6 $MgCl_2$, 0.1 $CaCl_2$, 1 EGTA, 10 HEPES, 4 Mg-ATP, 0.4 Na-GTP, pH 7.3; adjusted to 295 mOsm with sucrose). The micropipettes were positioned with a Burleigh PCS™ 5000 micromanipulator. Whole-cell, voltage clamp recordings were acquired using an AxoPatch 200B™ amplifier (Molecular Devices), filtered at 2 kHz with the internal Bessel™ filter, and digitized with a National Instruments PCIE-6323™ acquisition board at 10 kHz. Ambient 60 Hz noise was removed using a HumBug Noise Eliminator™ (AutoMate Scientific). For experiments requiring rapid modulation of transmembrane potential, series resistance and whole-cell capacitance were predicted to 95% and corrected to ~50%. Electrical stimuli were generated using the PCIE-6323 acquisition board and sent to the AXOPATCH™, which then applied these signals in either constant current or constant voltage mode.

[00542] Measurements of photocurrents were performed on HEK cells held in voltage clamp at 0 mV while being exposed to brief (200 ms) pulses of illumination at 640 nm at an intensity of 1800 W/cm^2.

[00543] All experiments were performed at room temperature.

Estimates of membrane potentials from fluorescence images

[00544] A common practice in characterizing fluorescent voltage indicators is to report a value of ΔF/F per 100 mV of membrane potential. We feel that this parameter is of limited use, for several reasons. First, the value of ΔF/F is highly sensitive to the method of background subtraction, particularly for indicators in which F approaches zero at some voltage. Second, ΔF/F contains no information about signal-to-noise ratio: one indicator may have small values of F and of ΔF and another may have large values, but the ratio ΔF/F might be the same. Third, the ratio ΔF/F contains no information about the temporal stability of the fluorescence. Fluctuations may arise due to intracellular transport, photobleaching, or other photophysics.

[00545] We therefore sought a measure of the performance of a voltage indicator which reported the information content of the fluorescence signal. We sought an algorithm to infer membrane potential from a series of fluorescence images. We used the accuracy with which the estimated membrane potential matched the true membrane potential (as reported by patch clamp recording) as a measure of indicator performance.

[00546] The estimated membrane potential, $\hat{V}_{FL}(t)$, was determined from the fluorescence in two steps. First we trained a model relating membrane potential to fluorescence at each pixel. We used the highly simplified model that the fluorescence signal, $S_i(t)$, at pixel i and time t, is given by:

[00547] $$S_i(t) = a_i + b_i V(t) + \varepsilon_i(t), \qquad [S1]$$

[00548] where a_i and b_i are position-dependent but time-independent constants, the membrane potential $V(t)$ is time-dependent but position independent, and $\varepsilon_i(t)$ is spatially and temporally uncorrelated Gaussian white noise with pixel-dependent variance:

[00549] $$\left\langle \varepsilon_i(t_1)\varepsilon_j(t_2)\right\rangle = \sigma_i^2 \delta_{i,j}\delta(t_1 - t_2),$$

[00550] where $\langle\ \rangle$ indicates an average over time.

[00551] This model neglects nonlinearity in the fluorescence response to voltage, finite response time of the protein to a change in voltage, photobleaching, cell-motion or stage drift, and the fact that if $\varepsilon_i(t)$ is dominated by shot-noise then its variance should be proportional to $S_i(t)$, and its distribution should be Poisson, not Gaussian. Despite these simplifications, the model of Eq. S1 provided good estimates of membrane potential when calibrated from the same dataset to which it was applied.

[00552] The pixel-specific parameters in Eq. 1 are determined by a least-squares procedure, as follows. We define the deviations from the mean fluorescence and mean voltage by

[00553] $$\delta S_i(t) = S_i(t) - \left\langle S_i(t)\right\rangle$$

[00554] $$\delta V(t) = V(t) - \left\langle V(t)\right\rangle.$$

[00555] Then the estimate for the slope $\hat{b}_i$ is:

[00556] $$\hat{b}_i = \frac{\left\langle \delta S_i \delta V\right\rangle}{\left\langle \delta V^2\right\rangle}$$, and the offset is:

[00557] $$\hat{a}_i = \left\langle S_i\right\rangle - \hat{b}_i\left\langle V\right\rangle.$$

[00558] A pixel-by-pixel estimate of the voltage is formed from:

[00559] $$\hat{V}_i(t) = \frac{S_i(t)}{\hat{b}_i} - \frac{\hat{a}_i}{\hat{b}_i}.$$

[00560] The accuracy of this estimate is measured by

[00561] $$\varsigma_i^2 = \left\langle \left(\hat{V}_i(t) - V(t)\right)^2\right\rangle.$$

[00562] A maximum likelihood weight matrix is defined by:

[00563] $$w_i \equiv \frac{1/\varsigma_i^2}{\sum_i 1/\varsigma_i^2}.$$ [S2]

[00564] This weight matrix favors pixels whose fluorescence is an accurate estimator of voltage in the training set.

[00565] To estimate the membrane potential, the pixel-by-pixel estimates are combined according to:

[00566] $$\hat{V}_{FL}(t) = \sum_i w_i \hat{V}_i(t)$$ [S3]

[00567] Within the approximations underlying Eq. S1, Eq. S3 is the maximum likelihood estimate of $V(t)$.

Ramp and step-response of Arch 3 WT and D95N

[00568] To measure fluorescence as a function of membrane potential, a triangle wave was applied, with amplitude from -150 mV to +150 mV and period 12 s, with video recording at 100 ms per frame. A pixel weight matrix was calculated according to Eq. S2 and applied to the movie images to generate a fluorescence number for each frame. These fluorescence values were divided by their minimum value (at V = -150 mV). The result is plotted as a function of V in Figs. 7 and 8. This procedure preferentially weighted data from pixels at the cell membrane, but did not entail any background subtraction. Comparable results were obtained by manually selecting pixels corresponding to a region of plasma membrane, and plotting their intensity as a function of V, without background subtraction. Background subtraction from the raw fluorescence would have yielded considerably larger values of $\Delta F/F$.

[00569] The step response was measured in a similar manner, except that test waveforms consisted of a series of voltage pulses, from -70 mV to +30 mV with duration 300 ms and period 1 s. Cells were subjected to 20 repetitions of the waveform, and the fluorescence response was averaged over all iterations.

Frequency-dependent response functions of Arch 3 WT and D95N

[00570] Test waveforms consisted of a concatenated series of sine waves, each of duration 2 s, amplitude 100 mV, zero mean, and frequencies uniformly spaced on a logarithmic scale between 1 Hz and 1 kHz (31 frequencies total). The waveforms were discretized at 10 kHz and applied to the cell, while fluorescence movies were acquired at a frame rate of 2 kHz.

[00571] The model parameters for extracting $\hat{V}_{FL}(t)$ were calculated from the fluorescence response to low frequency voltages. These parameters were then used to calculate an estimated voltage at all frequencies.

[00572] The applied voltage was downsampled to 2 kHz to mimic the response of a voltage indicator with instantaneous response. For each applied frequency, the Fourier transform of $\hat{V}_{FL}(t)$ was calculated and divided by the Fourier transform of the downsampled $V(t)$. The amplitude of this ratio determined the response sensitivity. It was crucial to properly compensate pipette resistance and cell membrane capacitance to obtain accurate response spectra. Control experiments on cells expressing membrane-bound GFP showed no voltage-dependent fluorescence.

[00573] The power spectrum of $\hat{V}_{FL}(t)$ under constant $V = 0$ was also measured to enable calculations of signal-to-noise ratio for any applied $V(t)$.

Molecular biology and virus production

[00574] Plasmids encoding Arch-EGFP (FCK:Arch-EGFP) were either used directly for experiments in HEK cells, or first used to produce VSVg-pseudotyped virus according to published methods (Chow, B. Y. et al. High-performance genetically targetable optical neural silencing by light-driven proton pumps. Nature 463, 98-102 (2010)). For pseudotyping, HEK-293 cells were co-transfected with pDelta 8.74, VSVg, and either of the Arch backbone plasmids using LIPOFECTAMINE and PLUS reagent (Invitrogen). Viral supernatants were collected 48 hours later and filtered using a 0.45 μm membrane. The virus medium was used to infect neurons without further concentration.

[00575] The D95N mutation was introduced using the QUICKCHANGE® kit (Stratagene), according to the manufacturer's instructions using the same primers as the *E. coli* plasmid.

Neuronal cell culture

[00576] E18 rat hippocampi were purchased from BrainBits and mechanically dissociated in the presence of 1 mg/mL papain (Worthington) before plating at 5,000 – 30,000 cells per dish on poly-L-lysine and Matrigel™-coated (BD Biosciences) glass-bottom dishes. Cells were incubated in N+ medium (100 mL Neurobasal medium, 2 mL B27 supplement, 0.5 mM glutamine, 25 μM glutamate, penicillin-streptomycin) for 3 hours. An additional 300 μL virus medium was added to the cells and incubated overnight, then brought to a final volume of 2 mL N+ medium. After two days, cells were fed with 1.5 mL N+ medium. Cells were fed with 1 mL N+ medium without glutamate at 4 DIV, and fed 1 mL every 3-4 days after. Cells were allowed to grow until 10-14 DIV at which point they were used for experiments.

[00577] Whole-cell current clamp recordings were obtained from mature neurons under the same conditions used for HEK cells recordings. Series resistance and pipette capacitance were corrected. Only neurons having resting potentials between -50 and -70 mV were used in the analysis.

Spike sorting

[00578] A spike identification algorithm was developed that could be applied either to electrically recorded $V(t)$ or to optically determined $\hat{V}(t)$. The input trace was convolved with a reference spike of duration 10 ms. Sections of the convolved waveform that crossed a user-defined threshold were identified as putative spikes. Multiple spikes that fell within 10 ms (a consequence of noise-induced glitches near threshold) were clustered and identified as one.

[00579] **Table 6** shows **Optical and electrical response of Arch 3 WT and Arch D95N.** [(1)] Excitation at $\lambda = 532$ nm. [(2)] Absorption spectra calibrated assuming the same peak extinction coefficient as Bacteriorhodopsin, 63,000 M^{-1} cm^{-1} (Ref. [32]). [(3)] Determined via comparison to Alexa 647 with excitation at $\lambda = 633$ nm. [(4)] Measured in a 1:1 fusion with eGFP. [(5)] Determined via singular

value decomposition on absorption spectra. [6] Determined from step-response. Arch D95N has a minor component of its response (20%) that is fast (< 0.5 ms). [7] $\hat{V}_{FL}$ is the membrane potential estimated from fluorescence. Noise determined at frequencies $f \geq 0.1$ Hz in HEK cells.

Table 6

	λ_{max} abs (nm)	λ_{max} em[1] (nm)	ε_{633} [2] ($M^{-1}cm^{-1}$)	QY [3]	Photostability relative to eGFP[4]	pK_a of SB [5]	$\tau_{response}$ [6] (ms)	Noise in $\hat{V}_{FL}$ [7] ($\mu V/Hz^{1/2}$)	Photo-current
Arch WT	558	687	6,300	9×10^{-4}	0.25	10.1	< 0.5	625	yes
Arch D95N	585	687	37,500	4×10^{-4}	0.1	8.9	41	260	no

What is claimed is:

1. A method for measuring the membrane potential in a cell expressing a nucleic acid encoding a microbial rhodopsin protein, the method comprising the steps of:

a. exciting, *in vitro*, at least one cell comprising a nucleic acid encoding one of SEQ ID NOs: 67, 69, 71, or 73, with light of at least one wavelength; and

b. detecting, *in vitro*, at least one optical signal from the at least one cell, wherein the level of fluorescence emitted by the at least one cell compared to a reference is indicative of the membrane potential of the cell.

2. The method of claim 1, wherein the at least one wavelength is a wavelength between 594-645 nm.

3. The method of any one of claims 1 or 2, wherein the cell is a prokaryotic cell.

4. The method of any one of claims 1 or 2, wherein the cell is a eukaryotic cell.

5. The method of claim 4, wherein the eukaryotic cell is a mammalian cell.

6. The method of claim 4, wherein the eukaryotic cell is a stem cell, a pluripotent cell, or a progenitor cell.

7. The method of claim 4, wherein the eukaryotic cell is an induced pluripotent cell.

8. The method of claim 4, wherein the eukaryotic cell is a neuron.

9. The method of claim 4, wherein the eukaryotic cell is a cardiomyocyte.

10. The method of any one of claims 1 to 9 further comprising a step of transfecting, *in vitro*, the at least one cell with a vector comprising the nucleic acid encoding the microbial rhodopsin protein.

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11. The method of any one of claims 1 to 10, wherein the nucleic acid encoding the microbial rhodopsin protein is operably linked to a cell-type specific promoter.

12. The method of any one of claims 1 to 11, wherein the nucleic acid encoding the microbial rhodopsin protein is operably linked to a membrane-targeting nucleic acid sequence.

13. The method of claim 12, wherein the membrane-targeting nucleic acid sequence is a plasma membrane targeting nucleic acid sequence.

14. The method of claim 12, wherein the membrane-targeting nucleic acid sequence is a subcellular compartment-targeting nucleic acid sequence.

15. The method of claim 14, wherein the subcellular compartment is selected from a mitochondrial membrane, an endoplasmic reticulum, a sarcoplastic reticulum, a synaptic vesicle, an endosome, and a phagosome.

16. The method of any one of claims 1 to 15, wherein the nucleic acid encoding the microbial rhodopsin protein is operably linked to a nucleic acid encoding at least one additional fluorescent protein or a chromophore.

17. The method of claim 16, wherein the at least one additional fluorescent protein is a fluorescent protein capable of indicating the ion concentration in the cell.

18. The method of claim 17, wherein the at least one additional fluorescent protein capable of indicating ion concentration is a calcium indicator.

19. The method of claim 17, wherein the at least one additional fluorescent protein capable of indicating ion concentration is a pH indicator.

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20. The method of claim 16, wherein the at least one additional fluorescent protein is capable of undergoing nonradiative fluorescence resonance energy transfer to the microbial rhodopsin protein, with a rate of energy transfer dependent on the membrane potential of the cell.

21. The method of claim 16, wherein the at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

22. The method of any one of claims 16 to 21, wherein brightness of the at least one additional fluorescent protein is insensitive to membrane potential and local chemical environment.

23. The method of any one of claims 16 to 22 further comprising steps of exciting, *in vitro*, the at least one cell with light of at least a first and a second wavelength; and detecting, *in vitro*, at least the first optical signal resulting from the excitation with the first wavelength and the second optical signal resulting from the excitation with the second wavelength from the at least one cell.

24. The method of claim 23, wherein the at least second wavelength is between 447-594 nm.

25. The method of any one of claims 22 to 24 further comprising a step of calculating the ratio of the fluorescence emission from the microbial rhodopsin to the fluorescence emission of the at least one additional fluorescent protein to obtain a measurement of membrane potential independent of variation in expression level.

26. The method of any one of claims 1 to 25 further comprising the step of exposing, *in vitro*, the at least one cell to a stimulus capable of, or suspected of being capable of, changing membrane potential.

27. The method of claim 26, wherein the stimulus is a candidate agent.

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28. The method of claim 26, wherein the stimulus is a change to the composition of the cell culture medium.

29. The method of claim 26, wherein the stimulus is an electrical current.

30. The method of any one of claims 1 to 29 further comprising the step of measuring, *in vitro*, the at least one optical signal at a first and at least at a second time point.

31. The method of any one of claims 26-29 further comprising the step of measuring, *in vitro*, the at least one optical signal at a first and at least at a second time point, wherein the first time point is before exposing the at least one cell to the stimulus and the at least second time point is after exposing the at least one cell to the stimulus.

32. An isolated and purified nucleic acid encoding a protein defined by one of SEQ ID NOs: 67, 69, 71, or 73, wherein the isolated and purified nucleic acid is operably linked to a nucleic acid encoding at least one additional fluorescent protein or a chromophore.

33. The isolated and purified nucleic acid of claim 32, wherein the at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

34. The isolated and purified nucleic acid of claim 32, wherein the at least one additional fluorescent protein is a fluorescent protein capable of indicating ion concentration in a cell.

35. The isolated and purified nucleic acid of claim 34, wherein the at least one additional fluorescent protein capable of indicating ion concentration is a calcium indicator.

36. The isolated and purified nucleic acid of claim 34, wherein the at least one additional fluorescent protein capable of indicating ion concentration is a pH indicator.

37. The isolated and purified nucleic acid according to any one of claims 32 to 33, wherein the at least one additional fluorescent protein is capable of undergoing nonradiative fluorescence

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resonance energy transfer to the protein defined by one of SEQ ID NOs: 67, 69, 71, or 73, with a rate of energy transfer dependent on a membrane potential of a cell.

38. A vector comprising the isolated and purified nucleic acid of any one of claims 32 to 37.

39. The vector of claim 38, wherein the vector is a viral vector.

40. The vector of claim 39, wherein the viral vector is a lentiviral vector.

41. The vector of claim 39, wherein the viral vector is an adeno-associated viral vector.

42. A kit comprising the isolated and purified nucleic acid of any one of claims 32 to 41 in a container.

43. An isolated eukaryotic cell comprising a nucleic acid encoding a protein defined by one of SEQ ID NOs: 67, 69, 71, or 73, wherein the nucleic acid is operably linked to a nucleic acid encoding at least one additional fluorescent protein or chromophore.

44. The isolated cell of claim 43, wherein the at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

45. The isolated cell of claim 43, wherein the at least one additional fluorescent protein is capable of undergoing nonradiative fluorescence resonance energy transfer to the protein defined by one of SEQ ID NOs: 67, 69, 71, or 73, with a rate of energy transfer dependent on the membrane potential of the cell.

46. The isolated cell of claim 43, wherein the least one additional fluorescent protein is a fluorescent protein whose brightness is insensitive to membrane potential and local chemical environment.

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47. The isolated cell of claim 43, wherein the at least one additional fluorescent protein is capable of indicating ion concentration in the cell.

48. The isolated cell of claim 47, wherein the at least one additional fluorescent protein capable of indicating ion concentration is a calcium indicator.

49. The isolated cell of claim 47, wherein the at least one additional fluorescent protein capable of indicating ion concentration is a pH indicator.

50. The isolated cell of any one of claims 43 to 49, wherein the cell is a stem cell, a pluripotent cell, an induced pluripotent cell, or a differentiated or undifferentiated progeny thereof.

51. The isolated cell of any one of claims 43 to 49, wherein the cell is a differentiated cell.

52. The isolated cell of claim 51, wherein the differentiated cell is a neuron.

53. The isolated cell of claim 51, wherein the differentiated cell is a cardiomyocyte.

54. An isolated cell comprising the isolated and purified nucleic acid of any one of claims 32 to 41.

55. A kit comprising a plurality of cells of any one of claims 43 to 54 in a suitable cell culture medium and a container.

56. A method of making an engineered cell for optical measurement of membrane potential, the method comprising: transducing, *in vitro*, a cell with a nucleic acid encoding a microbial rhodopsin protein with a sequence of one of SEQ ID NO: 67, 69, 71, or 73, wherein the microbial rhodopsin protein is an optical sensor to sense voltage across a membrane of the cell.

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57. The method of claim 56, wherein the nucleic acid encoding the microbial rhodopsin protein is operably linked to a membrane targeting nucleic acid sequence.

58. The method of claim 57, wherein the membrane targeting nucleic acid sequence is a plasma membrane targeting nucleic acid sequence.

59. The method of claim 57, wherein the membrane targeting nucleic acid sequence is a subcellular membrane targeting nucleic acid sequence.

60. The method of claim 59, wherein the subcellular membrane is a mitochondrial membrane, an endoplasmic reticulum, a sarcoplastic reticulum, a synaptic vesicle, an endosome, or a phagosome.

61. The method of any one of claims 56 to 60, wherein the nucleic acid encoding the microbial rhodopsin protein is operably linked to a cell-type specific promoter.

62. The method of any one of claims 56 to 61, wherein the nucleic acid encoding the microbial rhodopsin protein is operably linked to an additional nucleic acid encoding at least one additional fluorescent protein or a chromophore.

63. The method of claim 62, wherein the at least one additional fluorescent protein is a green fluorescent protein or a homolog thereof.

64. The method of claim 62, wherein the at least one additional fluorescent protein is capable of indicating ion concentration in the cell.

65. The method of claim 64, wherein the at least one additional fluorescent protein capable of indicating ion concentration in the cell is a calcium indicator.

66. The method of claim 64, wherein the at least one additional fluorescent protein capable of indicating ion concentration in the cell is a pH indicator.

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67. The method of any one of claims 56 to 66, wherein the cell is a differentiated cell.

68. The method of claim 67, wherein the differentiated cell is a neuron.

69. The method of claim 67, wherein the differentiated cell is a cardiomyocyte.

70. The method of any one of claims 56 to 66, wherein the cell is a pluripotent cell, a stem cell, or an induced pluripotent stem cell.

71. The method of claim 70 further comprising a step of differentiating the pluripotent cell, the stem cell, or the induced pluripotent stem cell into a differentiated cell.

72. The method of any one of claims 56 to 71, wherein the transducing is performed using a transient transfection.

73. The method of any one of claims 56 to 71, wherein the transducing is performed using a stable transfection.

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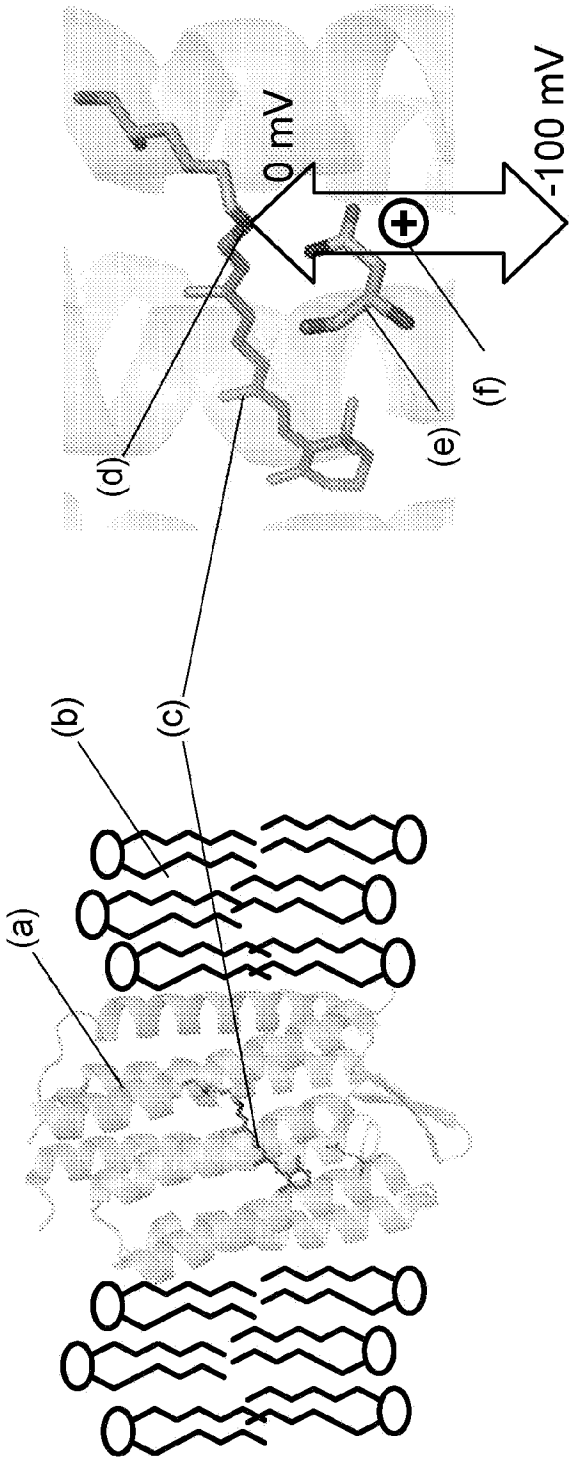


FIG. 1

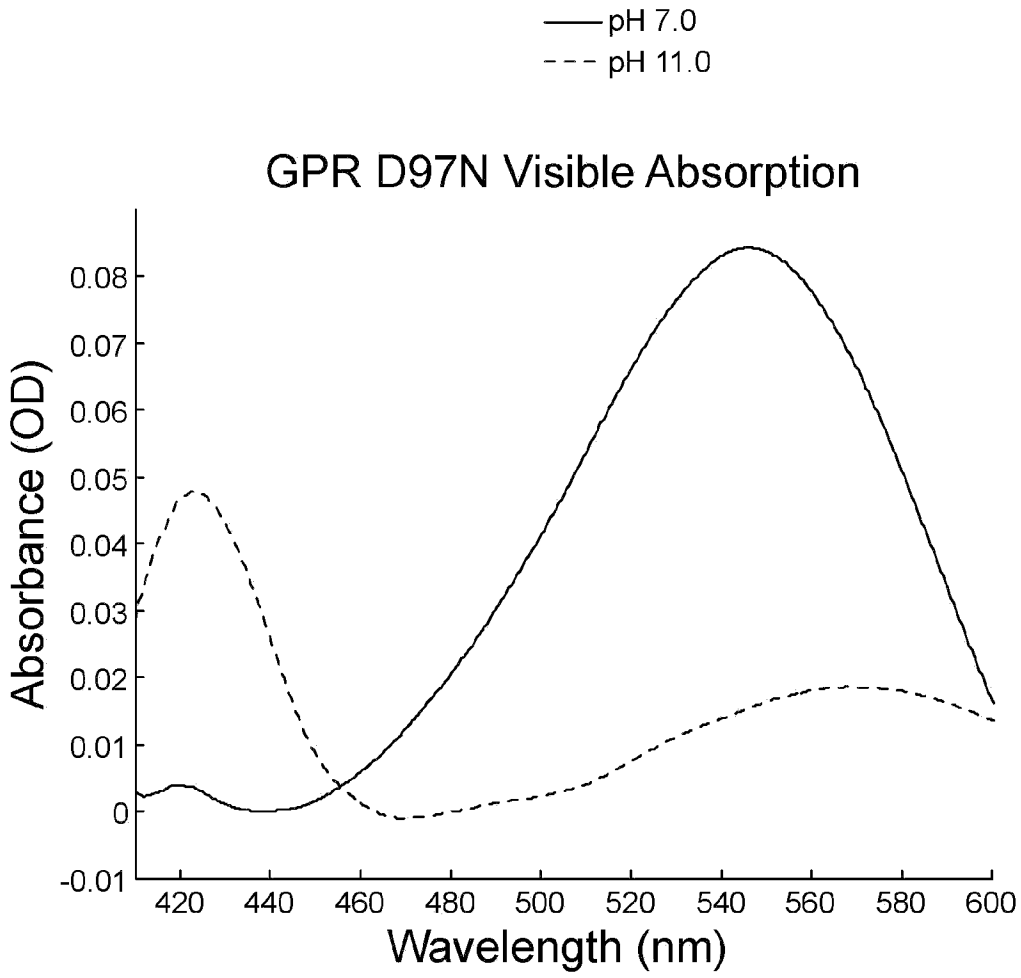

pH 7.0
pH 11.0
GPR D97N Visible Absorption
Absorbance (OD)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
420
440
460
480
500
520
540
560
580
600
Wavelength (nm)

FIG. 2A

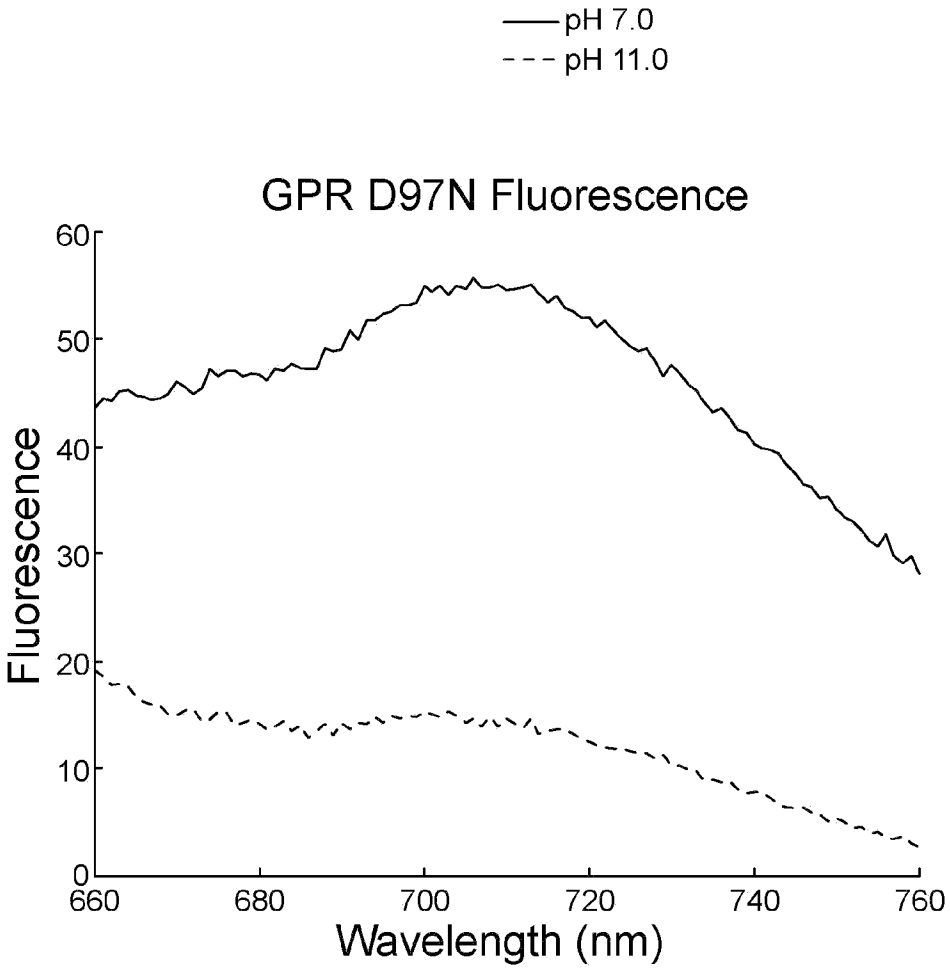

pH 7.0
pH 11.0
GPR D97N Fluorescence
Fluorescence
0
10
20
30
40
50
60
660
680
700
720
740
760
Wavelength (nm)

FIG. 2B

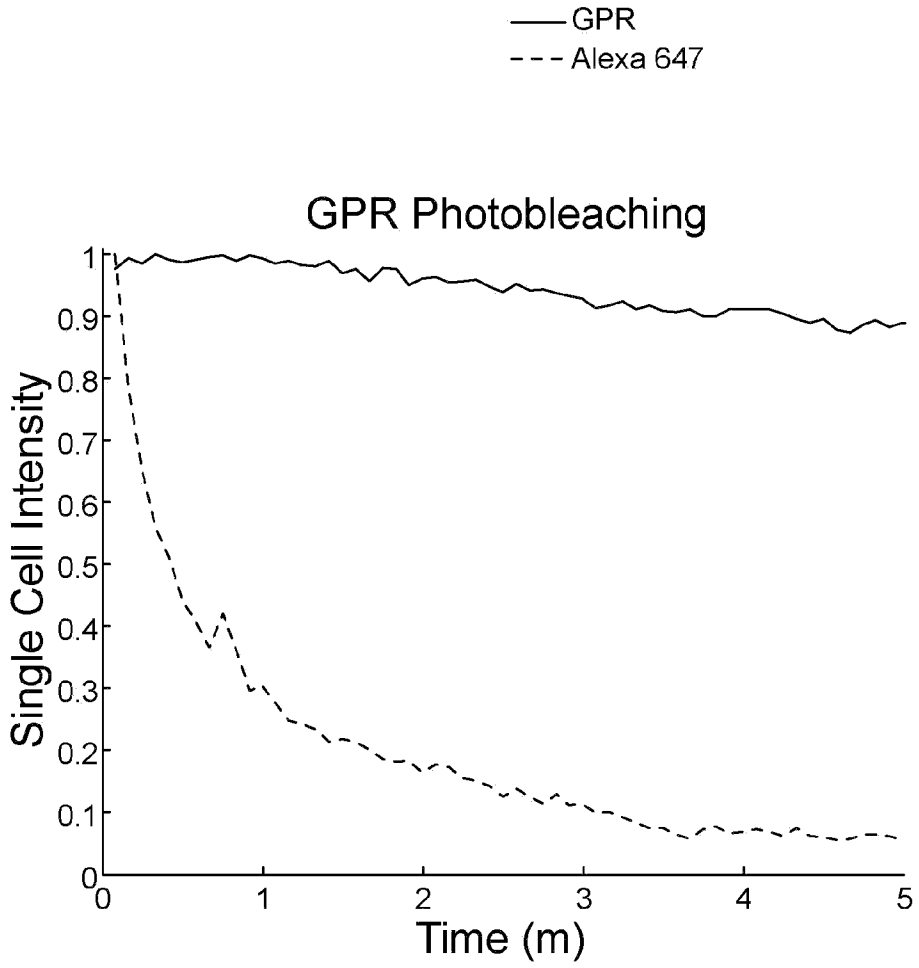

GPR
Alexa 647
GPR Photobleaching
Single Cell Intensity
Time (m)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
1
2
3
4
5

FIG. 2C

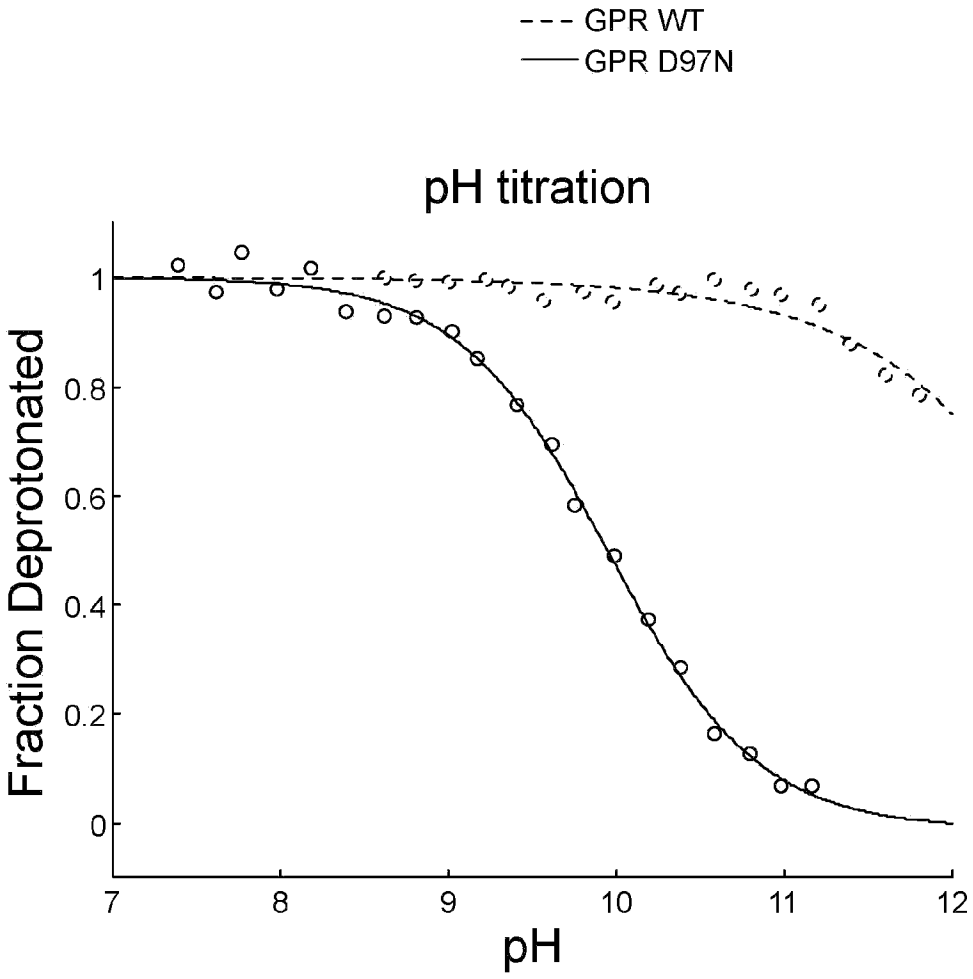

GPR WT
GPR D97N
pH titration
Fraction Deprotonated
1
0.8
0.6
0.4
0.2
0
7
8
9
10
11
12
pH

FIG. 2D

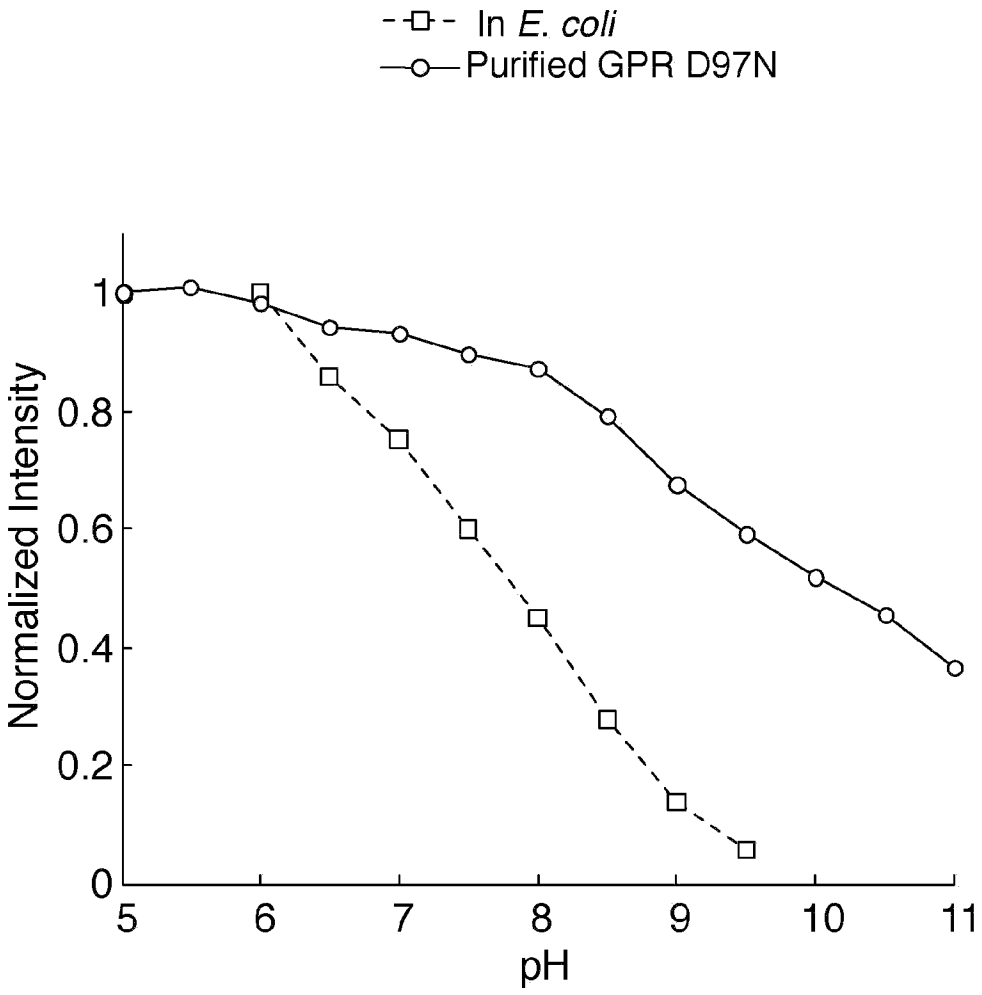

In E. coli
Purified GPR D97N
Normalized Intensity
0
0.2
0.4
0.6
0.8
1
5
6
7
8
9
10
11
pH

FIG. 3

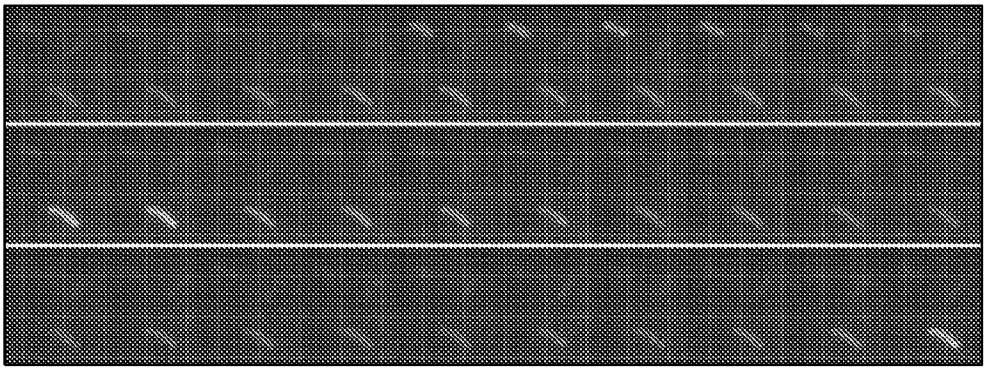


FIG. 4A

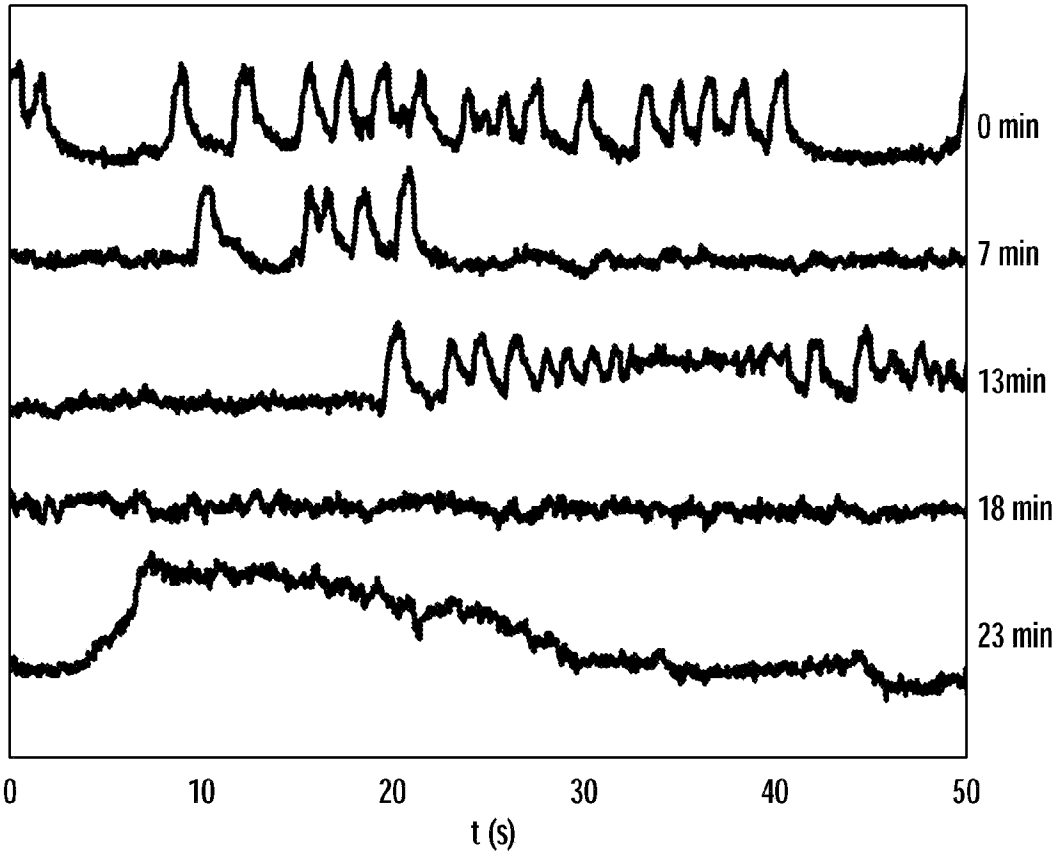


FIG. 4B

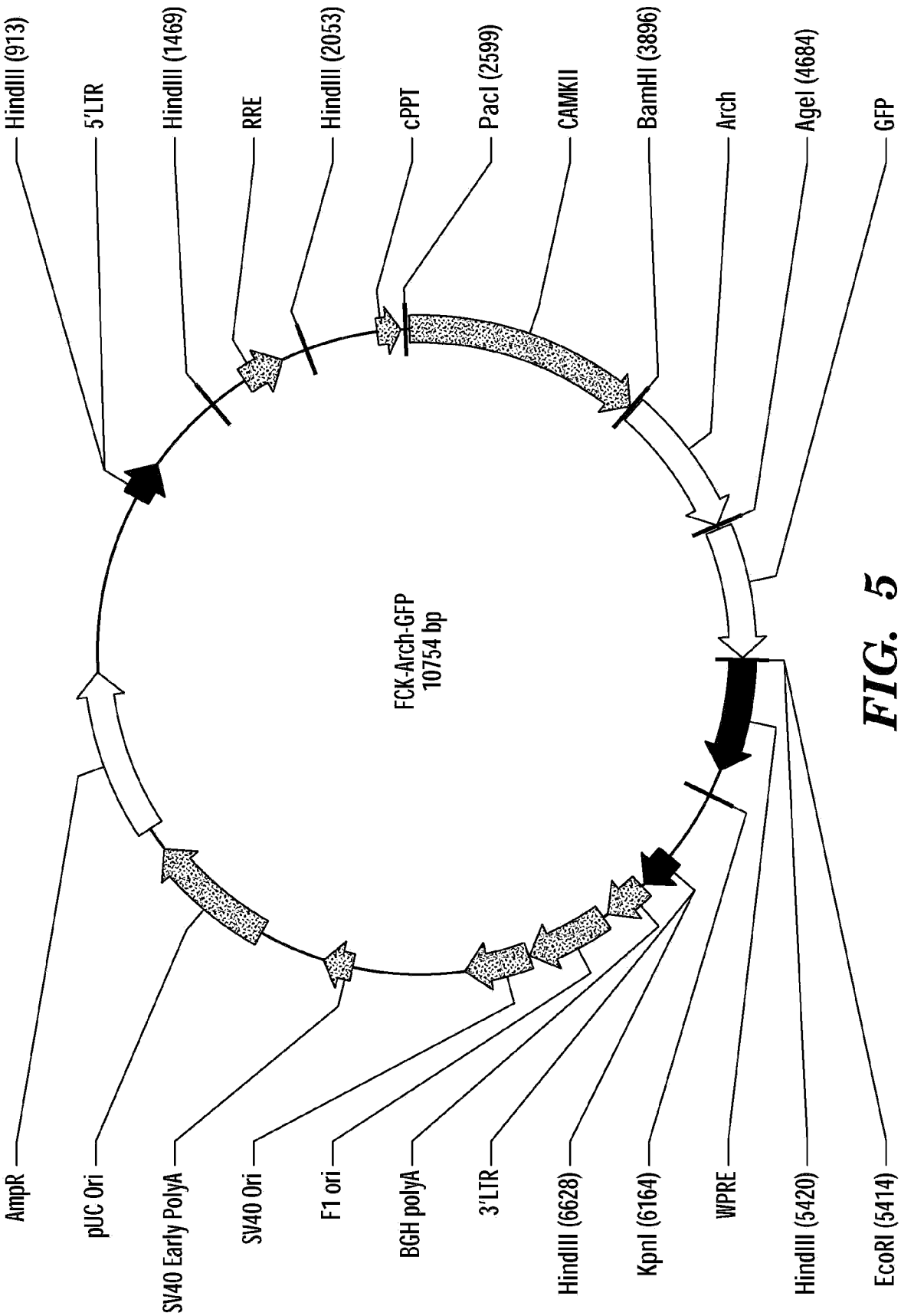

HindIII (913)
5'LTR
HindIII (1469)
RRE
HindIII (2053)
cPPT
PacI (2599)
CAMKII
BamHI (3896)
Arch
AgeI (4684)
GFP
FCK-Arch-GFP
10754 bp
AmpR
pUC Ori
SV40 Early PolyA
SV40 Ori
F1 ori
BGH polyA
3'LTR
HindIII (6628)
KpnI (6164)
WPRE
HindIII (5420)
EcoRI (5414)

FIG. 5

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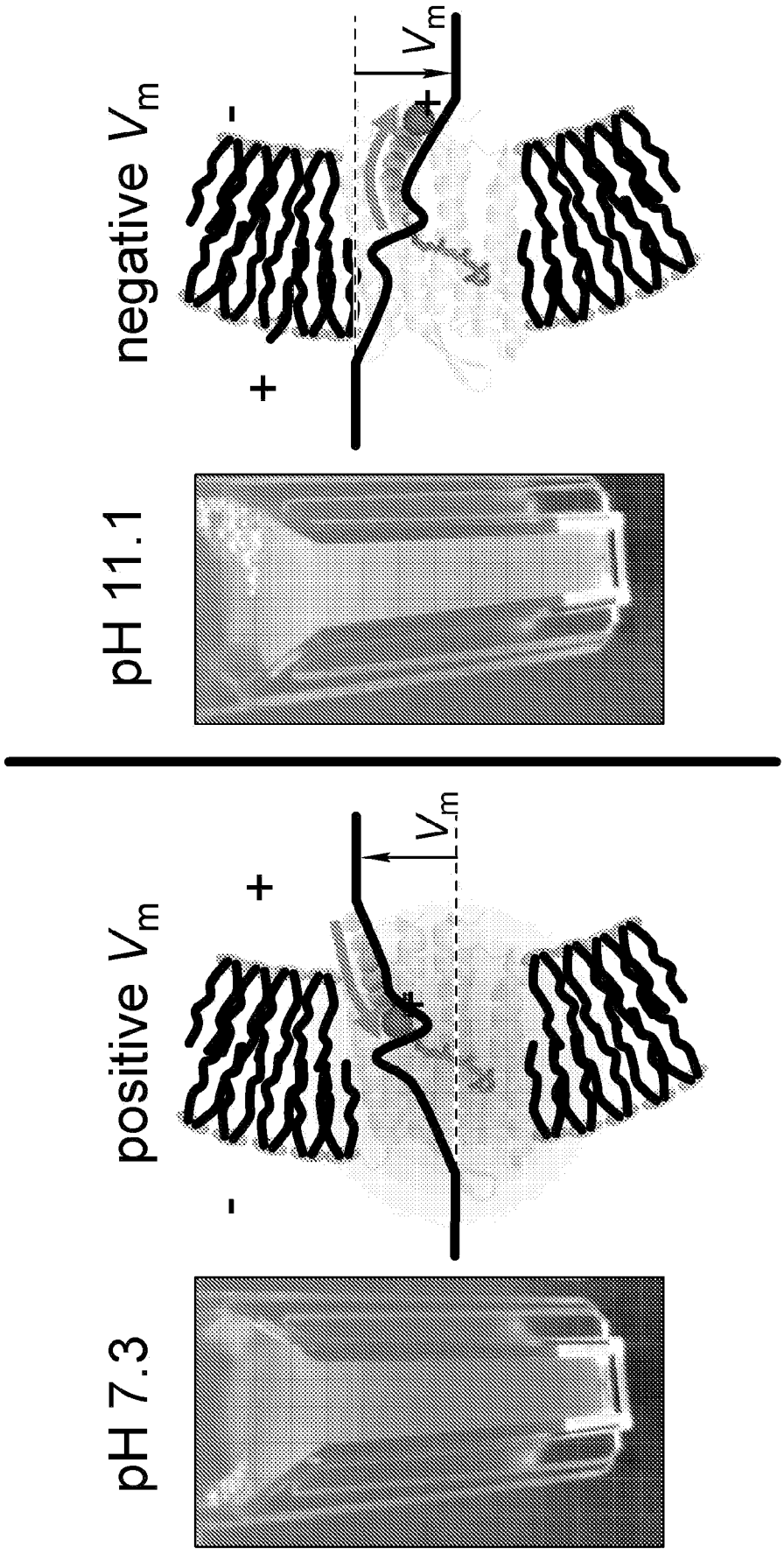


FIG. 6A

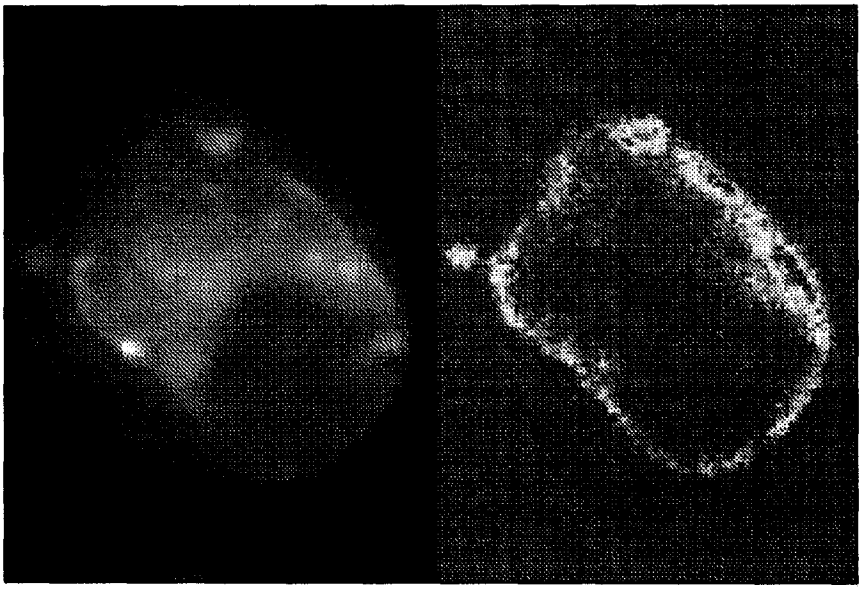


FIG. 6C

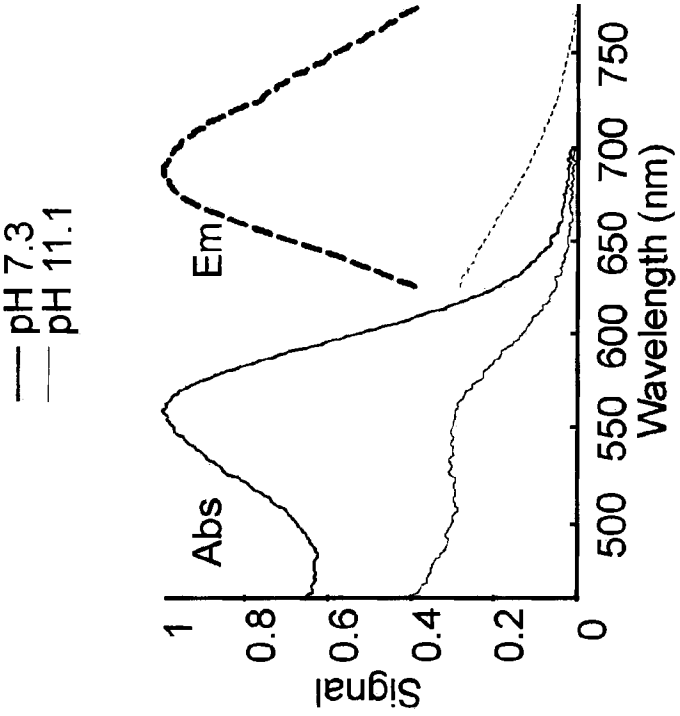

pH 7.3
pH 11.1
Em
Abs
Signal
1
0.8
0.6
0.4
0.2
0
500
550
600
650
700
750
Wavelength (nm)

FIG. 6B

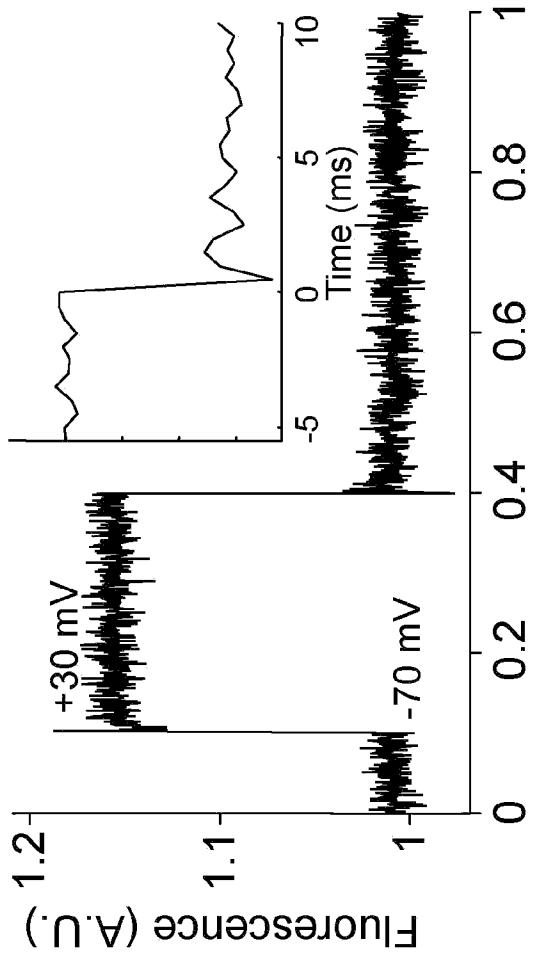

Fluorescence (A.U.)
1.2
1.1
1
+30 mV
-70 mV
0
0.2
0.4
0.6
0.8
1
Time (s)
-5
0
5
10
Time (ms)

FIG. 6E

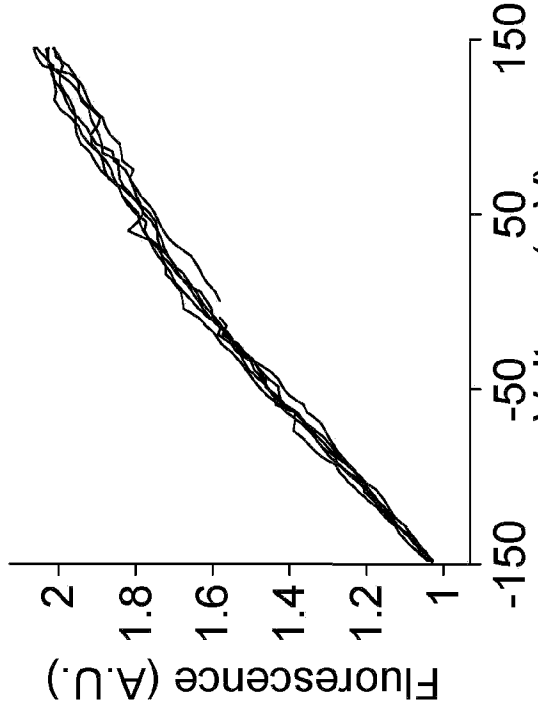

Fluorescence (A.U.)
2
1.8
1.6
1.4
1.2
1
-150
-50
50
150
Voltage (mV)

FIG. 6D

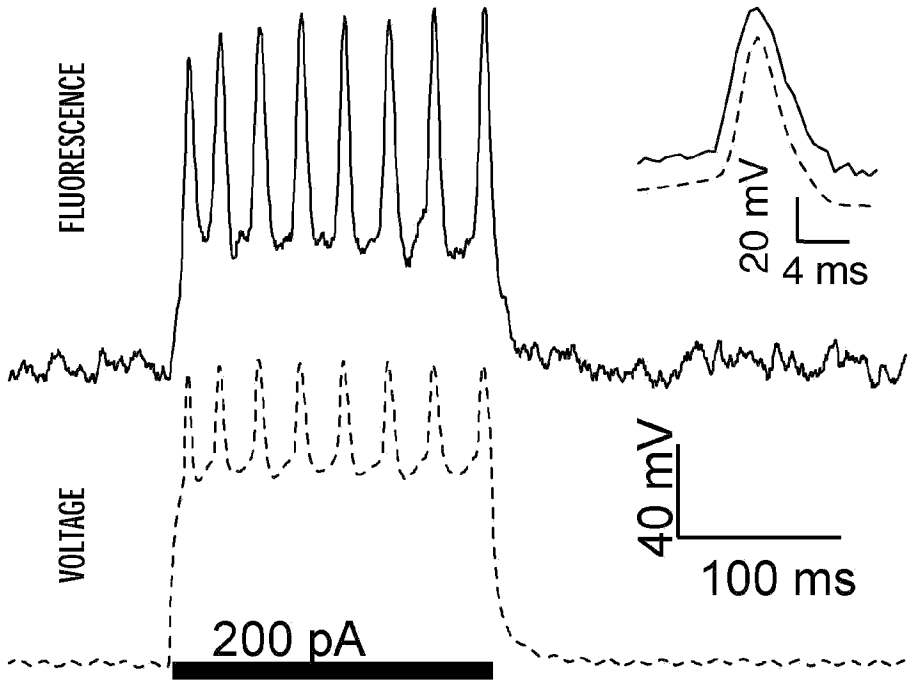


FIG. 7A

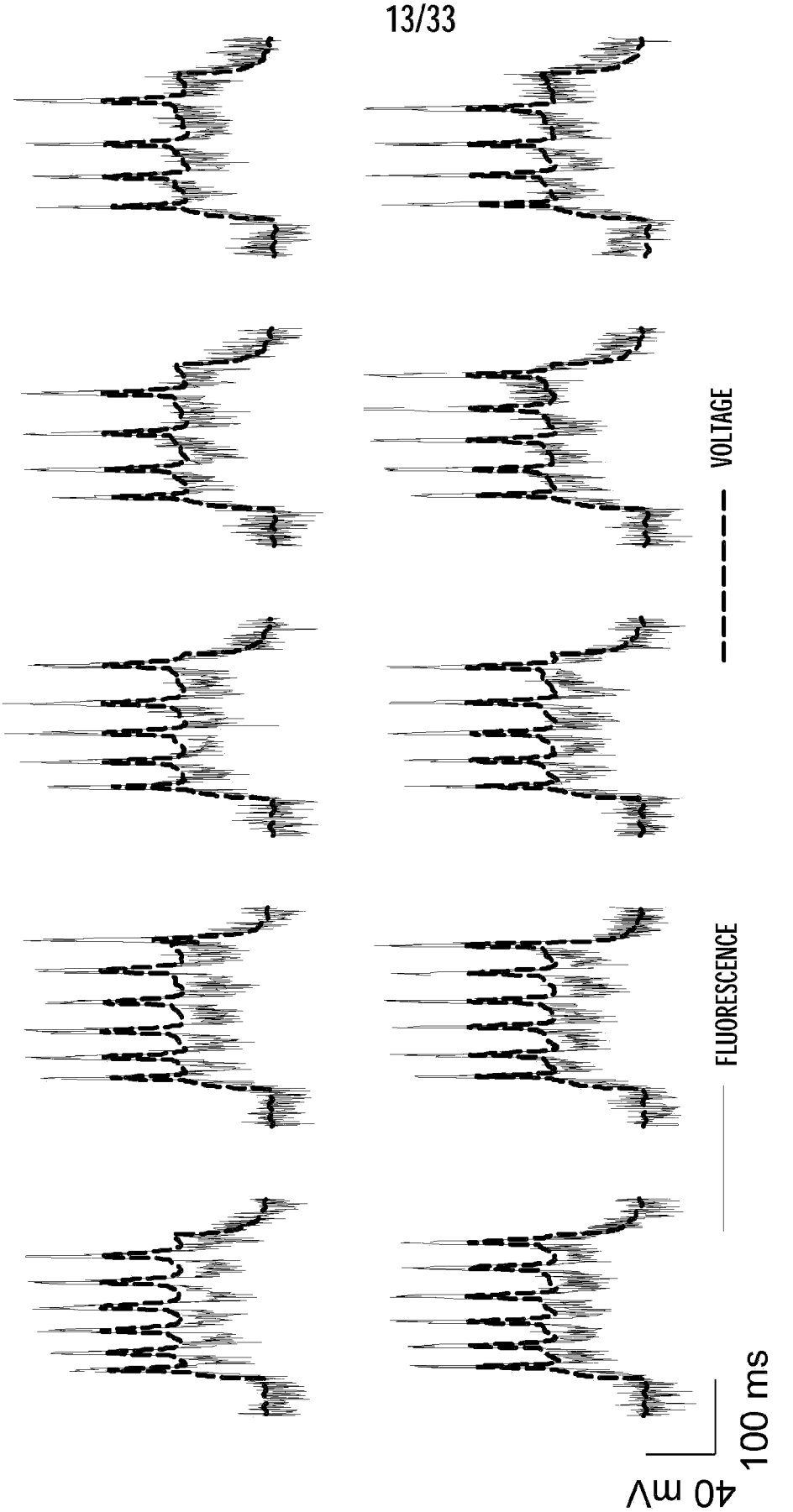


FIG. 7B

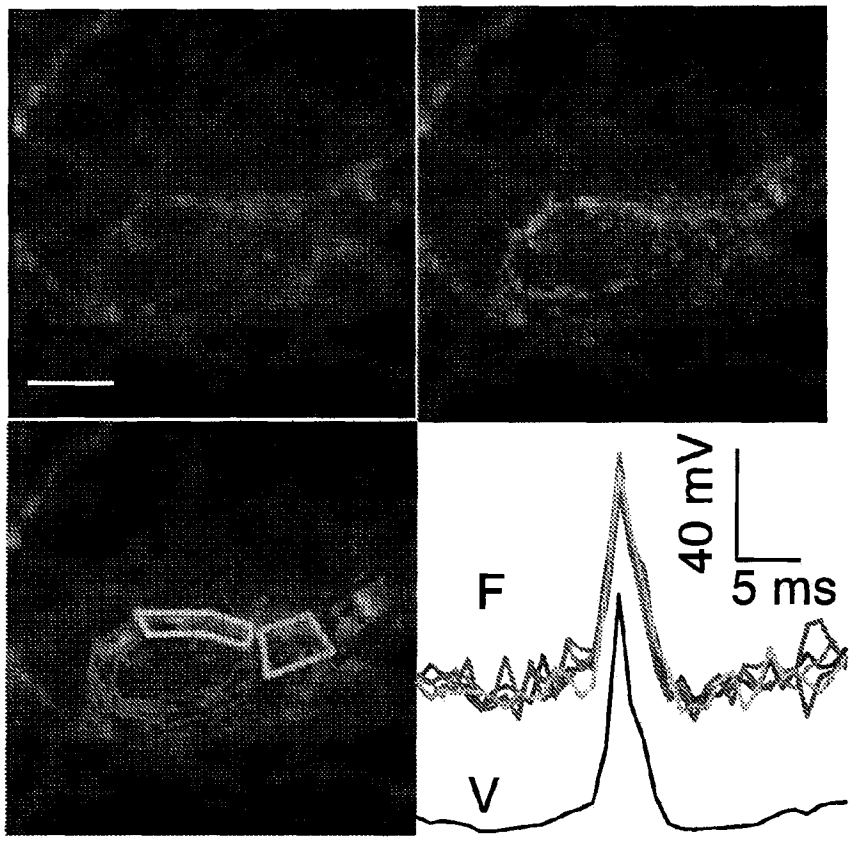


FIG. 7C

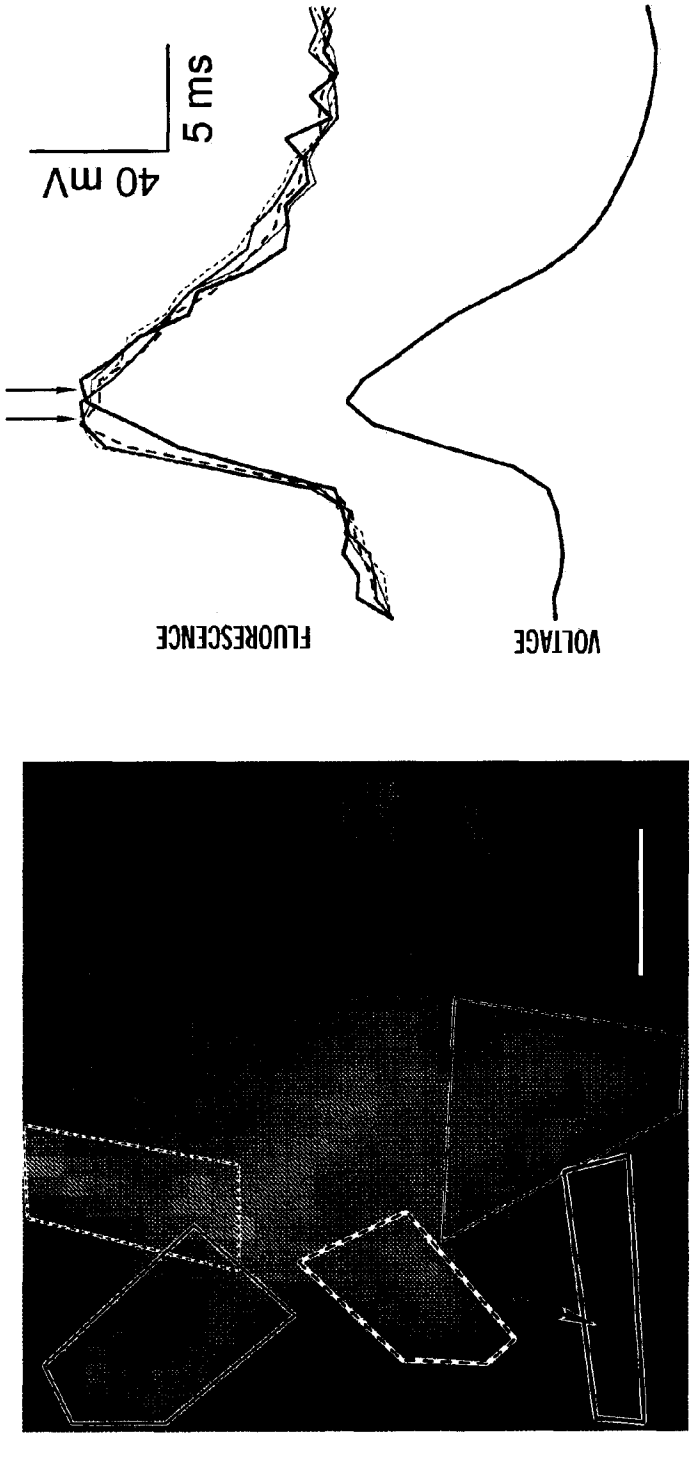


FIG. 7D

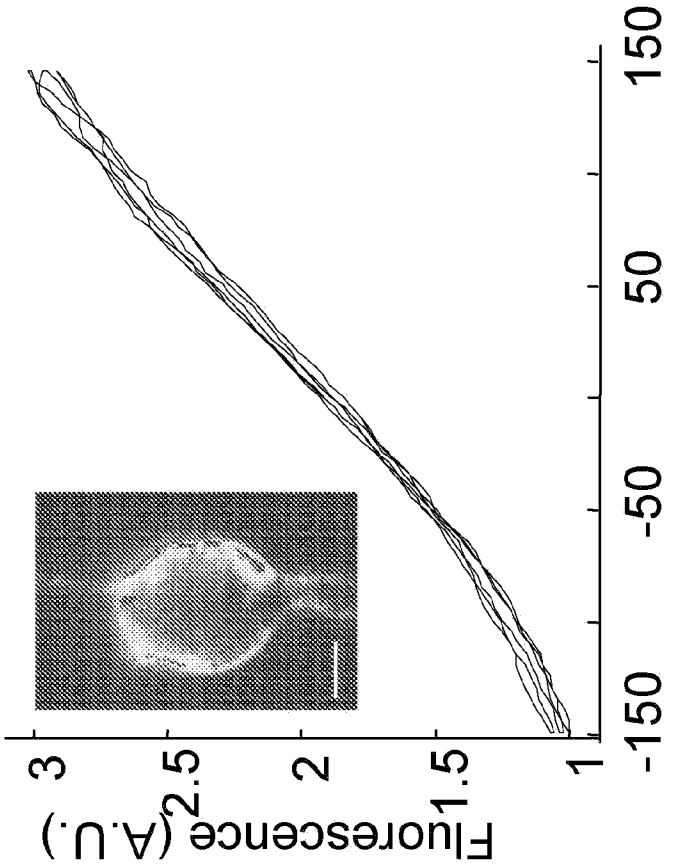

Fluorescence (A.U.)
3
2.5
2
1.5
1
-150
-50
50
150
Voltage (mV)

FIG. 8B

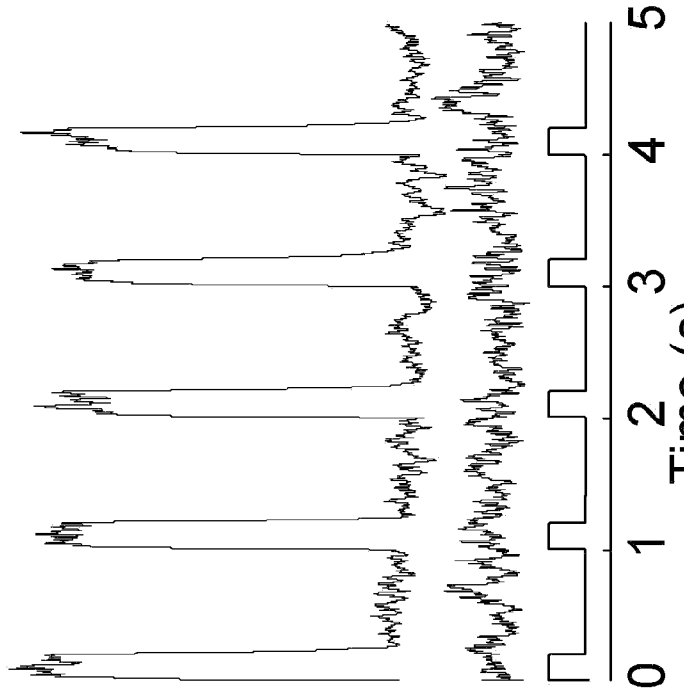

10 pA
WT
D95N
Laser
0
1
2
3
4
5
Time (s)

FIG. 8A

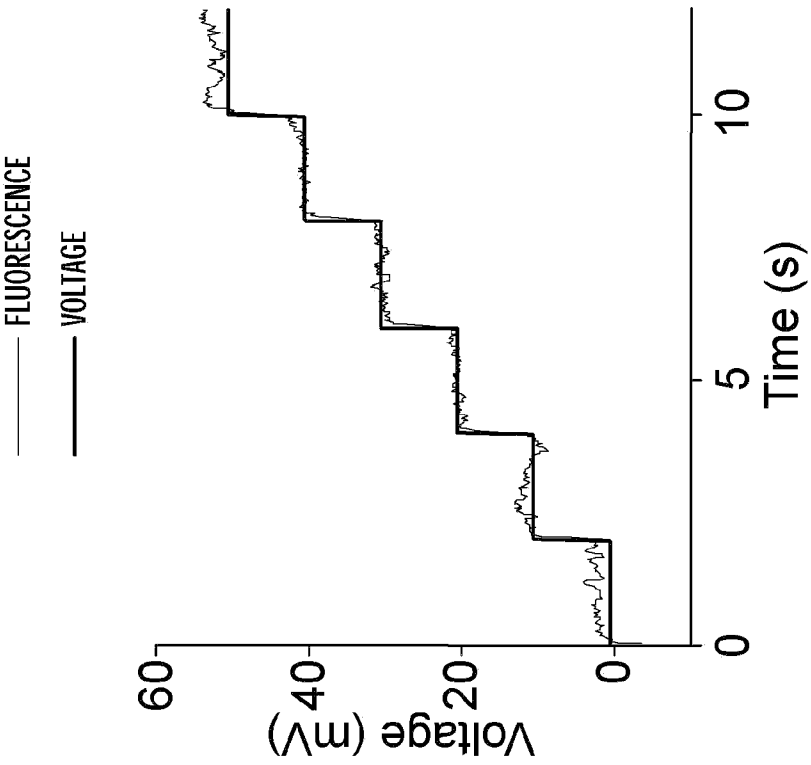

FLUORESCENCE
VOLTAGE
Voltage (mV)
60
40
20
0
0
5
10
Time (s)

FIG. 8D

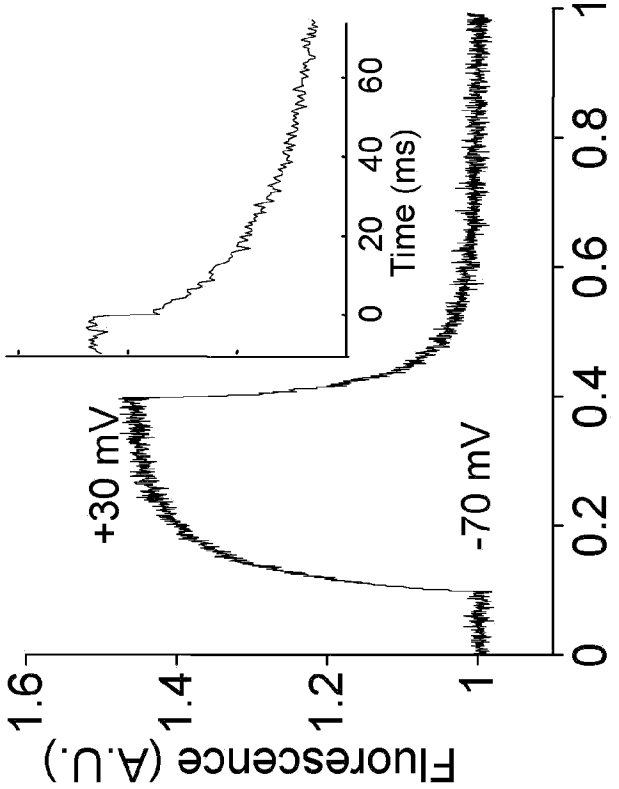

Fluorescence (A.U.)
1.6
1.4
1.2
1
+30 mV
-70 mV
0
0.2
0.4
0.6
0.8
1
Time (s)
0
20
40
60
Time (ms)

FIG. 8C

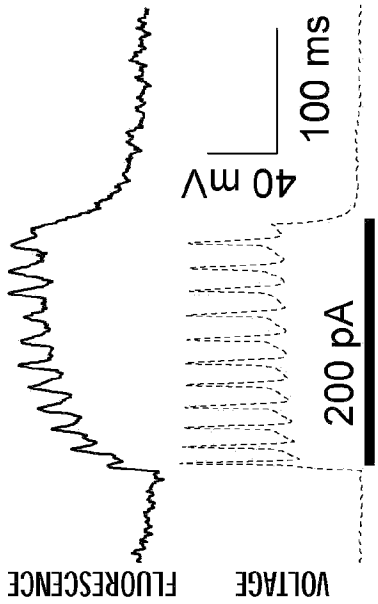


FIG. 9C

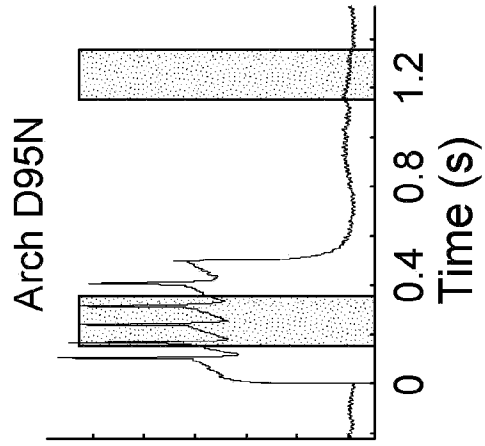


FIG. 9B

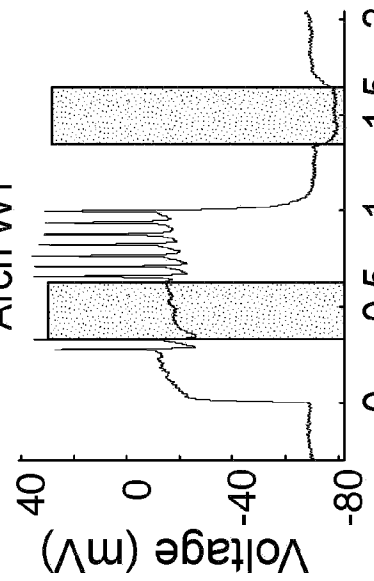


FIG. 9A

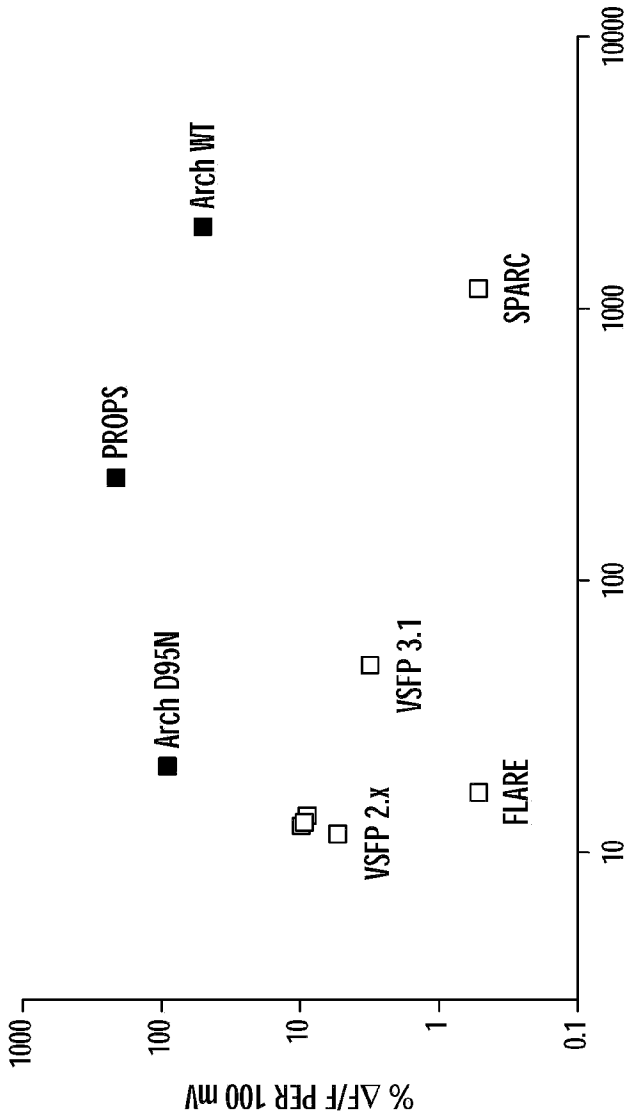

COMPARISON OF FLUORESCENT VOLTAGE INDICATORS
% ΔF/F PER 100 mV
1000
100
10
1
0.1
10
100
1000
10000
RESPONSE SPEED (1/s)
PROPS
Arch WT
Arch D95N
SPARC
VSFP 3.1
VSFP 2.x
FLARE

FIG. 10

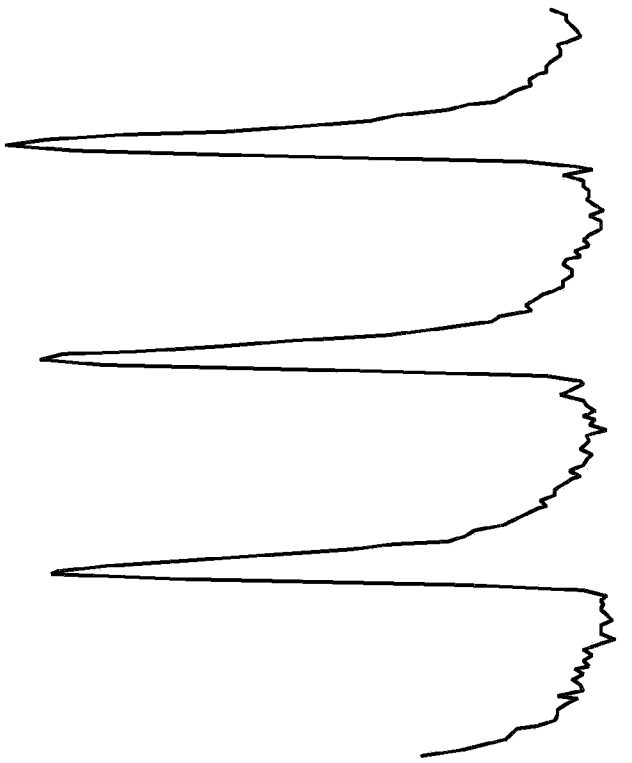


FIG. 11B

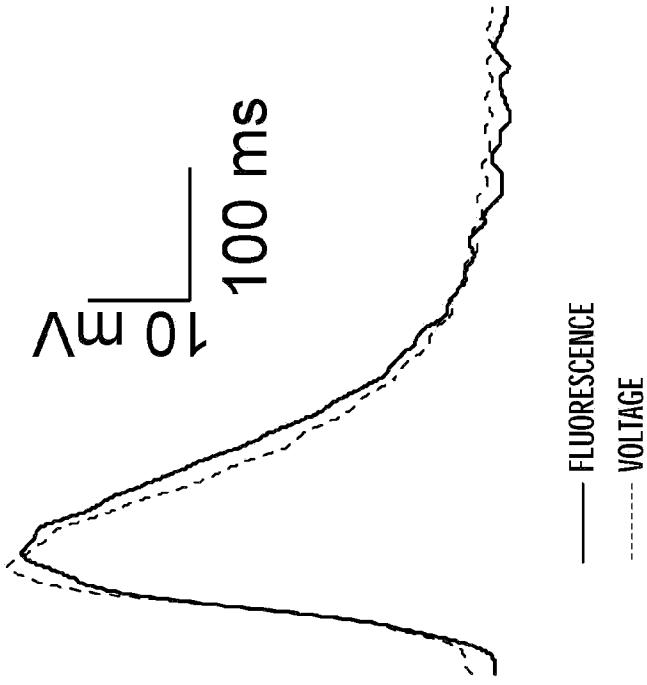


FIG. 11A

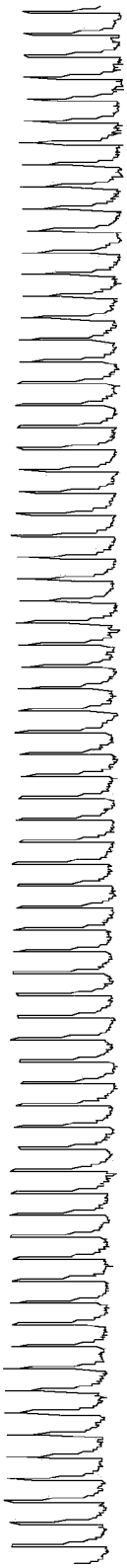


FIG. 11C

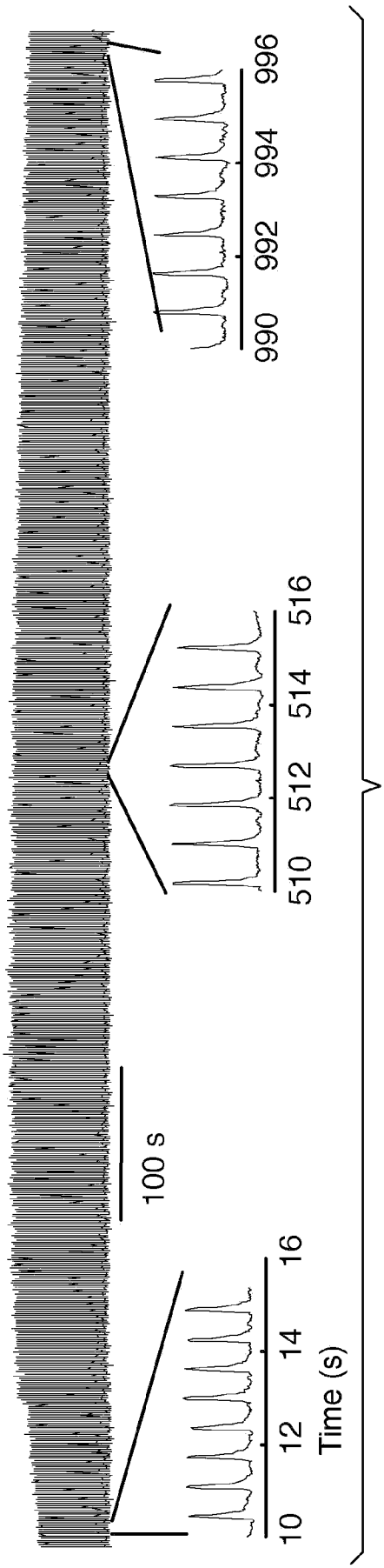


FIG. 11D

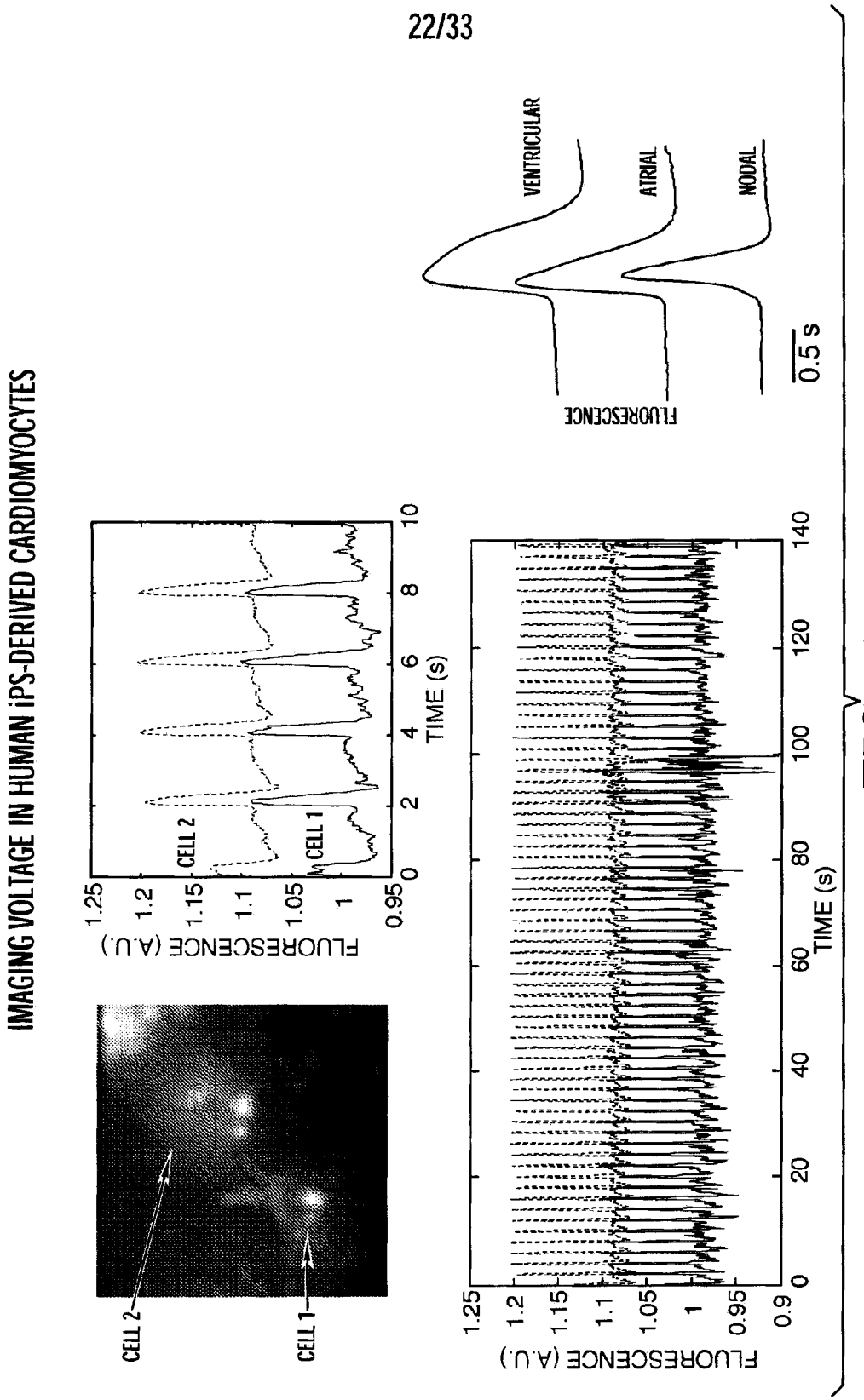

IMAGING VOLTAGE IN HUMAN iPS-DERIVED CARDIOMYOCYTES
CELL 2
CELL 1
FLUORESCENCE (A.U.)
TIME (s)
VENTRICULAR
ATRIAL
NODAL
FLUORESCENCE
0.5 s

FIG. 12

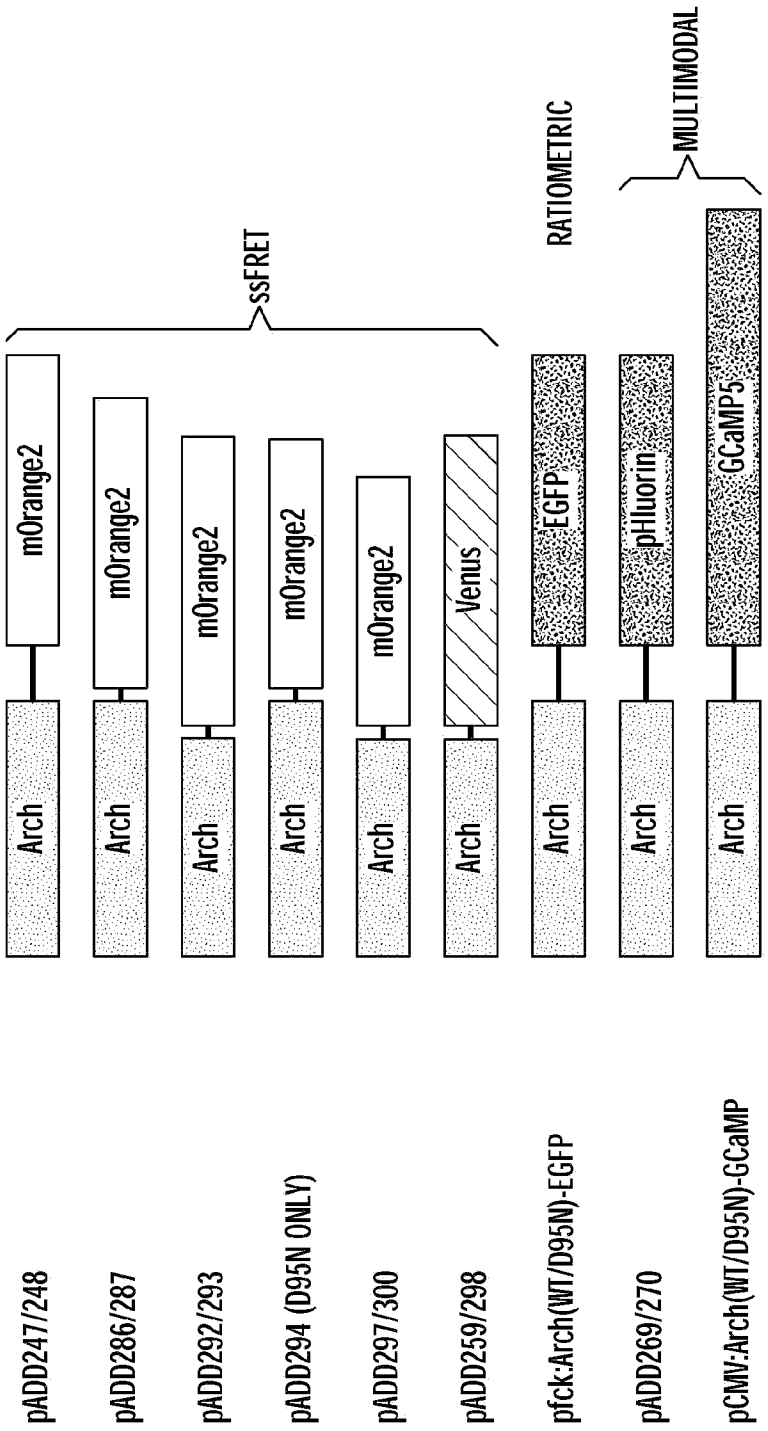

SSFRET
RATIOMETRIC
MULTIMODAL
mOrange2
Venus
EGFP
pHluorin
GCaMP5
Arch
pADD247/248
pADD286/287
pADD292/293
pADD294 (D95N ONLY)
pADD297/300
pADD259/298
pfck:Arch(WT/D95N)-EGFP
pADD269/270
pCMV:Arch(WT/D95N)-GCaMP

FIG. 13

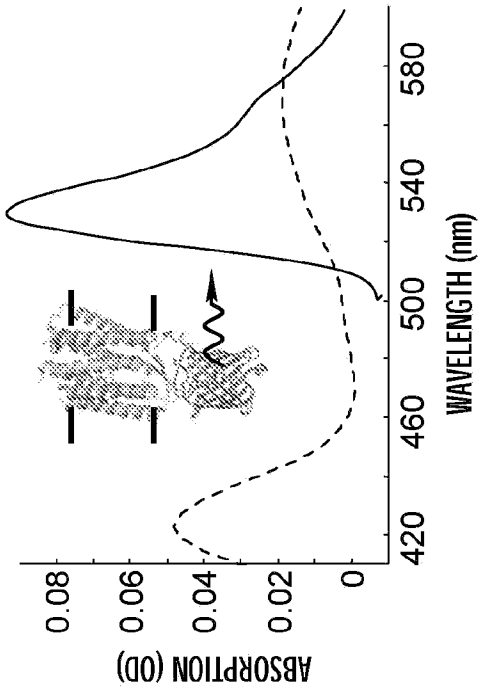


FIG. 14 B

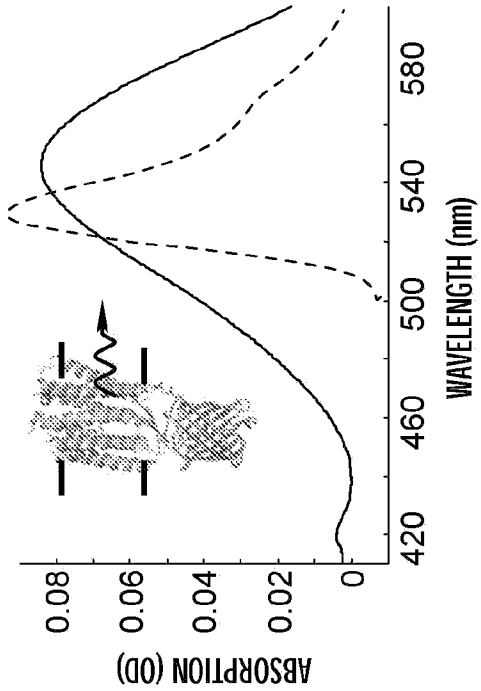


FIG. 14A

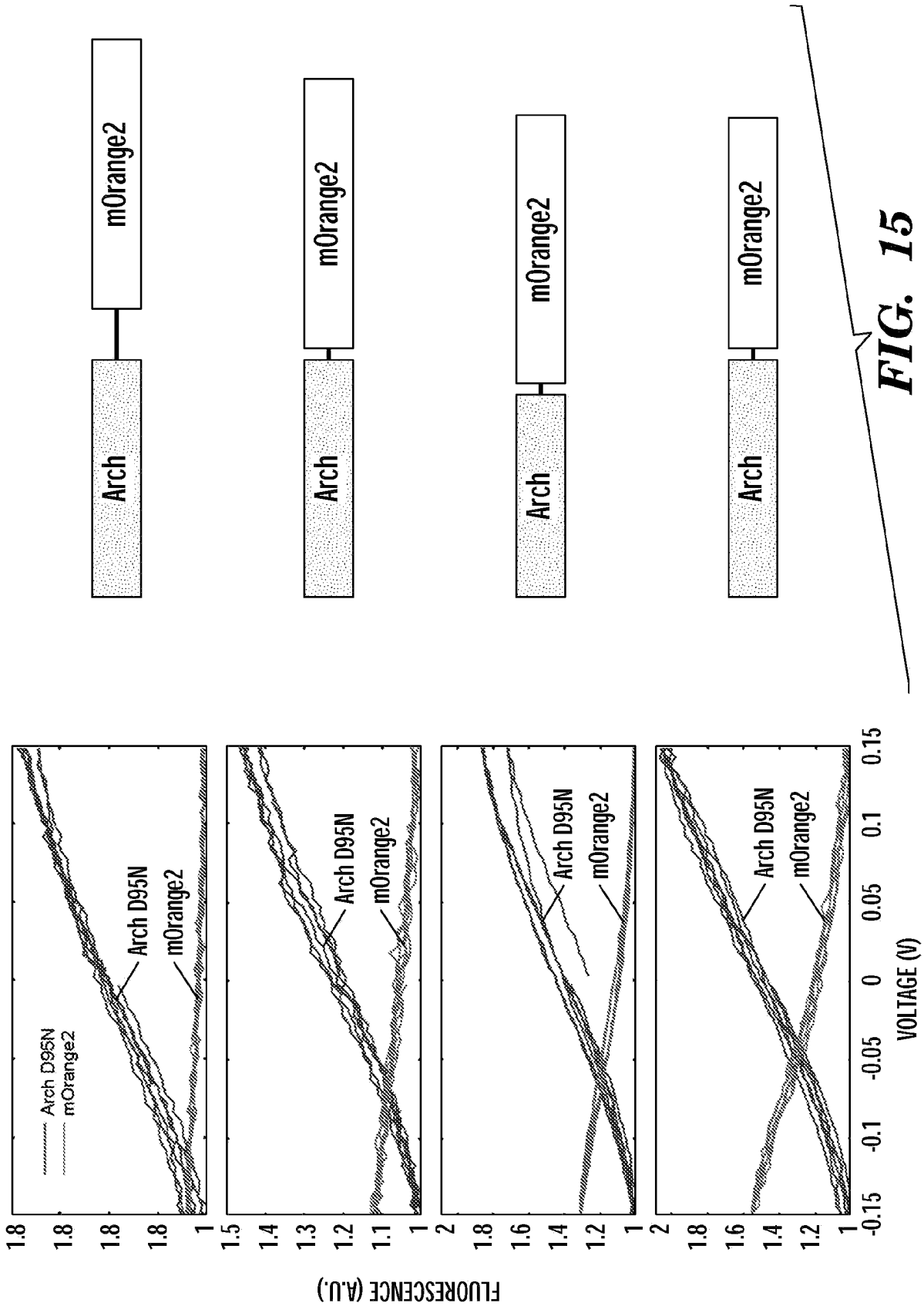

Arch
mOrange2
Arch
mOrange2
Arch
mOrange2
Arch
mOrange2
Arch D95N
mOrange2
FLUORESCENCE (A.U.)
VOLTAGE (V)

FIG. 15

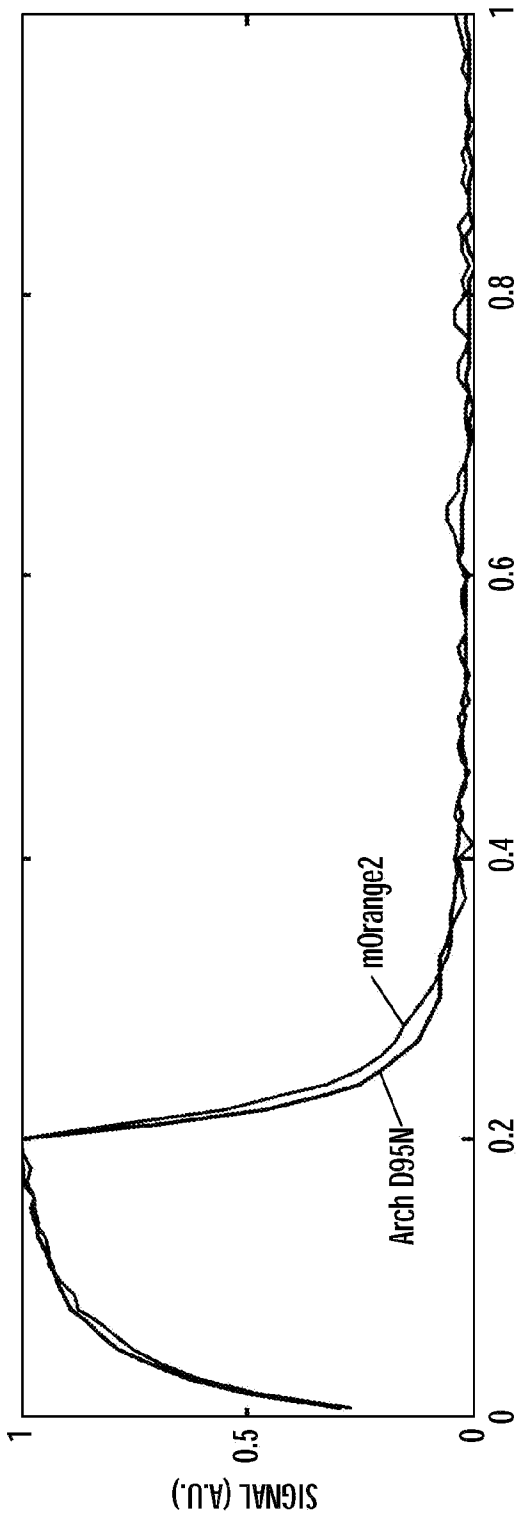


FIG. 16

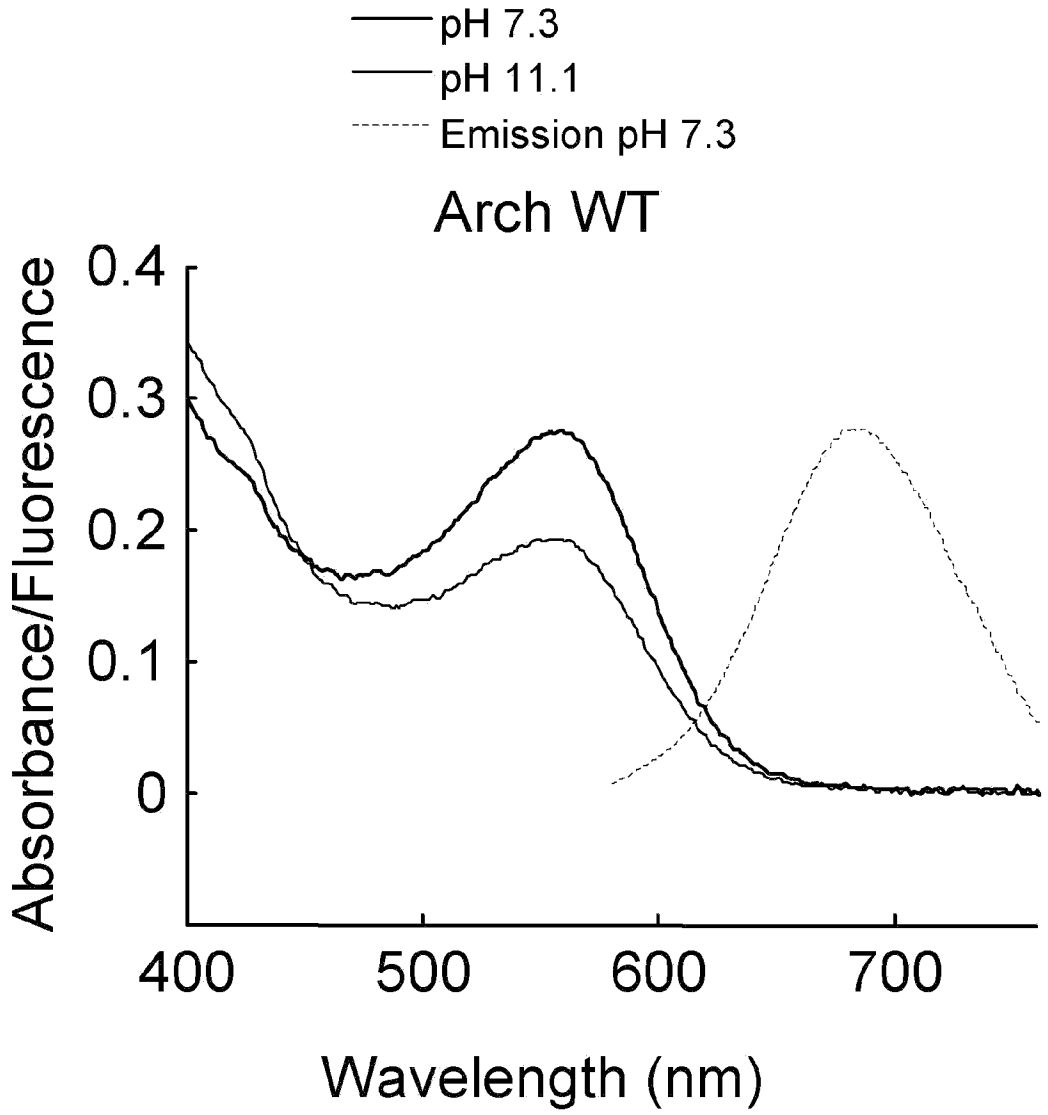

pH 7.3
pH 11.1
Emission pH 7.3
Arch WT
Absorbance/Fluorescence
0.4
0.3
0.2
0.1
0
400
500
600
700
Wavelength (nm)

FIG. 17A

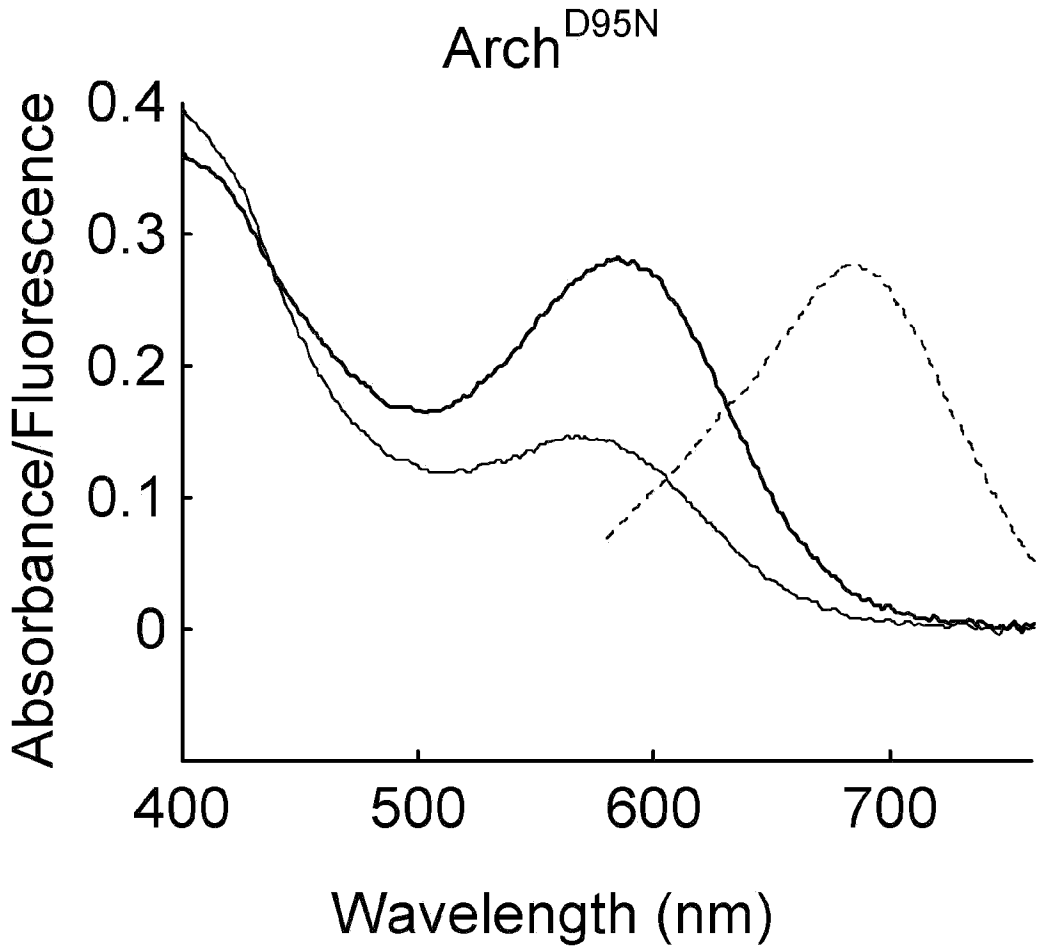

Arch D95N
Absorbance/Fluorescence
0.4
0.3
0.2
0.1
0
400
500
600
700
Wavelength (nm)

FIG. 17B

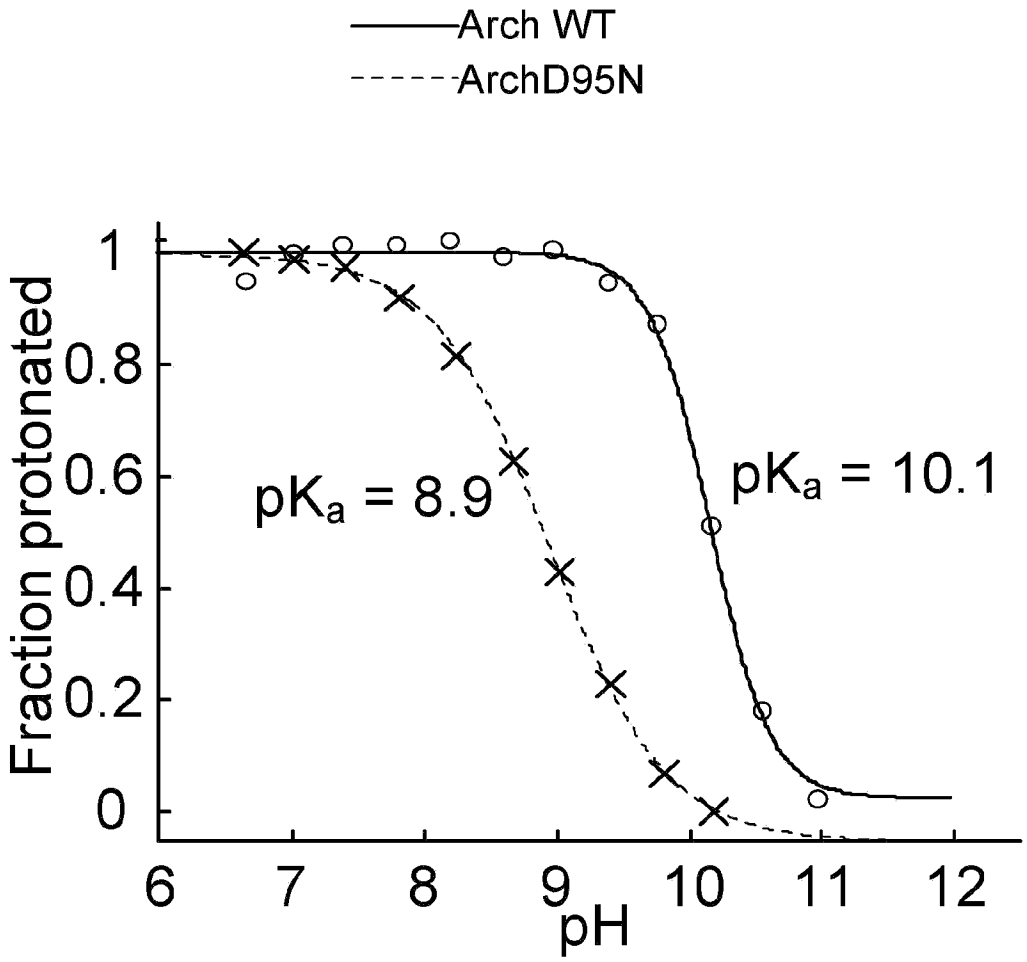

Arch WT
ArchD95N
Fraction protonated
1
0.8
0.6
0.4
0.2
0
pKa = 8.9
pKa = 10.1
6
7
8
9
10
11
12
pH

FIG. 17C

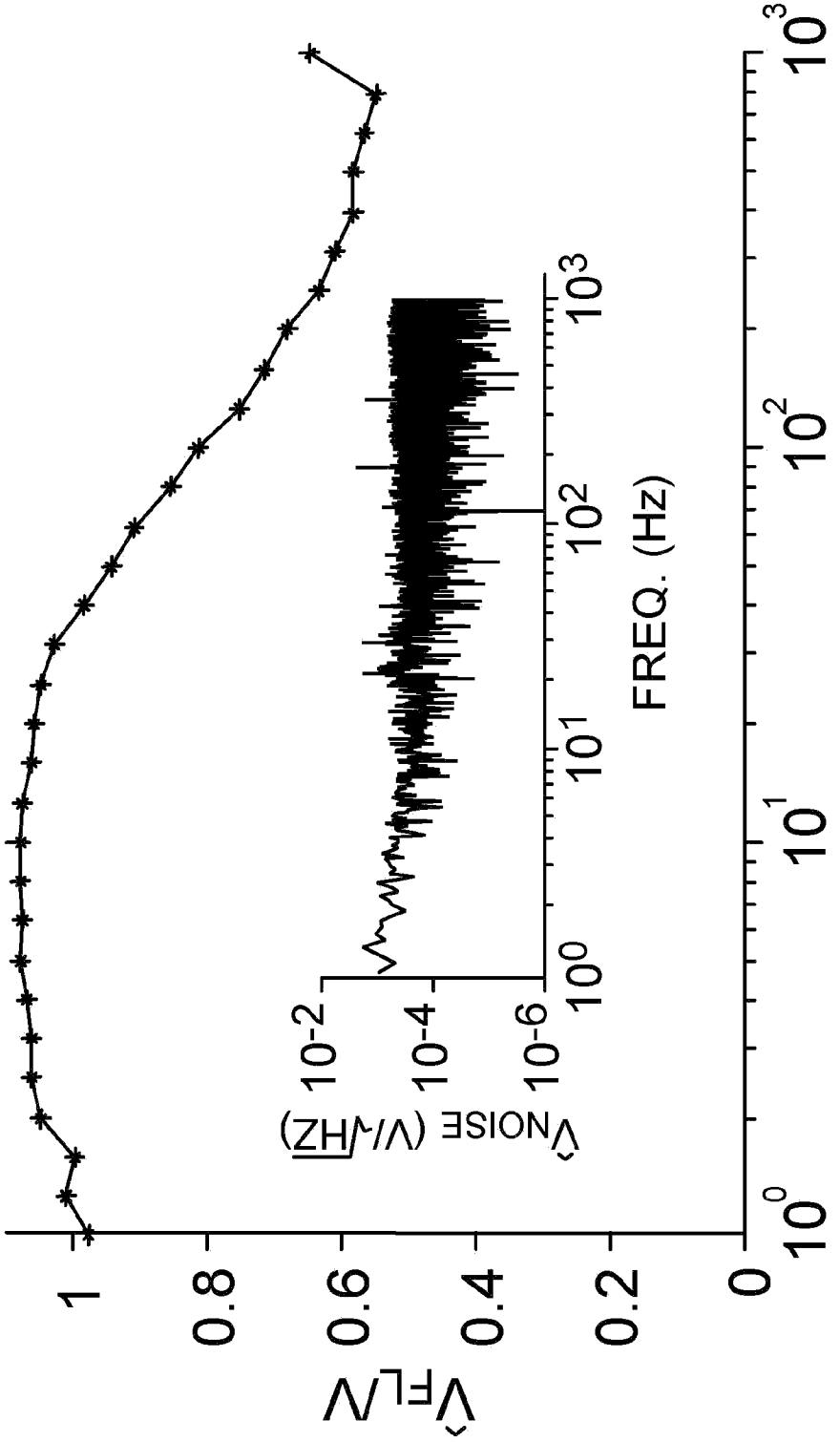

V_{FL}/V
1
0.8
0.6
0.4
0.2
0
10^0
10^1
10^2
10^3
Frequency (Hz)
V_{NOISE} (V/√HZ)
10^{-2}
10^{-4}
10^{-6}
10^0
10^1
10^2
10^3
FREQ. (Hz)

FIG. 18

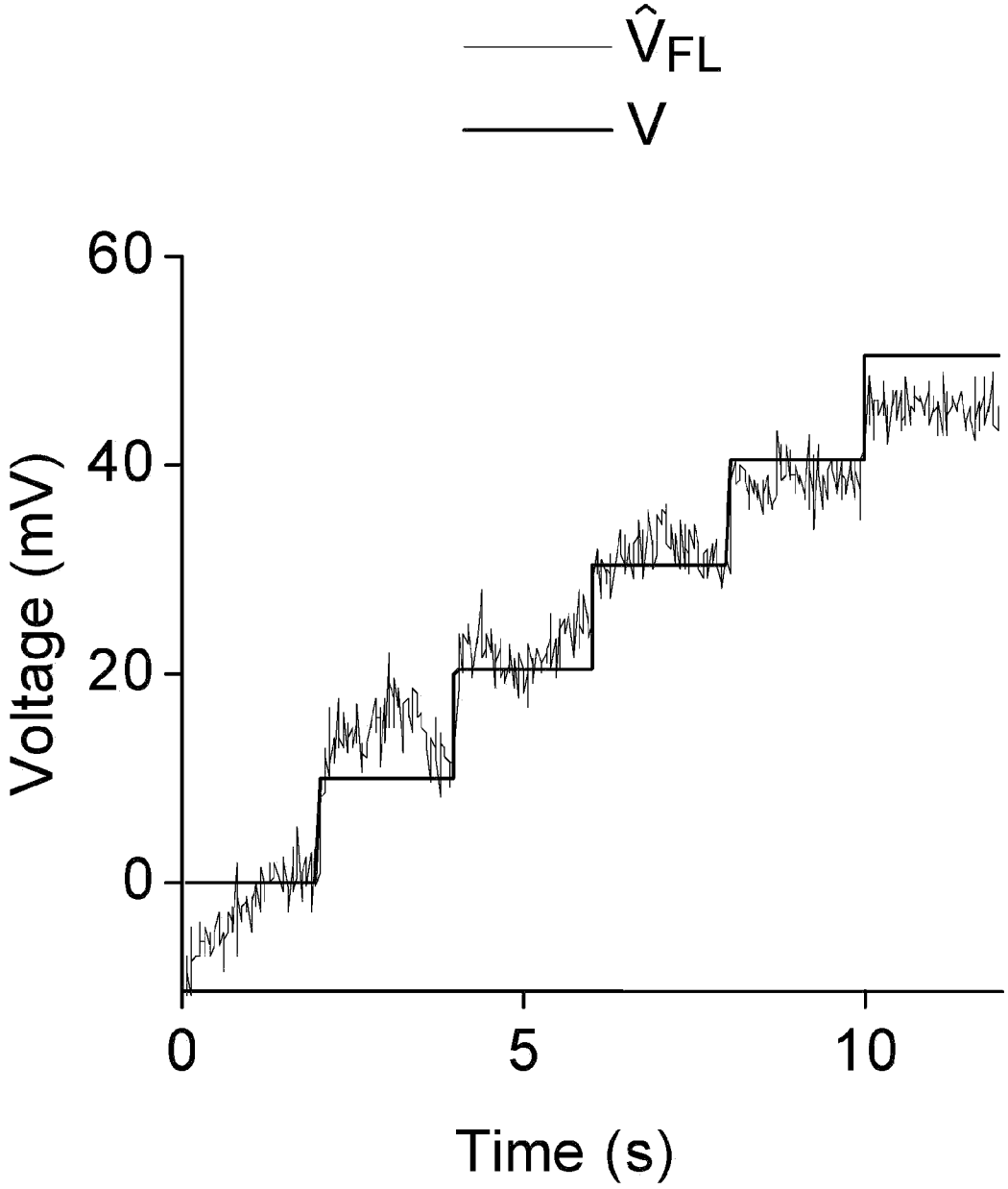


FIG. 19

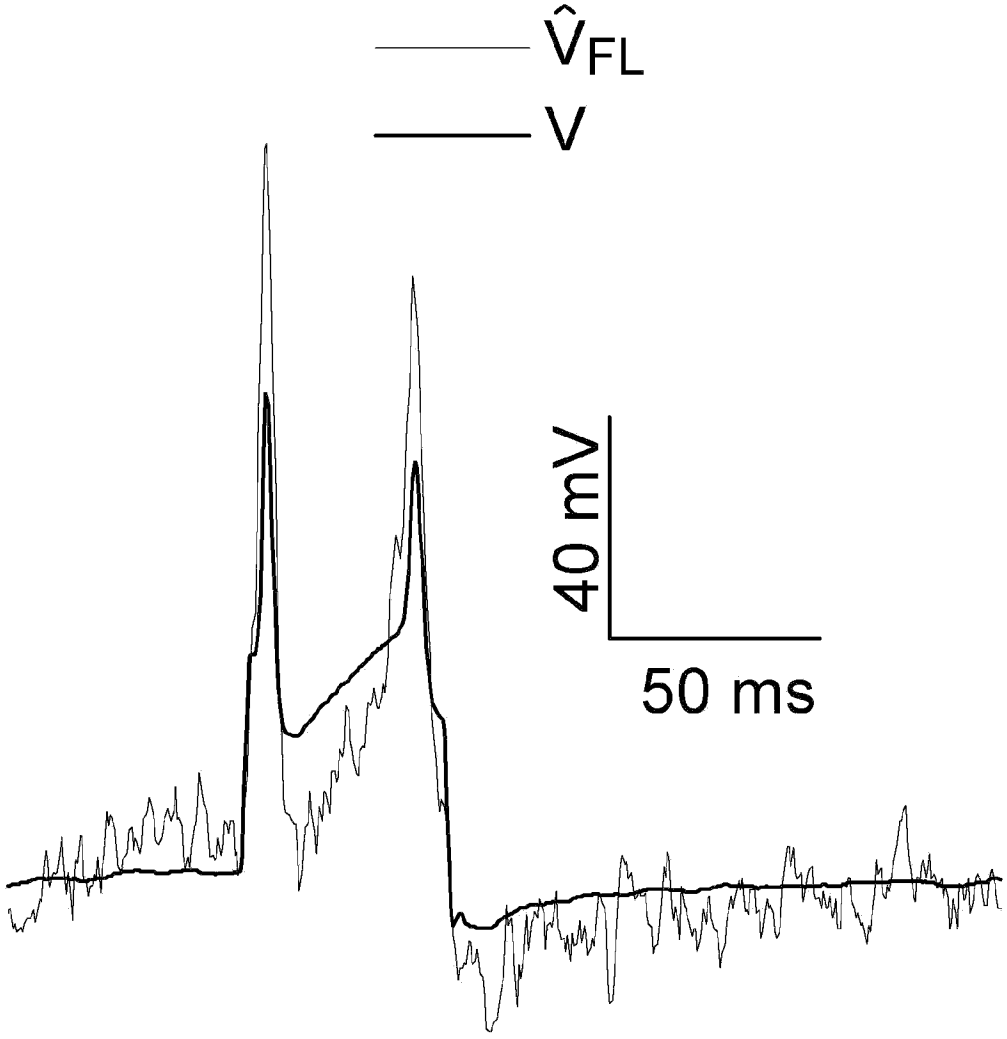


FIG. 20

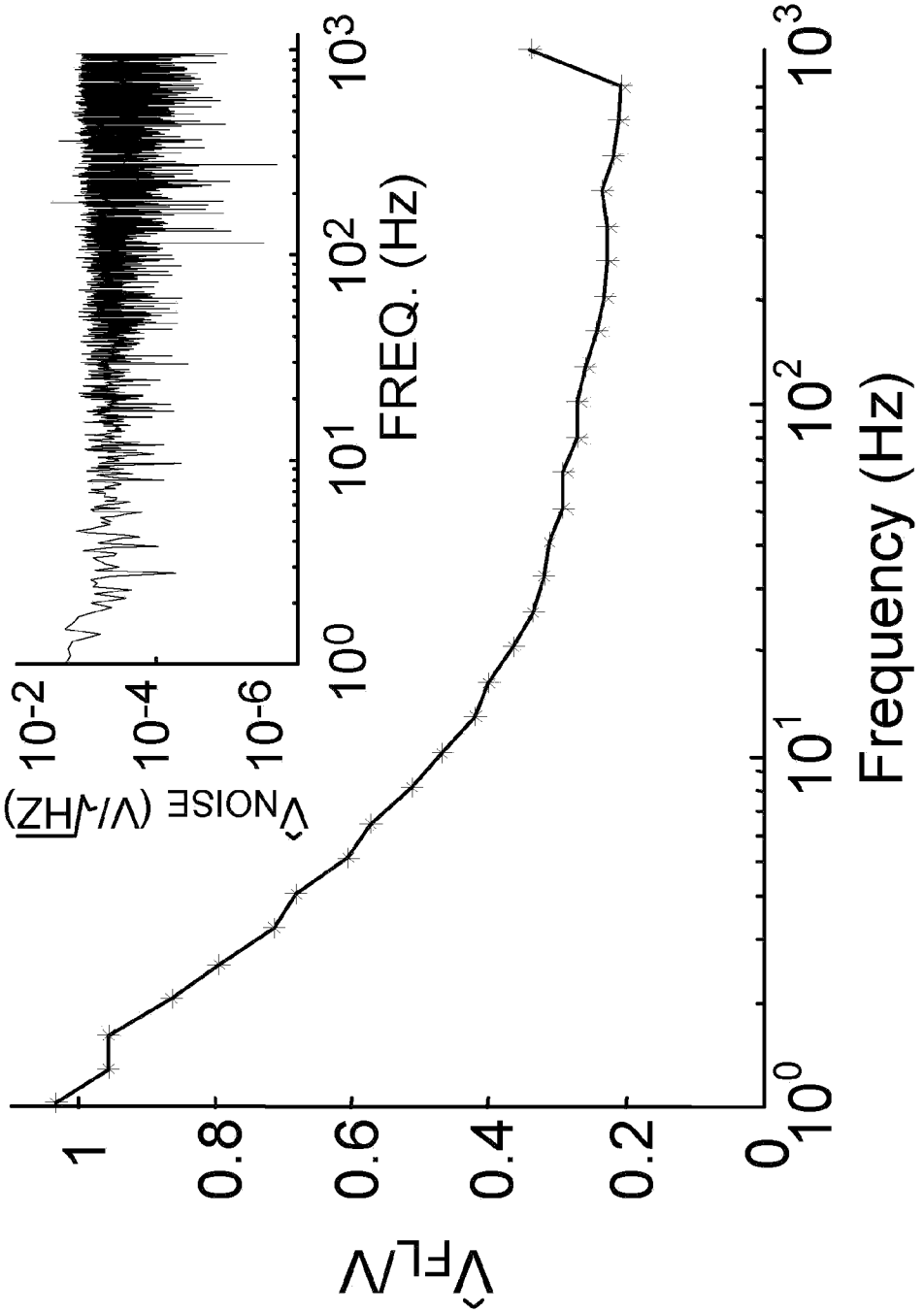
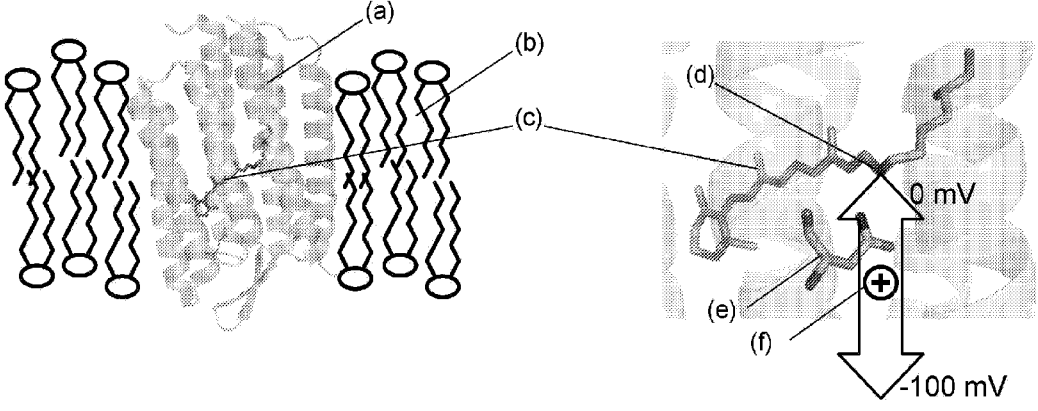

V_{FL}/V
1
0.8
0.6
0.4
0.2
0
10^0
10^1
10^2
10^3
Frequency (Hz)
V_{NOISE} (V/√HZ)
10^{-2}
10^{-4}
10^{-6}
10^0
10^1
10^2
10^3
FREQ. (Hz)

FIG. 21


(a)
(b)
(c)
(d)
0 mV
(e)
(f)
-100 mV