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(54) Title: VOLTAGE SENSITIVE COMPOSITION AND METHOD OF USE THEREOF

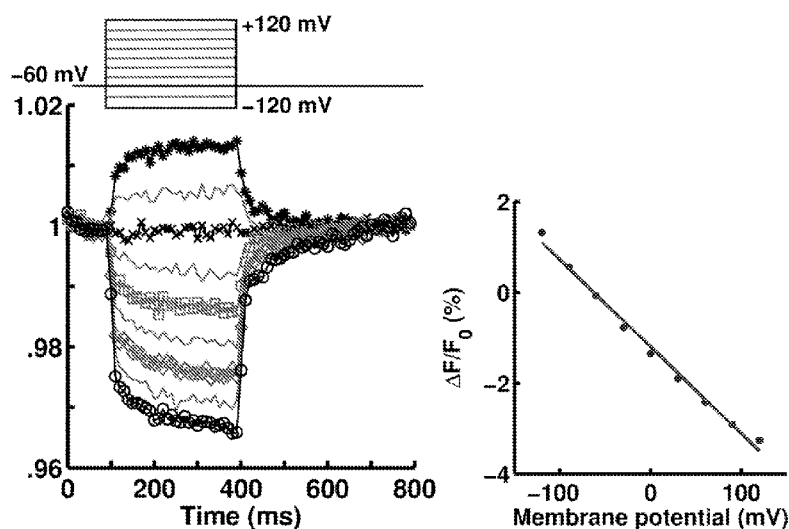


Figure 3

(57) Abstract: The present invention relates generally to voltage-sensitive compositions and to methods of using such compositions. One aspect of the invention provides a method of monitoring the electrical activity of a cell, for example, a human cell. In certain embodiments, the cell is a nerve cell or a cardiac cell.



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Voltage Sensitive Composition and Method of Use Thereof

RELATED APPLICATIONS

[0001] This patent application claims the benefit of United States provisional patent application numbers 61/917,091, filed December 17, 2013 and 62/021,832, filed July 8, 2014, the entire contents of which applications are hereby incorporated by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with U.S. government support under grant number and NIH GM030376. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention relates generally to voltage-sensitive compositions and to methods of using such compositions.

BACKGROUND

[0004] Membrane potential is the difference in electric potential between the interior and the exterior of a biological cell. With respect to the exterior of the cell, typical values of membrane potential range from -40 mV to -80 mV. In non-excitable cells, and in excitable cells in their baseline states, the membrane potential is held at a relatively stable value, called the resting potential. In excitable cells, a sufficiently large depolarization can evoke an action potential, in which the membrane potential changes rapidly and significantly for a short time (on the order of 1 to 100 milliseconds), often reversing its polarity. Action potentials are generated by the activation of certain voltage-gated ion channels.

[0005] Cellular regulation of membrane potential is an essential feature of any electrically excitable cell. Abnormal regulation of these potentials

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underlies many diseases of the heart, skeletal muscles, and central and peripheral nervous systems. Most clinical electrophysiological tests of excitable tissue, such as electrocardiograms (ECGs) and electroencephalograms (EEGs), use external electrodes to measure the summed electrical field produced by large populations of cells. This makes these techniques convenient and non-invasive, but dramatically limits their spatial resolution. In contrast, electrophysiological techniques that monitor individual cells or small cell populations typically require the surgical placement of *in situ* electrodes that are limited in number and thus provide little information about large populations of excitable cells. These barriers limit the clinical utility of these diagnostic tests, so a need remains for a technique that can screen for abnormal electrical activity across large regions of tissue yet still localize those anomalies to a small area.

SUMMARY

[0006] Indocyanine green (ICG) is an infrared fluorescent dye with widespread use in clinical applications, including hepatic function tests, cardiac output monitoring, and ophthalmic angiography. Using an *in vivo* preparation, the applicants have found that ICG is voltage-sensitive and capable of responding to changes in cellular membrane potential with a roughly linear voltage dependency. ICG's voltage response is not electrochromic; however, it is faster than many so-called slow-response voltage probes and can clearly follow action potentials produced at approximately 70 Hz in an oocyte. ICG has low expected cytotoxicity; an infrared wavelength amenable to deep tissue imaging; and the ability to label excitable cells, including cardiac myocytes and neurons. Consequently, the voltage-sensitivity of ICG has significant implications for new applications in research and the clinic.

[0007] One aspect of the invention provides a method of determining electrical activity in a cell. One embodiment of the method includes

labeling the cell with a composition including ICG and illuminating the cell with electromagnetic radiation having a first frequency range, where the first frequency range is within a spectral absorption frequency range of the ICG. This embodiment also includes measuring a variation in intensity of spectral emission of the ICG, where the variation in intensity of spectral emission is indicative of the electrical activity in the cell. The measuring of the variation in intensity of spectral emission of the ICG includes measuring the intensity of spectral emission at least 10 times within a period of one second.

[0008] Another aspect of the invention provides a method of diagnosing a disease of the eye in a subject. One embodiment of the method includes labeling a retinal ganglion cell present in the retina of the subject with a composition comprising ICG and illuminating the retinal ganglion cell with electromagnetic radiation having a first frequency range, where the first frequency range is within a spectral absorption frequency range of the ICG. This embodiment also includes illuminating the retinal ganglion cell with electromagnetic radiation having a second frequency range, where the second frequency range is within a frequency range of visible light and measuring a variation in intensity of spectral emission of the ICG. In one embodiment, the measuring of the variation in intensity of spectral emission of the ICG includes measuring the intensity of spectral emission at least 10 times within a period of one second. The variation in intensity of spectral emission is indicative of presence or absence of the disease of the eye.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] The invention may be more fully understood by reading the following description in conjunction with the drawings, in which:

[0010] Figure 1 is an illustration showing that ICG fluorescence accurately traces low (left hand) and high (right hand)-frequency action potentials triggered sequentially in a single cell, expressing voltage-gated sodium and voltage-gated potassium channels. The ICG fluorescence is capable of following action potentials firing at a rate of 70 Hz. Traces shown were taken from a single sweep, without averaging.

[0011] Figure 2 is a Fundus image of eye of patient with retinitis pigmentosa, one day after a one minute exposure to 100 μ M ICG. Retinal ganglion cells are white from retained ICG fluorescence. The image was taken with a Heidelberg scanning laser ophthalmoscope. The arc shows an example of a binning procedure that could be used to examine particular bundles of retinal ganglion cells for dysfunction, using ICG.

[0012] Figure 3 are graphs showing how ICG fluorescence responds to changes in membrane potential. (left) Simultaneous electrophysiological and optical recordings of a *Xenopus laevis* oocyte briefly labeled with ICG show that ICG fluorescence is voltage-sensitive. Oocyte membrane potential was held at -60 mV and then pulsed for 300 ms to potentials ranging from -120 mV to +120 mV (top, pulse protocol); Holding potential, -60 mV, black, crosses. +120 mV, black, circles; -120 mV, black, stars; +80 mV, gray, diamonds, +40 mV, gray squares. Optical recordings (bottom; black circles, is +120 mV, black stars is -120 mV) of ICG fluorescence displayed a clear decrease in fluorescence as membrane potential increased and an increase in fluorescence as membrane potential decreased. (right) The voltage-sensitive fluorescence change of ICG is roughly linear (line fit to black circles data points) with respect to change in membrane potential, with an amplitude change of approximately 1.9% per 100 mV of membrane potential change. The holding potential for this experiment was -60 mV.

[0013] Figure 4 are graphs showing how ICG distinguishes normal and diseased action potentials in oocytes. (a) *Xenopus laevis* oocytes

coinjected with voltage-gated sodium and potassium channel cRNA behave like an excitable cell and fire action potentials (bottom) when held under current clamp. ICG fluorescence changes (top) can detect these action potentials at a rate of 107 Hz. The left-most arrow represents the onset of stimulating current injection, while the right-most arrow indicates the end of stimulation. (lower left, right) ICG fluorescence changes (black line, inverted) are rapid and precise enough to distinguish between healthy action potentials (left, dashed line) produced from wild-type sodium channels and diseased action potentials (right, gray dashed line) produced from sodium channels with the myotonic substitution G1306E.

Fluorescence slightly lags behind membrane potential due to the response time of the dye. The cells are being stimulated for the entire time course of these panels. (d) In cells expressing myotonic sodium channels, a brief stimulus (top, current clamp protocol) was sufficient to elicit a train of action potentials that only ceased upon significant hyperpolarization of the membrane (bottom), as expected in a myotonia. ICG fluorescence (middle) successfully followed each one of these action potentials.

[0014] Figure 5 are graphs showing how ICG follows electrical activity in mammalian cells. (a) Rat cultured dorsal root ganglion cells fired action potentials (dotted gray line; black and gray arrows – stimulus start, end) under current-clamp that were tracked by ICG fluorescence changes (black, inverted, low-pass filtered at 225 Hz; black arrow – relative fluorescence change of 0.2%). ICG fluorescence is shown here inverted and low-pass filtered at 225 Hz. The stimulating current injection occurred between the black and red arrows. The blue arrow shows the size of a relative fluorescence change of 0.2%. (b) Spontaneous membrane potential changes resulting in contractions of cardiomyocyte syncytia were followed by ICG fluorescence changes. Like all voltage-dependent traces in this work, data in this figure are single acquisitions with no averaging of different recordings.

[0015] Figure 6 includes a photograph and graphs showing how ICG responds to stimulation of living brain tissue. (a) Cultured hippocampal brain slices from neonatal rats maintain glial cells and the three-dimensional structure of the hippocampus. In the lower left of the image, the bipolar stimulating microelectrodes can be seen. The scale bar is 0.5 mm. (b) In a hippocampal slice, ICG responds differentially to no stimulus (top curve) and stimuli of increasing intensity (top to bottom curves, in order of increasing stimulus amplitude). Smaller stimulus traces (i.e. second from top) show complete fluorescence recovery while traces with larger stimuli (i.e. bottom curve) do not fully recover within this time course. Traces are offset vertically for clarity. For this and subsequent panels, the arrowhead marks the time of the stimulus, while the scale bar shows the size of a given relative fluorescence change. (c) Application of tetrodotoxin reduces the response of ICG to stimuli of a fixed intensity (light gray – before TTX; ; gray, dark gray, black – increasing time post-TTX; low-pass filtered at 40 Hz). Black arrow denotes stimulus. These traces were taken over a period of 12 minutes. (d) Washout of TTX recovers excitability and ICG response (the black curve corresponds to before washout and the dark gray curve corresponds to 5 minutes after washout). Fluorescence traces in c and d have been low-pass filtered at 40 Hz.

[0016] Figure 7 are photographs showing how ICG labels RGCs in patients after a common ophthalmological procedure. (a) ICG intraoperatively applied to the retina of a patient with retinitis pigmentosa clearly stained RGC axons when observed with a scanning laser ophthalmoscope on post-operative day 1. Blood vessels converging at the optic disc (dark region on right) show little to no ICG fluorescence, due to the rapid rate of ICG clearance from blood. The dark region in the center of the image is the macula with lower RGC density. ICG fluorescence is clearly seen in numerous RGCs extending from the macula to form the optic nerve, as expected. The scale bar is 800 micrometers. Observation

of ICG voltage dependence in human patients has not yet been attempted.
(b) A higher magnification image of the same retina. The scale bar is 800 micrometers.

[0017] Figure 8 is a graph showing kinetics of the ICG voltage-sensitive response. The kinetics of the change in fluorescence of ICG in response to a change in membrane potential can be fit by a double exponential function (circles, see inset for residuals). The time constant of the faster component, which comprises the majority of the fluorescence change, was 4.0 ms, while the time constant of the slower component was 62 ms.

[0018] Figure 9 includes graphs showing that ICG follows the shapes of action potentials. ICG fluorescence (top and third from top, Normalized fluorescence) follows action potentials (membrane potential) fired by excitocytes in response to an injection of current (beginning at left-most arrow and ending at right-most arrow). The fluorescence follows the shape of the action potentials, discriminating between action potentials generated by wild-type Nav1.4 (top pair, full time course of Fig. 6b) and action potentials generated by mutant Nav1.4 with the G1306E myotonia substitution (bottom pair, full time course of Fig. 6c).

[0019] Figure 10 includes graphs showing unprocessed data from cultured rat neurons demonstrates voltage sensitivity. Unprocessed data of ICG fluorescence from Fig. 7a in the main text (top). This is an average of all pixels in the field of view, including those outside of the neuron, causing the reduction in signal to noise. Additionally, no bleach correction or filtering has been applied. An injection of depolarizing current was given from the left-most arrow until the right-most arrow, resulting in an action potential (bottom curve, identical to Fig. 7a).

[0020] Figure 11 includes graphs and an image showing fluorescence changes in cardiomyocyte syncytia are not due to contractile movement (a,b,c) Motion in the z-axis that takes the syncytium out of the

microscope's focal plane would result in fluorescence signals similar to what we see in (a), the unprocessed ICG fluorescence from a cardiomyocyte syncytium (same data as Fig. 7b). However, intentionally focusing the microscope above (b) or below (c) the syncytium should produce the opposite effect, as the syncytium would move into the new focal plane, increasing fluorescence during each contraction. Instead, all focal planes display similar behavior, suggesting that no prominent z-axis motions are responsible for observed ICG fluorescence changes. (d,e) Similarly, motion in the x-y plane could decrease fluorescence by removing bright areas or bringing dim areas into the field of view. In this case, different parts of the field of view should have drastically different signals with some regions displaying net increases in fluorescence by chance. Instead, we observe in (d) a systematic decrease in fluorescence in each individual syncytial quadrant; the quadrants are mapped in (e): left-upper, lowest curve in (d); right-upper second curve from bottom in (d); right-lower top curve in (d); left-lower third curve from bottom in (d); center fourth curve from bottom in (d). In all panels, ADU stands for analog-to-digital units, the camera output. The scale bar is 40 micrometers.

[0021] Figure 12 includes graphs showing that in rat brain slices, ICG responds differently to no stimulus (black) and stimuli of increasing intensity (dark gray, gray, light gray, dark gray dotted; increasing stimulus amplitude). Smaller stimulus traces (i.e. dark gray) show complete fluorescence recovery while traces with larger stimuli (i.e. dark gray dotted) do not fully recover within this time course. On the right is the same data prior to bleach correction and spatial filtering. Note the improvement in signal to noise ratio accomplished by appropriate filtering unprocessed ICG fluorescence traces from hippocampal slices demonstrate voltage sensitivity. (a) Unprocessed data of slice stimulations at 200 ms (bottom trace) and 400 ms (top trace,) after recording. Changing the timing of the stimulus pulse (arrows for the bottom and top traces) changes the timing of

the ICG fluorescence change. (b) The data shown in Fig. 8b, before processing. All unprocessed data in this figure is an average of all pixels in the image and has no filtering or bleach correction. (c) Similar to Fig. 8b in the main text, but the duration of the stimulus pulse is varied rather than the intensity (In rat brain slices, ICG responds differently to no stimulus (black) and stimuli of increasing intensity (dark gray, gray, light gray, dark gray dotted; increasing stimulus amplitude). Smaller stimulus traces (i.e. dark gray) show complete fluorescence recovery while traces with larger stimuli (i.e. dark gray dotted) do not fully recover within this time course. On the right is the same data prior to bleach correction and spatial filtering. Note the improvement in signal to noise ratio accomplished by appropriate filtering.'). (d) As in c, but unprocessed data. (e, f) The data shown in Fig. 7c and d in the main text, shown here without processing. As the unprocessed traces (b, d, e and f) demonstrate, potential artifacts that could arise from our data processing are not responsible for the observed changes in ICG fluorescence. All panels in this figure have traces offset vertically for clarity; for b-f, the time point of stimulation for all conditions is marked by the black arrow.

[0022] Figure 13 is a graph showing an example of a change in fluorescence across the entire field of view of the barrel cortex in a living rat brain. The data were post processed to apply a baseline subtraction.

[0023] Figure 14 are images of the data illustrated in Figure 13. The images show that neuronal activity in response to whisker stimulation can be spatially localized within a region of the barrel cortex. Imaging is approximately 3.5 mm in each direction, and black corresponds to increased electrical activity.

DEFINITIONS

[0024] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of

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ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

[0025] As used herein the terms “comprise(s),” “include(s),” “having,” “has,” “can,” “contain(s),” and variants thereof, are intended to be open-ended transitional phrases, terms, or words that do not preclude the possibility of additional acts or structures. The present invention also contemplates other embodiments “comprising,” “consisting of” and “consisting essentially of,” the embodiments or elements presented herein, whether explicitly set forth or not.

DETAILED DESCRIPTION

[0026] For the purposes of promoting an understanding of the principles of the invention, reference will now be made to the embodiments illustrated in the drawings and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, and alterations and modifications in the illustrated device, and further applications of the principles of the invention as illustrated therein are herein contemplated as would normally occur to one skilled in the art to which the invention relates.

[0027] Indocyanine green (ICG) is a fluorescent dye with FDA approval for numerous uses in medical imaging, including cardiac output monitoring and ophthalmic angiography. One aspect of the present invention provides a method of measuring the electrical activity of a cell by a method including labeling the cell with ICG and detecting changes in the membrane potential

of the cell by measuring changes in the fluorescence intensity of the ICG label. This method has many uses for monitoring the electrical activity of human cells *in vivo*, such as in muscle, heart and brain tissue. In certain embodiments, ICG is used to label cardiac myocytes or neurons.

[0028] One aspect of the present method allows the diagnosis of pathologies in such cells, for example retinal ganglion nerve cells, by observation of abnormal or nonexistent excitation patterns. Cells, such as retinal ganglion nerve cells, can be labeled by ICG and subsequently imaged *in vivo* in human or veterinary patients using equipment and methodologies allowing for the detection and analysis of ICG fluorescence signals produced by the natural electrical activity of these cells. In certain embodiments, the ICG is administered to the retina by topical or intravenous administration.

[0029] In various embodiments, the present method offers advantages over currently available methods. Current techniques for measuring electrical activity, such as electroencephalography or electrocardiography, suffer from low spatial resolution, while other techniques such as fMRI are costly and indirect, and intracellular implantation of electrodes is highly invasive. At present, no other voltage-sensitive fluorescent dyes are approved for use in humans, and many are highly toxic. Therefore, the use of the voltage sensitivity of ICG and its application in a clinical setting provides a useful tool for diagnosing diseases of excitable cells.

[0030] In various embodiments, voltage-sensitive dyes localize to cell membranes where their fluorescence intensity changes in response to changing membrane potential. Voltage-sensitive dyes thus provide a way to observe cellular electrical activity without the physical limitations imposed by electrodes; this method is commonly used in research laboratories to monitor membrane potential with a resolution of a few microns from large populations of cells. In complex tissues, these imaging methods can then be used to produce spatiotemporal maps of excitable

cell physiology. Despite this capability, there are three main obstacles that currently prevent the widespread use of these dyes in a clinical setting. First, prior to this report, no voltage-sensitive dyes have ever been available for administration in humans. Second, most voltage-sensitive dyes use visible wavelengths of light that prevent imaging of tissues beneath the surface of the skin. Third, many of these dyes produce significant toxicity or off-target effects.

[0031] We show that ICG is voltage sensitive. ICG has been FDA-approved for use in ophthalmic angiography, as well as in tests of cardiac output and hepatic function and is additionally used off-label in a number of surgical applications. Since ICG absorbs and fluoresces in tissue-penetrating infrared wavelengths, it may be imaged through the skin in tissues up to two centimeters deep. Finally, it has been used as an intravenous contrast agent in patients for over 50 years and is known to have low toxicity.

[0032] Imaging ICG allows for the measurement of large populations of cells at high resolution with different optical acquisition systems optimized for specific applications. For instance, in ophthalmic angiography, a section of the retina several millimeters wide can be imaged with a resolution of approximately 20 microns at video framerates. Consequently, when used as a voltage sensitive dye, ICG may allow for wide-field spatiotemporal imaging of retinal ganglion cell and photoreceptor function for the clinical assessment of common retinal degenerative diseases such as glaucoma. More generally, ICG voltage-sensitivity may serve an important role in extending the capabilities of modern electrophysiological techniques for disease diagnosis and monitoring.

[0033] In one embodiment, recordings of ICG voltage sensitivity are performed using a cooled CCD camera acquiring at a typical frame rate of 500 Hz. Excitation is performed using a light source having near-infrared emission, for example a 780 nm laser (WorldTechLabs) at power levels at

or below 20 microW. In other embodiments, excitation is performed with a broadband halogen lamp with an infrared filter.

[0034] Emission may be captured with a T810lpxr dichroic (Chroma) and an emission filter ET845/55m (Chroma). Cells are typically labeled for 10 minutes in 20 microM ICG, and then rinsed in dye-free solution for at least five minutes. The detector may be a photodetector with a fast imaging rate and good sensitivity in the near infrared. For example, a camera or photodiode array may allow an image to be produced and different parts of the image separately analyzed. But for convenience or other reasons, some embodiments may include only a simple detector, such as a photodiode or photomultiplier tube.

[0035] A mechanism of stimulating neuronal activity in a time-locked fashion is beneficial, although not necessary to the technique. This method would, of course, depend on the anatomy being imaged. In the case of the eye, for example, while ICG is being continuously imaged with invisible infrared light, one may also want a visible light source that can be targeted to particular locations on the retina to provide selective stimulation of those specific locations. If one does not use a particular stimulus, one would simply rely on changes in membrane potential, and the resulting change in dye emission, due to intrinsic processes.

[0036] A number of currently available instruments may be adapted for the ICG imaging/diagnostic method. One example is instrumentation by BrainVision/SciMedia, including imaging instrumentation and systems for imaging membrane potential, calcium concentration and other high speed imaging.

[0037] In other embodiments, a scanning laser ophthalmoscope, typically used for imaging the retina during ophthalmological procedures, may be used. Such a system may be modified for ICG imaging by including a time-locked stimulus to stimulate the area being imaged. Typically, this stimulus will be of visible light. Furthermore, in

ophthalmological settings, a simple light detector such as a photodiode may be used in place of a camera.

[0038] Other possible refinements to available instrumentation include: improvement of image processing algorithms, especially to deal with factors like bleaching and movement, and using pulses of stimulus light rather than constant excitation of ICG to better preserve fluorescence signal for a longer time duration.

[0039] For example, in a clinical application, ICG is typically applied topically to the retina for one minute in concentrations ranging from 100-500 microM, and can be applied intravenously in concentrations as high as 30 mM. For staining of the retinal ganglion cell neurons, ICG fluorescence persists for as long as weeks or even months after dye application but can be clearly seen one day after application.

[0040] In one embodiment, the voltage-sensitivity of fluorescence recorded begins diminishing after one hour, and is effectively zero by 24 hours later. It is believed, but not relied upon for the purposed of the present embodiments, that long-term ICG staining may be due to aggregates of the dye. In one embodiment, recordings of changes in membrane potential are taken more closely to the time of dye application than ophthalmologists are typically imaging the retina post-dye application. For example, such changes may be recorded within 1, 2, 3, 4, 5, 6, 12, 18 or 24 hours post-dye application.

[0041] The voltage-sensitivity of ICG is found to be relatively rapid, with kinetics on the tens of milliseconds time scale (Figure 3), and to respond to changes in membrane potential over a physiological range with a near-linear response (Figure 3). In one embodiment, such recordings image individual action potentials produced in oocytes at 70 Hz (Figure 1), about two to three times the typical basal firing rate found in the retinal ganglion cells of the macaque or mouse. Such recordings also demonstrate that ICG voltage sensitivity can accurately measure action potential rate

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changes over time in a single cell. In one embodiment, ICG fluorescence can track changes in firing rate in excitable cells over time, *in vivo*.

[0042] In the eye, action potential imaging can take place at a multicellular level. In one embodiment, lower wavelength visible light is used to stimulate a specific area of the eye, and simultaneous ICG imaging is performed using a scanning laser ophthalmoscope (SLO). SLOs typically use a CCD and excite at 790 nm, very close to the 780 nm typically used for imaging experiments. Currently available SLOs image at a resolution between 5 and 20 microns at a rate of 10-26 images per second (Table 1). This imaging rate is limited largely by the relatively large area of retina being imaged, as the maximum image refresh rate is 60 Hz.

[0043] Table 1.

Name	Wavelength (nm)	Optical Resolution (μm)	Speed (Hz)
Nidek F-10	790	16-20	10-26
Heidelberg Spectralis HRA	790	5-11	~16

[0044] Two major forms of imaging are provided: a high-speed, low-area imaging mode (detail) and a low-speed, high-area imaging mode (map). Map mode will produce images of ICG fluorescence over a large area of the retina, as is seen in current standard ICG images taken with SLOs (for example, see figure 2). Even if obtained at the current relatively slow rates of ten to twenty images per second, these images distinguish abnormal responses to visible light applied in concert with the ICG imaging. This is because retinal ganglion cells are comprised of cells that increase their firing rate in response to light, and cells that decrease their firing rate in response to light. Reductions in firing rate can only go from the basal level of 20 to 30 Hz to 0 Hz, while increases in firing rate go to 75-100 Hz or even higher. Thus, the application of light should produce a net increase

in the firing rate, and a corresponding net decrease in the ICG fluorescence compared to when the light is not applied.

[0045] Post-processing image analysis of map mode will improve resolution by binning pixels corresponding to estimated bundles of retinal ganglion neurons (see the arc, figure 2). In this way, clinicians can more precisely define the pathological area of the retina.

[0046] When using detail mode, acquisition will focus in on a particular region of interest, called out by results from map mode or other clinical data. This mode will improve temporal resolution by reducing the area examined, although the spatial resolution can remain similar. Current ophthalmological instrumentation is incapable of imaging single spikes, but acceleration of sampling to 1 to 2 kHz should be sufficient to allow for single spike imaging even when retinal ganglion neurons are firing at their fastest rates. However, in certain embodiments, single spike imaging is not necessary for clinical applications, which are concerned with grosser defects. Single spike imaging can also prove unfeasible due to small neuronal size and the heterogeneous firing patterns of neurons within the population that is being imaged. Population data at an improved spatial resolution, as acquired in detail mode, can help in the diagnosis and understanding of numerous pathologies.

[0047] These pathologies include numerous optical diseases. A common disease that could benefit from ICG voltage sensitive imaging is glaucoma, which affects an estimated 44 million people worldwide. Models of glaucoma and its presentation in the clinic suggest that the disease begins with damage to the axons of the retinal ganglion neurons, which then spreads. The voltage-sensitive fluorescent signal from ICG applied to the retina should be virtually entirely carried by these axons, providing a unique and novel method for diagnosing early signs of dysfunction. Rather than measuring substitutions for neuronal dysfunction, such as the thickness of the nerve fiber layer, ophthalmologists can more

directly measure neuronal function using ICG fluorescence recordings. Earlier diagnosis can allow for earlier treatment; if diagnosis is early enough, new medications may be found to be more effective at reducing disease progression and symptoms.

[0048] Other retinal ganglion cell neuropathies that ICG imaging can aid in diagnosing include hereditary optic neuropathy and optic disc drusen. Additionally, retinal ganglion cell dysfunction is an early marker of active multiple sclerosis. ICG voltage-sensitive fluorescence imaging can provide an additional marker of multiple sclerosis, promoting earlier diagnosis and treatment to slow disease progression, and testing treatment efficacy. Finally, retinal ganglion cell death increases in diabetes.

[0049] Other potential clinical applications include measuring electrical activity in the brain or heart, if appropriate staining of these tissues can be achieved. This could aid clinicians in the localization of dysfunctions of brain or heart firing, such as seizures or arrhythmias.

[0050] We have shown for the first time that the FDA-approved fluorescent dye ICG is voltage-sensitive and can measure electrical activity in excitable frog oocytes, as well as in rat neurons, cardiomyocytes, and brain tissue. Given the long history of ICG in clinical use, its voltage sensitivity provides a foundation for numerous novel diagnostic clinical tools. ICG has proven itself safe in a large number of clinical contexts, including angiography, ophthalmic surgery, and neurosurgery. Despite this track record, concerns of toxicity persist, particularly when high concentrations of the dye are used. Prior studies that reported ICG toxicity required concentrations ranging from 0.25 mg/ml to 5 mg/ml. In contrast, the highest concentration of dye used to observe electrical activity in this study was 0.062 mg/ml. This suggests that excitable cell function can be assessed with low ICG concentrations; it should be practical to achieve these doses in clinical settings with few side effects.

[0051] Another potential limitation is the well-known aggregation of ICG in aqueous solution. While we observed this phenomenon, preparing the dye immediately prior to use, as is done in clinical settings, and using lower concentrations of dye minimized aggregation during labeling. ICG aggregation or endocytosis can also occur once a cell membrane has been labeled. This phenomenon creates a critical period of time when the voltage sensitivity of the fluorescence can be tracked. We examined the time course of aggregation in oocytes and found that it became significant between one and two hours in oocytes. As ICG washout from the blood occurs fully in 10 to 15 minutes⁸, ICG voltage sensitivity should be visible for half an hour or more when ICG is applied to tissue intravenously. Applying ICG through other routes, such as topically or intravitreally, likely results in different durations of voltage sensitivity. The voltage sensitivity of ICG described here is not limited to particular labeling or recording conditions. A voltage-sensitive signal was observed when exciting the dye with both monochromatic and broad-band illumination sources. Labeling occurred in three different salt solutions with various additives, at different concentrations, for varying amounts of time. Furthermore, some labeling procedures took place in the presence of either the glycoprotein fibronectin or the cationic polymer polylysine; neither one appeared to label or fluoresce significantly. Similarly, recording of ICG fluorescence from plasma membranes was achieved through a variety of substances, including the natural vitelline membrane of the oocyte and coated plastic cell culture plates. Finally, recordings were taken at temperatures ranging from 19 °C to 30 °C with no apparent changes in ICG's voltage sensitivity. The robustness of ICG voltage sensitivity in different situations suggests its use could be broadly applicable.

[0052] Within a few years after the discovery that voltage-sensitive dyes could be used to track changes in invertebrate nerve membrane potentials,

these dyes were successfully applied to a diverse range of tissues, including cardiac muscle, skeletal muscle, and individual neurons. However, prior to the present embodiments, no voltage-sensitive fluorescent dye has been described that is also safe for human clinical use. The voltage sensitivity of ICG reported here appears similarly applicable to a wide range of excitable tissues, and thus could provide the basis for the development of new tools to diagnose human diseases.

[0053] One area of immediate interest is assessing retinal ganglion cell (RGC) activity since these cells are easily accessible to light. ICG dye would be ideal for imaging the voltage activity elicited by stimulating light within the retina, since the excitation and emission wavelengths for ICG are invisible to rods and cones and thus will not interfere with the visual stimuli used to gauge retinal function. ICG has previously been shown to stain RGCs in rats and rabbits.

[0054] Furthermore, studies in humans have incidentally demonstrated that ICG labels RGCs following routine retinal surgical procedures, although the methods used were not optimized to observe these cells. While we have not yet been able to test for voltage dependence in human patients, here we find that distinct RGC imaging can be obtained by labeling with a low concentration of ICG (Figure 7). For this image, the retina was stained with 0.08 mg/ml of ICG for one minute and examined one day after dye exposure. Under the conditions used for obtaining these images, no clinical evidence of retinal toxicity was observed. Imaging the voltage sensitivity of ICG could allow the degenerative processes of retinal diseases such as retinitis pigmentosa, age-related macular degeneration, and glaucoma to be directly mapped. Physiological changes during retinal vascular occlusive disease, a common sequela of diabetes and hypertension, would also likely be observable. ICG fluorescence should provide an improvement in both spatial and temporal resolution over the

multifocal electroretinogram, the only option currently available for localizing dysfunctions of retinal electrical activity.

[0055] The prospective utility of ICG fluorescence as a readout of membrane potential is not limited to the retina. We have also established that ICG can track electrical activity in mammalian heart and brain tissue. As a result, when these tissues are exposed during surgery, ICG may prove useful in providing real-time readouts of activity by spatially locating the loci of abnormal or absent activity. In addition, although these tissues are not as optically accessible as the retina, it has been shown that the lymphatic system, for example, can be imaged through human skin using ICG fluorescence. Looking towards the future, if imaging techniques improve to the point that similar fluorescence readouts are attainable from ICG-stained excitable tissues, many cardiac and neural diseases could potentially become diagnosable in a clinical setting by imaging the voltage sensitivity of ICG.

[0056] In certain embodiments of the present method, observations are taken after the blood within the imaging region has been essentially cleared of ICG. Thus, only the dye that remains in the tissues themselves is imaged. Thus, a constant concentration of ICG is observed and changes in ICG fluorescence are indicative of changes in membrane potential (electrical activity) of the stained tissues. Such relatively-smaller changes may be masked by the large fluorescence changes produced by the diminishing ICG concentrations that are imaged by a method of measuring tissue perfusion rate.

[0057] The present method may be used to measure voltage sensitivity over a period of many minutes at a time. However, seconds or milliseconds are frequently sufficient to observe the desired changes in electrical activity. Typically, the frame rates used are around 100-500 Hz. Dye may be delivered to the imaging area by, for example, intravenous injections, intrathecal injections into the brain or spinal cord, intravitreous

injections into the eyeball, and direct topical staining of exposed tissues of interest (such as an exposed brain during neurosurgery).

[0058] Although a stimulus may not be necessary in some embodiments of the present method, in many embodiments measurement of ICG voltage sensitivity will involve a specific stimulus to trigger excitation in defined patterns for observation with ICG. Many embodiments involve stimuli, including electrical, olfactory, and tactile stimuli when using different preparations. However, as we have shown with spontaneously beating cardiomyocytes (heart tissue), such stimuli is not required for ICG to appropriately follow electrical activity changes.

Example 1 - Optical Equipment

[0059] ICG fluorescence was monitored using an Evolve 128 EMCCD camera (Photometrics, Tucson, Arizona) attached to an Olympus IX71 inverted microscope (Center Valley, Pennsylvania). The camera CCD is 128x128 pixels and can acquire images at up to 500 Hz full-frame. Binning pixels allowed us to acquire images at 1.8 kHz for preparations in which high spatial resolution was unnecessary, such as oocytes and individual neurons. The dye was excited with a 780 nm diode laser (World Star Technologies, Toronto, Canada), and the ICG filter set from Chroma Technology Corp. (49030 ET, Bellows Falls, Vermont) was used to isolate fluorescence emission. Various microscope objectives were used for different preps ranging from a 20X/0.45NA air lens to a 60X/1.45NA oil immersion lens. Raw excitation power varied widely depending on the objective lens in use, but illumination intensity was typically about 0.5 W/cm². Retinal imaging was performed using a scanning laser ophthalmoscope (HRA-2, Heidelberg Engineering, Heidelberg, Germany). Excitation of ICG was performed at 795 nm and emission was imaged at wavelengths longer than 810 nm using a 30 degree field of view.

Example 2 - Electrophysiological Equipment

[0060] Two-electrode voltage clamp was performed with a Warner Instruments OC-725A amplifier (Hamden, Connecticut). For current clamp, a 100 M Ω resistance was placed in series with the oocyte. Micropipettes were pulled to approximately 0.5 M Ω on a Flaming/Brown micropipette puller (Sutter Instruments, Novato, California, model P-87) and filled with 3 M KCl. Stimulation of rat brain tissue was accomplished via a homemade platinum bipolar field electrode. Patch-clamp of cultured cells was performed using a Dagan 3900 integrating patch clamp amplifier (Minneapolis, Minnesota). Patch pipettes were pulled to approximately 5 M Ω on a CO₂ laser micropipette puller (Sutter Instruments, model P-2000), fire polished immediately prior to use, and filled with patch pipette solution (see Materials and Solutions below). Amplifier outputs were filtered through an 8-pole low-pass Bessel filter (Frequency Devices, Ottawa, Illinois, model 950L8L) and digitized with an SBC-6711-A4D4 data acquisition board (Innovative Integration, Simi Valley, CA). Filter cutoff frequency was set to approximately one fourth of the digitizing sample frequency. The acquisition board was also used to control the clamp and synchronize optical and electrical acquisitions. Software programs for both electrical control and camera control were written in-house.

Example 3 - Tissue preparation protocols

[0061] Oocytes were digested in OR2 with collagenase and bovine serum albumin added and incubated in SOS. Excitocytes were prepared by coinjecting cRNAs of Nav1.4 α and β subunits with cRNA of a clone of the Shaker Kv channel with fast inactivation removed (as described by Shapiro, M. G., Homma, K., Villarreal, S., Richter, C.-P. & Bezanilla, F. "Infrared light excites cells by changing their electrical capacitance." *Nat. Commun.* 3, 736 (2012) and Shapiro, M. G., Priest, M. F., Siegel, P. H. & Bezanilla, F. "Thermal Mechanisms of Millimeter Wave Stimulation of

Excitable Cells.” Biophys. J. 104, 2622–2628 (2013) and waiting 2-4 days for channels to express sufficiently. Uninjected oocytes were labeled in 0.0155 mg/ml (20 microM) ICG in SOS for 5-15 minutes. Labeled oocytes were then rinsed and recorded in SOS. Labeling and recording protocols for excitocytes were identical to those for uninjected oocytes apart from the recording solution which had reduced calcium in excitocyte recordings. Oocyte recordings were performed at room temperature of around 19 °C. Excitocyte recordings were performed at room temperature of around 19 °C, except for tests of high frequency firing, which were performed in a bath at around 30 °C.

[0062] Dorsal root ganglia were harvested from spinal cords taken from P2-P6 Sprague-Dawley rats and digested to isolate individual neurons. These cells were then cultured in DMEM (supplemented with 10% fetal bovine serum and 100 U/ml of penicillin and 100 micro g/ml of streptomycin) for 24 hours prior to recording. Immediately prior to use, cells were rinsed in patch bath solution, labeled in 0.00775 mg/ml (10 micro M) ICG for one to two minutes and rinsed in patch bath solution again for recording. Neuron recordings were performed at room temperature.

[0063] Cardiomyocytes were cultured from hearts harvested from P1 neonatal Wistar rats. Hearts were digested in EBSS with 0.08% trypsin similar to prior descriptions. Modifications to the protocol included shaking in an incubator rather than on a magnetic stirrer and substituting EBSS with fetal bovine serum for cold culture media with a trypsin inhibitor. Cells were plated on glass coverslips coated in fibronectin. Cardiomyocytes were incubated in DMEM with 10% fetal bovine serum and 100 U/ml of penicillin and 100 micro gm/ml of streptomycin added. Cardiomyocytes were labeled in 0.00155 mg/ml (2 micro M) ICG in DMEM for 1-10 minutes. Cardiomyocyte recordings were performed at 24 °C.

[0064] Hippocampal cultured brain slices were prepared as described by Pusic, A. D., Pusic, K. M., Clayton, B. L. L. & Kraig, R. P. "IFN γ -stimulated dendritic cell exosomes as a potential therapeutic for remyelination." *J. Neuroimmunol.* 266, 12–23 (2014); Pusic, A. D. & Kraig, R. P. "Youth and environmental enrichment generate serum exosomes containing miR-219 that promote CNS myelination." *Glia* 62, 284–299 (2014) and Pusic, A. D., Grinberg, Y. Y., Mitchell, H. M. & Kraig, R. P. "Modeling neural immune signaling of episodic and chronic migraine using spreading depression in vitro." *J. Vis. Exp. JoVE* (2011). doi:10.3791/2910.

[0065] During labeling and prior to recording, slices were kept in a humidified carbogen (95% O₂ / 5% CO₂) atmosphere in a pipette tip box, similar to prior descriptions⁶. Slices were labeled in .031 mg/ml (40 micro M) ICG in GBSS for 15-20 minutes. Slices were subsequently rinsed in GBSS and recorded in fresh GBSS. Tetrodotoxin stock was diluted to 1 micro M in GBSS immediately before use. After application, it was washed out with fresh GBSS. All brain slice solutions were bubbled with carbogen prior to use. Brain slice recordings were performed at room temperature; activity was stimulated with a field electrode using symmetric bipolar pulses to prevent electrode polarization. The maximum stimulus duration was 6 ms in total (a 3 ms pulse in one polarity immediately followed by a 3 ms pulse in the opposite polarity).

[0066] All tissue harvesting was performed in accordance with protocols approved by the University of Chicago Animal Care and Use Committee.

[0067] For the retinal images, a 0.08 mg/ml ICG in a 5% dextrose solution was directly applied to the retina of a patient with retinitis pigmentosa to aid in peeling the epiretinal membrane during a surgical procedure. When dye exposure was limited to 60 seconds under low light conditions, no clinical evidence of retinal toxicity was observed. The patient's retina was imaged one day after this procedure.

Example 4 - Materials and Solutions

[0068] ICG was obtained in both the clinical formulation as IC-Green (Akorn, Lake Forest, Illinois) and non-clinical formulation as Cardiogreen (Sigma-Aldrich, St. Louis, Missouri). No appreciable differences were found between the two sources. Dye was dissolved in DMSO at a concentration of 20 mM and stored in aliquots at -80 °C. Immediately prior to use, aliquots were thawed and sonicated briefly.

[0069] Antibiotics and other salt solution additives: Streptomycin (Sigma-Aldrich), penicillin (Sigma-Aldrich), gentamicin (Sigma-Aldrich), trypsin-TRL3 (Worthington, Lakewood, New Jersey), fetal bovine serum (ATCC, Manassas, Virginia), and DNaseI (Sigma-Aldrich)

[0070] Tetrodotoxin (Abcam, Cambridge, United Kingdom) was stored in a 3 mM stock solution in water.

[0071] Concentrations are in mM unless otherwise stated

[0072] OR2: 82.5 NaCl, 2.5 KCl, 2 MgCl₂, 10 HEPES, pH 7.4

[0073] Standard oocyte solution (SOS): 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, pH 7.4, 50 micro gm/ml of gentamicin

[0074] Excitocyte recording solution: Same as SOS but with 0.6 CaCl₂ and no gentamicin.

[0075] EBSS: 132 NaCl, 5.3 KCl, 1 NaH₂PO₄, 10 HEPES, 5.5 glucose, pH 7.4

[0076] DMEM: HyClone-DMEM/High Glucose with high glucose, L-glutamine, sodium pyruvate, and phenol red (LifeTechnologies, Carlsbad, California, currently GELifeSciences, Piscataway, New Jersey)

[0077] Patch bath solution: 132 NaCl, 6 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 HEPES, 5 glucose, pH 7.4

[0078] Patch pipette solution: 150 KF, 10 NaCl, 4.5 MgCl₂, 2 ATP, 9 EGTA, 10 HEPES, pH 7.4, filtered

[0079] GBSS (Sigma) or made with 137 NaCl, 5 KCl, 1.5 CaCl₂, 1 MgCl₂, 2.7 NaHCO₃, 0.22 KH₂PO₄, 0.28 MgSO₄, 0.85 Na₂HPO₄, 5.6 glucose, pH 7.4.

Example 5 - Data Analysis

[0080] Movies were converted into time-dependent traces by averaging pixel values for each frame of a given movie. Images of oocyte membranes and cardiomyocyte syncytia showed relatively little variation in signal size across different regions of the field of view, so all pixels of each frame were used. Dorsal root ganglion neurons, in contrast, occupied only about 30% of the field of view, so a level threshold was used to isolate neuronal pixels from the non-fluorescent background. Finally, the hippocampal slices contained regions that were largely quiescent.

[0081] Although the voltage-dependent signal was clearly visible when averaging all pixels, the signal-to-noise ratio was greatly improved by selecting only regions of the image that displayed the most voltage sensitivity. These regions were located by first identifying the single pixels which contained the largest signal and then using morphological image processing to create a binary mask for the image. Morphological operations were based on a diamond-shaped structuring element and utilized repeated openings and closings. Although the exact parameters used were arbitrarily chosen, the resulting signal was qualitatively insensitive to exact choices of morphological parameters. The net effect of these operations was to delineate regions enriched in active pixels and reject the remainder of the image. Once a mask was defined for an image, it was applied to all frames in the movie so that the same pixels would be averaged in each frame to create the time-dependent trace. All traces

shown in this work are derived from single acquisitions with no averaging of separate movies.

[0082] Once movies had been converted into time-dependent traces by averaging some or all pixels in each frame, the traces were corrected for photobleaching. This was achieved by fitting an exponential curve to all parts of the trace which did not exhibit a voltage-dependent signal and then subtracting this fitted curve from the original trace. In most cases a single exponential function was sufficient to provide a good fit of bleaching. Fluorescence traces from dorsal root ganglion neurons and hippocampal slices were digitally filtered with a low-pass Gaussian filter to reduce high-frequency noise.

[0083] Fluorescence traces were normalized by dividing all data points by the average fluorescence at the beginning of each trace before induction of any voltage change. To produce the fluorescence versus voltage graph, $\Delta F/F_0$ was found by taking the average of two regions, one before voltage induction (F_0) and another during voltage induction; the difference between these two averages (ΔF) was then divided by F_0 . This value was calculated for each voltage trace and plotted against the membrane potential commanded during voltage induction.

Example 6 - ICG Fluorescence Responds to changes in membrane potential

[0084] All fluorescence changes were obtained from single movies with no averaging of multiple acquisitions. Our initial experimental system utilized a single animal cell: an oocyte from the African clawed frog *Xenopus laevis*. Changes in the membrane potential of the cell induced by a two-electrode voltage clamp resulted in robust, consistent changes in the

fluorescence of ICG (Fig. 3a). ICG's voltage-sensitive fluorescence changes showed a large fast component and a smaller slow component; the fast component had a time constant of approximately 4 ms (Fig. 8). The amplitude of the ICG fluorescence changes under these excitation and emission parameters was typically around 1.9% of the baseline fluorescence value per 100 mV of membrane potential change (Fig. 3b). Furthermore, over a physiological range, ICG's voltage-sensitive fluorescence changes were roughly linear with respect to membrane potential change. These results demonstrated the voltage sensitivity of ICG and raised the possibility of using ICG to monitor action potentials.

Example 7 – ICG distinguishes normal and diseased action potentials

[0085] We transformed our oocytes into mimics of excitable cells, dubbed "excitocytes," by coinjecting them with cRNA of voltage-gated sodium and potassium channel components. Under suitable current-clamp conditions, these oocytes fire trains of action potentials similar to those in naturally excitable cells. With this preparation, we found that ICG's fluorescence clearly recapitulated action potentials repetitively firing at speeds above 100 Hz (Fig. 4a). Thus, ICG was capable of detecting action potentials at physiological firing rates.

[0086] In addition to gross detection of action potentials, we also questioned whether ICG could discriminate between normal and diseased action potentials by accurately following the action potential shapes. We compared excitocytes injected with wild-type voltage-gated sodium channel cRNA to those injected with cRNA coding for a version of the sodium channel containing a point mutation, G1306E, that produces episodic myotonia in patients. This disease is characterized by continued action potential firing in skeletal muscles after cessation of voluntary stimuli; the resulting prolonged muscular contractions are the hallmark of

myotonia. Compared to the wild-type sodium channel, the G1306E mutation causes a slowing of the fast inactivation of the sodium channels, which in turn results in broadened action potentials as the sodium channels stay open longer than normal.

[0087] Both versions of the excitocytes were injected with wild-type potassium channel cRNA. The electrical recordings and the ICG fluorescence response clearly distinguished the sharp action potentials produced by the healthy sodium channel (Fig. 4b, Fig. 9) from the wider peaks produced by the myotonic sodium channel (Fig. 4c, Fig. 9). Furthermore, a brief injection of current led to repetitive firing and hyperexcitability that persisted after the stimulus was stopped. ICG fluorescence clearly resolved every action potential of this myotonia-like behavior (Fig. 4d).

Example 8 – ICG Follows electrical activity in mammalian cells

[0088] We investigated whether ICG's voltage sensitivity extended to excitable mammalian tissue by measuring ICG fluorescence from cultured rat dorsal root ganglion neurons. Under whole-cell current clamp, we observed neurons firing in the stereotypical fashion of the nociceptive C-type fiber, and these action potentials were clearly visible in the ICG fluorescence (Fig. 5a, Fig. 10). We also examined syncytia of cultured cardiomyocytes from neonatal rats to further validate ICG's utility; these cells beat spontaneously and showed changes in ICG fluorescence indicative of changes in membrane potential (Fig. 5b). Although we cannot formally exclude the possibility that the physical motion of the cardiomyocytes produced changes in ICG fluorescence in addition to the voltage-dependent changes, several observations suggested that these effects were minimal (Fig. 11). Taken together, our results from frog oocytes and rat neurons and cardiomyocytes confirmed that ICG voltage

sensitivity was not confined to a particular animal model or cell lineage and could be broadly applicable across a range of tissues.

Example 9 – ICG responds to stimulation of living brain tissue

[0089] We tested whether ICG voltage sensitivity could be detected in a complex tissue as opposed to isolated excitable cells. Using rat hippocampal slice cultures (Fig. 6a), we demonstrated that brain excitation produced by field electrode stimulation was clearly accompanied by ICG fluorescence changes (Fig. 3a). Additionally, ICG fluorescence changes can discriminate between different neuronal responses caused by differing excitation intensity levels and durations (Fig. 6b, Fig. 12b, c, and d). After smaller stimuli, fluorescence recovered fully to baseline levels during the observed time course, while fluorescence subsequent to larger stimuli often did not fully recover during our observation windows (Fig. 6b). To further test whether these signals originated from the activity of excitable cells, we used tetrodotoxin (TTX), a neurotoxin that blocks voltage-gated sodium channels. We tested the effect of TTX using a stimulus intensity that allowed for fluorescence recovery. When TTX was applied to the brain slices, we observed a clear inhibition of electrical excitability (Fig. 6c, Fig. 12e). Furthermore, when the TTX was subsequently washed away from the slices, we saw a partial recovery of excitability (Fig. 6d, Fig. 12f). Partial recovery is not surprising; washing TTX-treated tissues sufficiently to completely remove toxin block is often impractical. Hippocampal slice cultures retain glial cells, including oligodendrocytes, astrocytes and microglia. Therefore, even with numerous non-excitable and weakly-excitable cells present, recordings of ICG fluorescence clearly responded to membrane potential changes of the excitable tissues, suggesting that ICG could be successfully used in complex physiological environments.

Example 10

[0090] The method is tested on animal models to image brain activity. ICG reveals neural activity in the barrel cortex of a live rat in response to whisker stimuli (the barrel cortex is a very widely-used neurological system in the literature). An additional feature of interest is that ICG is imaged through the intact skull of an adult rat. This has not been shown before using a voltage-sensitive dye, as the fluorescence emission wavelengths of other known voltage-sensitive dyes do not penetrate tissue deeply enough to permit imaging through the bone. This has significant implication for future human trials, as brain activity may be visualized by drilling only a tiny hole in the skull for dye injection, rather than carving out a large window to image through. This would make such a procedure considerable less invasive.

[0091] ICG was applied topically to a small section of the barrel (somatosensory, responds to whisker stimulation) cortex of the brain of a living rat. Figure 13 shows an example of a change in fluorescence we see taken across the entire field of view, with post processing baseline subtraction applied. Upon whisker stimulation, a decrease in overall fluorescence is observed, indicative of the brain region being more electrically active. Figure 14 is a different view of the same data, showing that neuronal activity in response to whisker stimulation can be spatially localized within this region of the barrel cortex. Imaging is approximately 3.5 mm in each direction. The red region corresponds to increased electrical activity. Thus, ICG can be used in a living animal to record neuronal electrical activity with high temporal (5 ms frame rate) and spatial (each pixel is ~ .03 mm) resolution. Although the invention has been described and illustrated with reference to specific illustrative embodiments thereof, it is not intended that the invention be limited to those illustrative embodiments. Those skilled in the art will recognize that variations and modifications can be made without departing from the true scope and spirit of the invention as defined by the claims that follow. It is therefore

intended to include within the invention all such variations and modifications as fall within the scope of the appended claims and equivalents thereof.

[0092] References

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WE CLAIM:

1. A method of determining electrical activity in a cell, the method comprising:
labeling the cell with a composition comprising indocyanine green;
illuminating the cell with electromagnetic radiation having a first frequency range, wherein the first frequency range is within a spectral absorption frequency range of the indocyanine green; and
measuring a variation in intensity of spectral emission of the indocyanine green, wherein the variation in intensity of spectral emission is indicative of the electrical activity in the cell.
2. The method of claim 1, wherein the measuring the of variation in intensity of spectral emission of the indocyanine green comprises measuring the intensity of spectral emission at least 10 times within a period of one second
3. The method of claim 1, wherein the cell is selected from the group consisting of a nerve cell and a cardiac cell.
4. The method of claim 1, wherein the intensity of spectral emission is indicative of the electrical activity in a single cell.
5. The method of claim 1 wherein the cell is a retinal ganglion cell and wherein the method further comprises illuminating the cell with electromagnetic radiation having a second frequency range, wherein the second frequency range is within a frequency range of visible light.

6. The method of claim 1, wherein the labeling comprises administering the composition to the retina of a human or veterinary subject.
7. The method of claim 6, wherein the administering is selected from the group consisting of topical and intravenous administering.
8. The method of claim 1, wherein the first frequency range is between 600 nanometers and 900 nanometers.
9. The method of claim 1, wherein the variation in intensity of spectral emission of indocyanine green is measured within 24 hours of the labeling.
10. The method of claim 9, wherein the variation in intensity of spectral emission of indocyanine green is measured within 6 hours of the labeling.
11. The method of claim 9, wherein the variation in intensity of spectral emission of indocyanine green is measured within 1 hour of the labeling.
12. A method of diagnosing a disease of the eye in a subject, the method comprising:
 - labeling a retinal ganglion cell present in the retina of the subject with a composition comprising indocyanine green;
 - illuminating the retinal ganglion cell with electromagnetic radiation having a first frequency range, wherein the first frequency range is within a spectral absorption frequency range of the indocyanine green;
 - illuminating the retinal ganglion cell with electromagnetic radiation having a second frequency range, wherein the second frequency range is within a

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frequency range of visible light; and
measuring a variation in intensity of spectral emission of the indocyanine green, wherein the measuring the of variation in intensity of spectral emission of the indocyanine green comprises measuring the intensity of spectral emission at least 10 times within a period of one second and wherein the variation in intensity of spectral emission is indicative of presence or absence of the disease of the eye.

13. The method of claim 12, wherein the labeling comprises administering the composition to the retina of the subject.

14. The method of claim 13, wherein the administering is selected from the group consisting of topical and intravenous administering.

15. The method of claim 12, where the disease is selected from the group consisting of a retinal ganglion cell neuropathy, hereditary optic neuropathy, optic disc drusen, multiple sclerosis and diabetes.

16. The method of claim 12, wherein the variation in intensity of spectral emission of indocyanine green is measured within 6 hours of the labeling.

17. The method of claim 16, wherein the variation in intensity of spectral emission of indocyanine green is measured within 1 hour of the labeling.

18. The method of claim 11, wherein the intensity of spectral emission is measured at least 20 times within a period of one second.

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19. The method of claim 11, wherein the retinal ganglion cell has a basal firing rate and wherein the intensity of spectral emission is measured at a frequency of at least 2 times a basal firing rate.

20. The method of claim 11, wherein the retina is illuminated with electromagnetic radiation having a first frequency range at a resolution of between 5 and 20 microns.

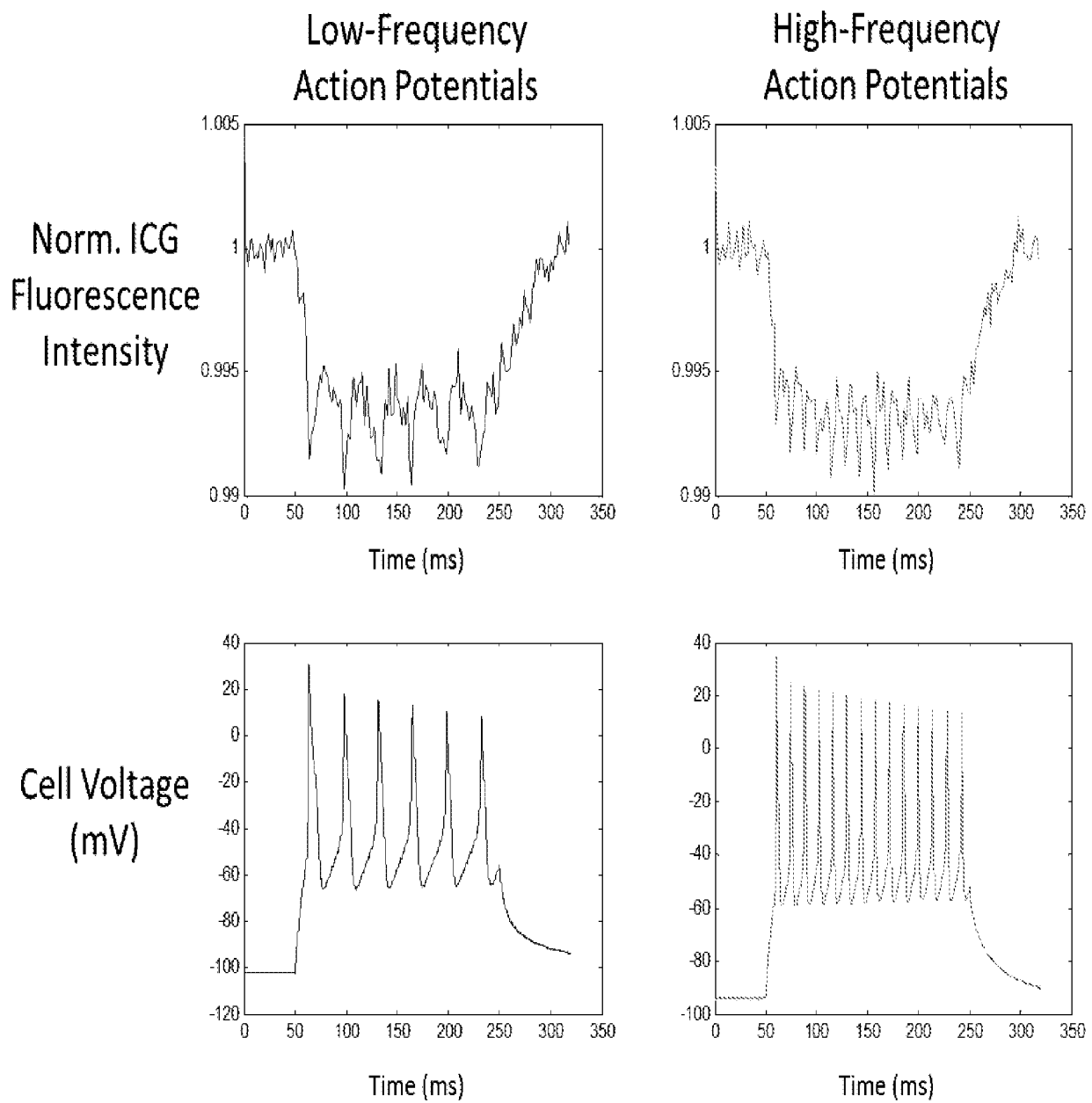
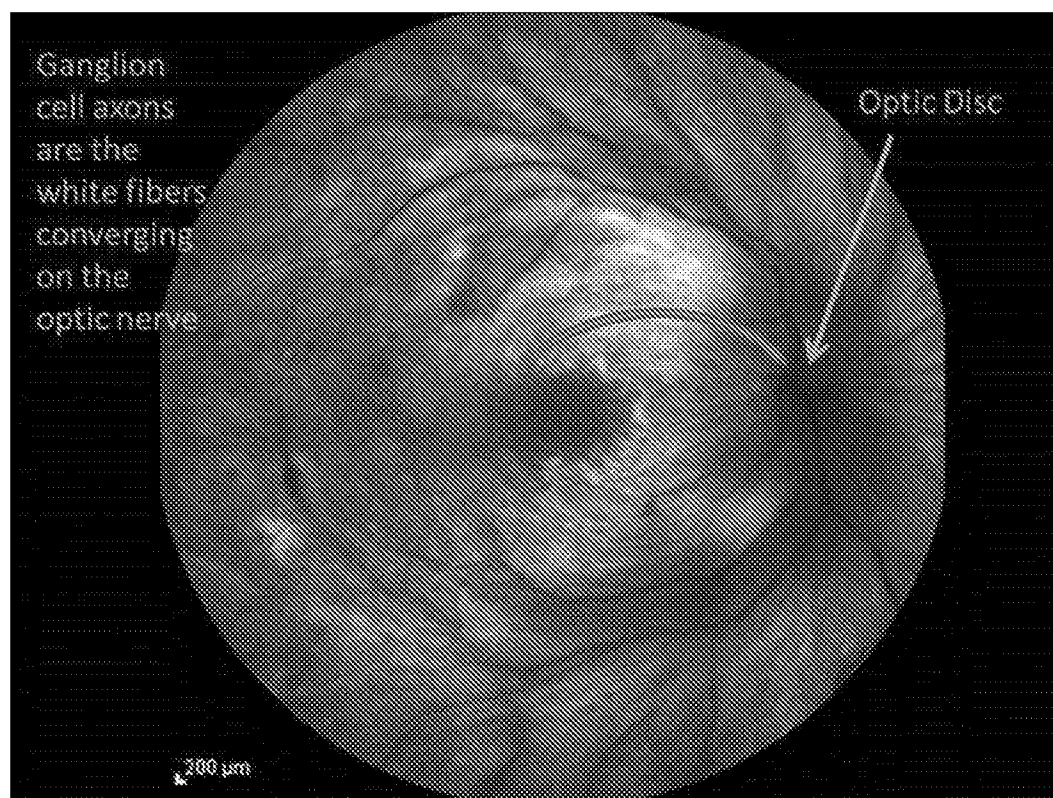
FIGURE 1

Figure 2



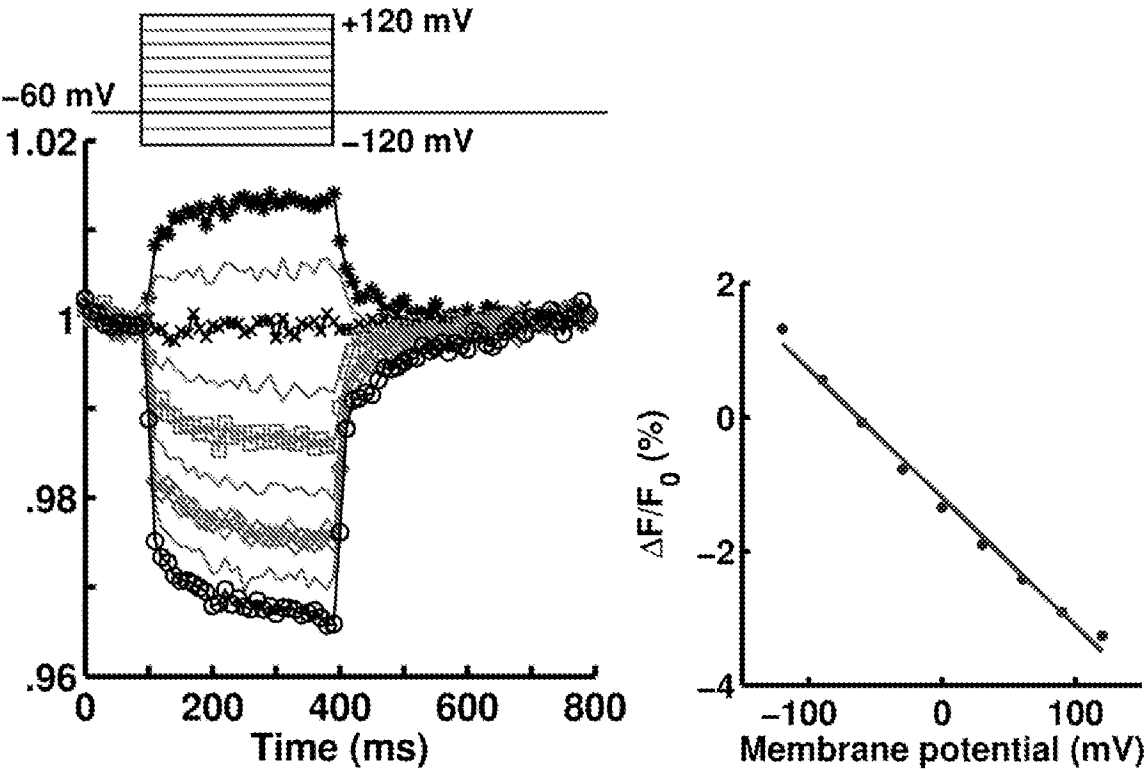


Figure 3

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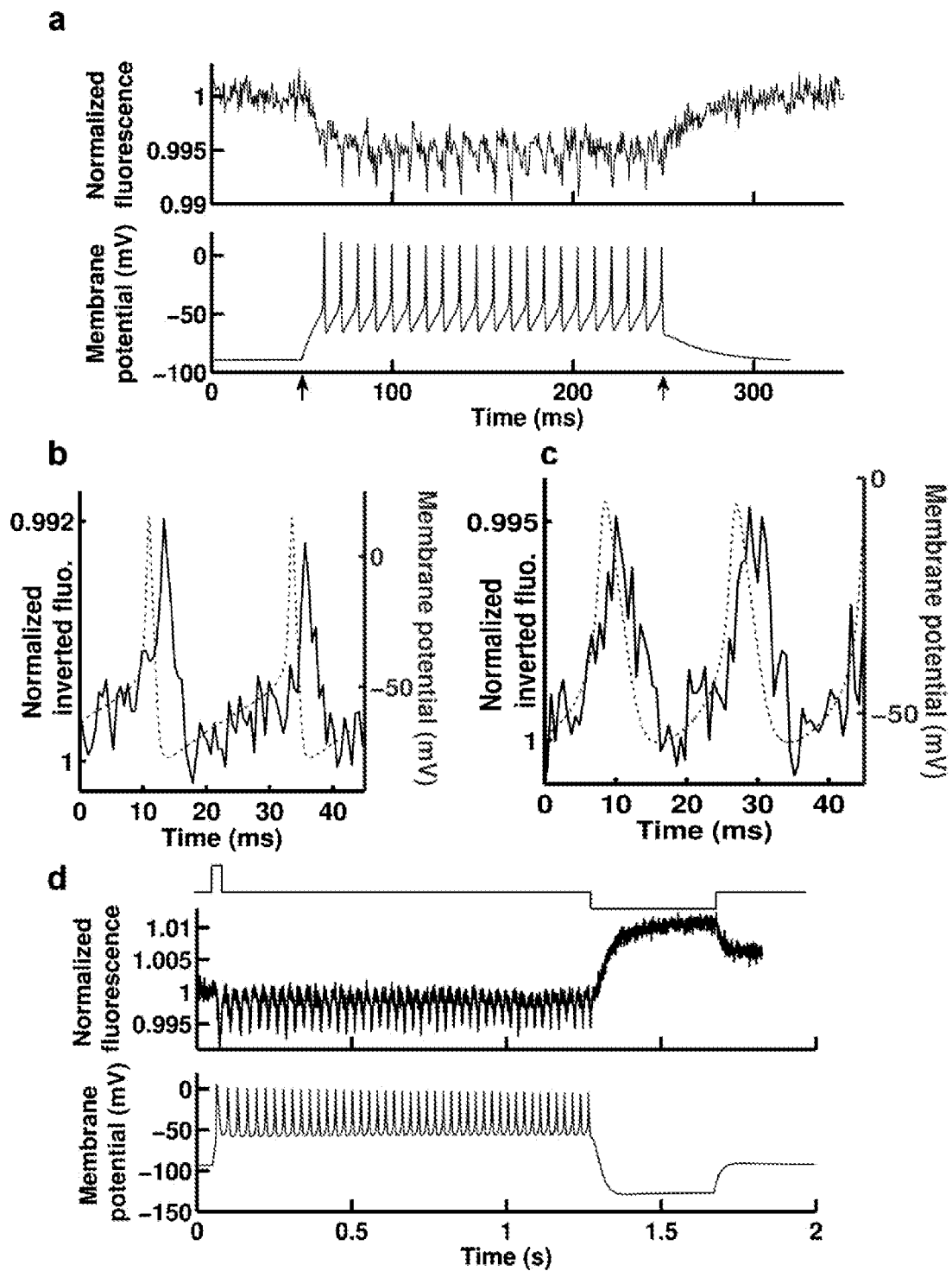


Figure 4

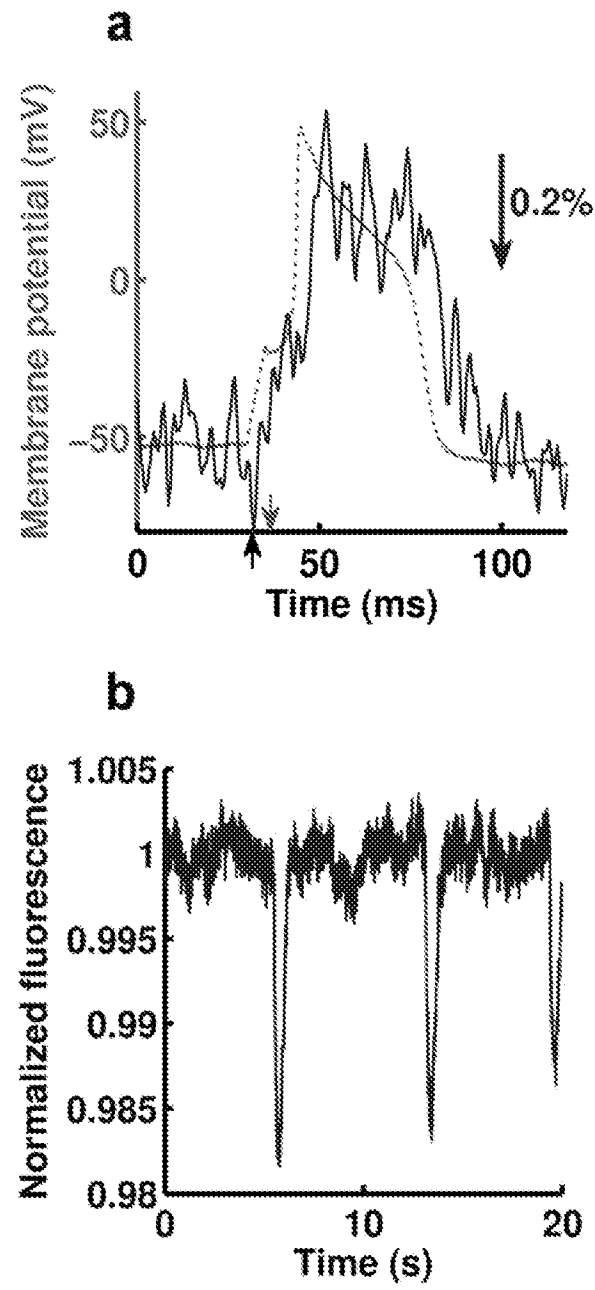


Figure 5

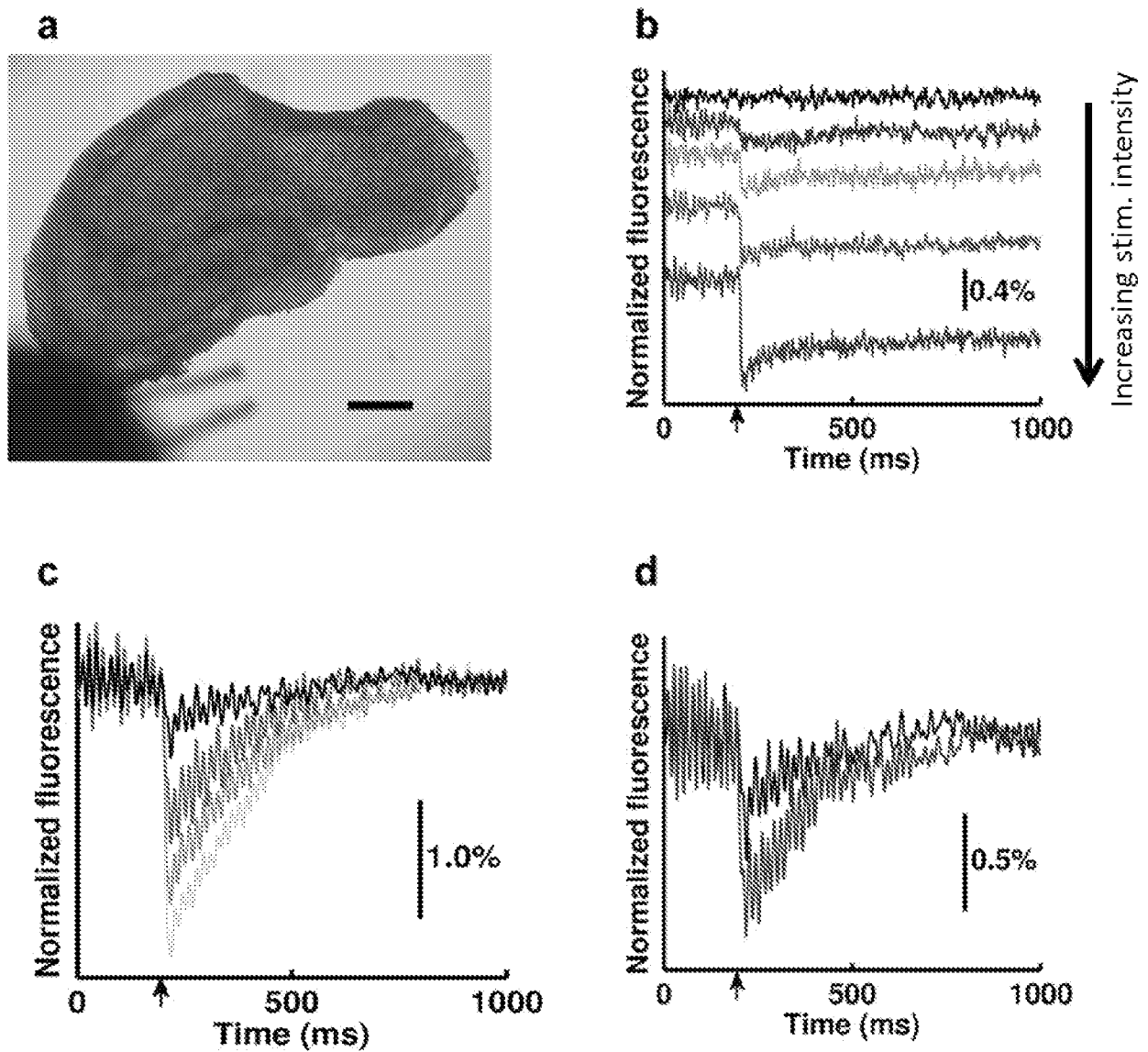


Figure 6

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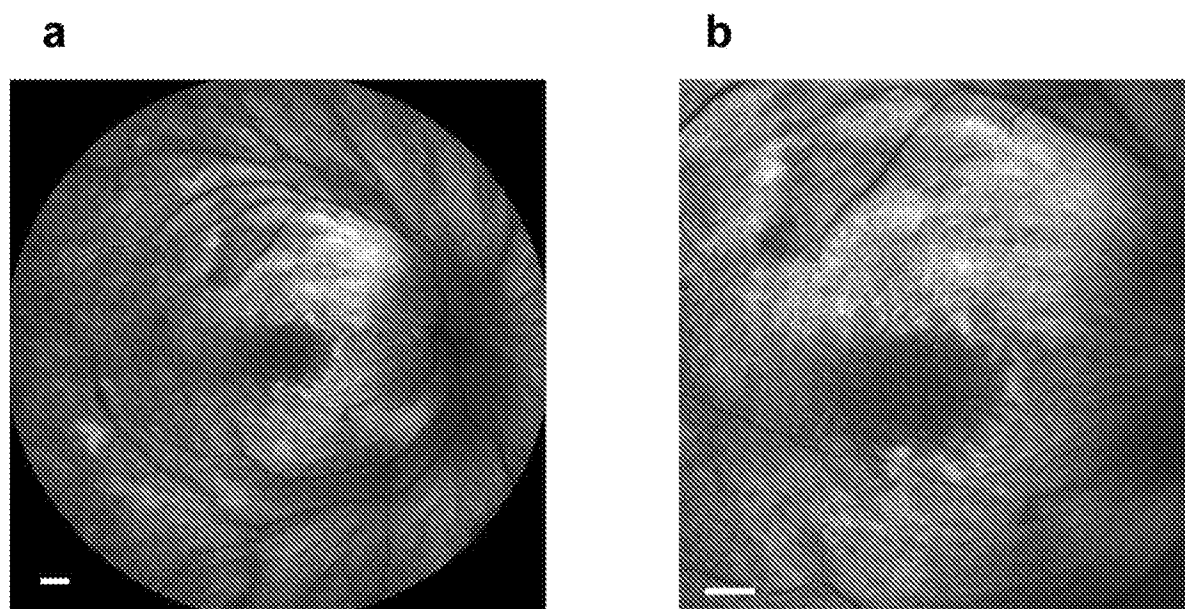


Figure 7

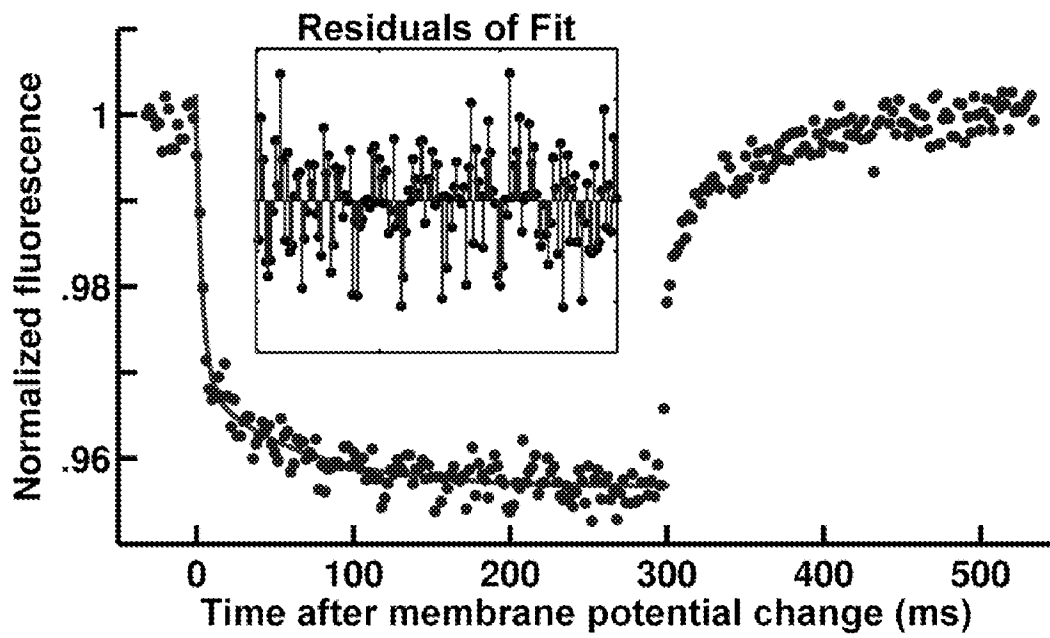


Figure 8

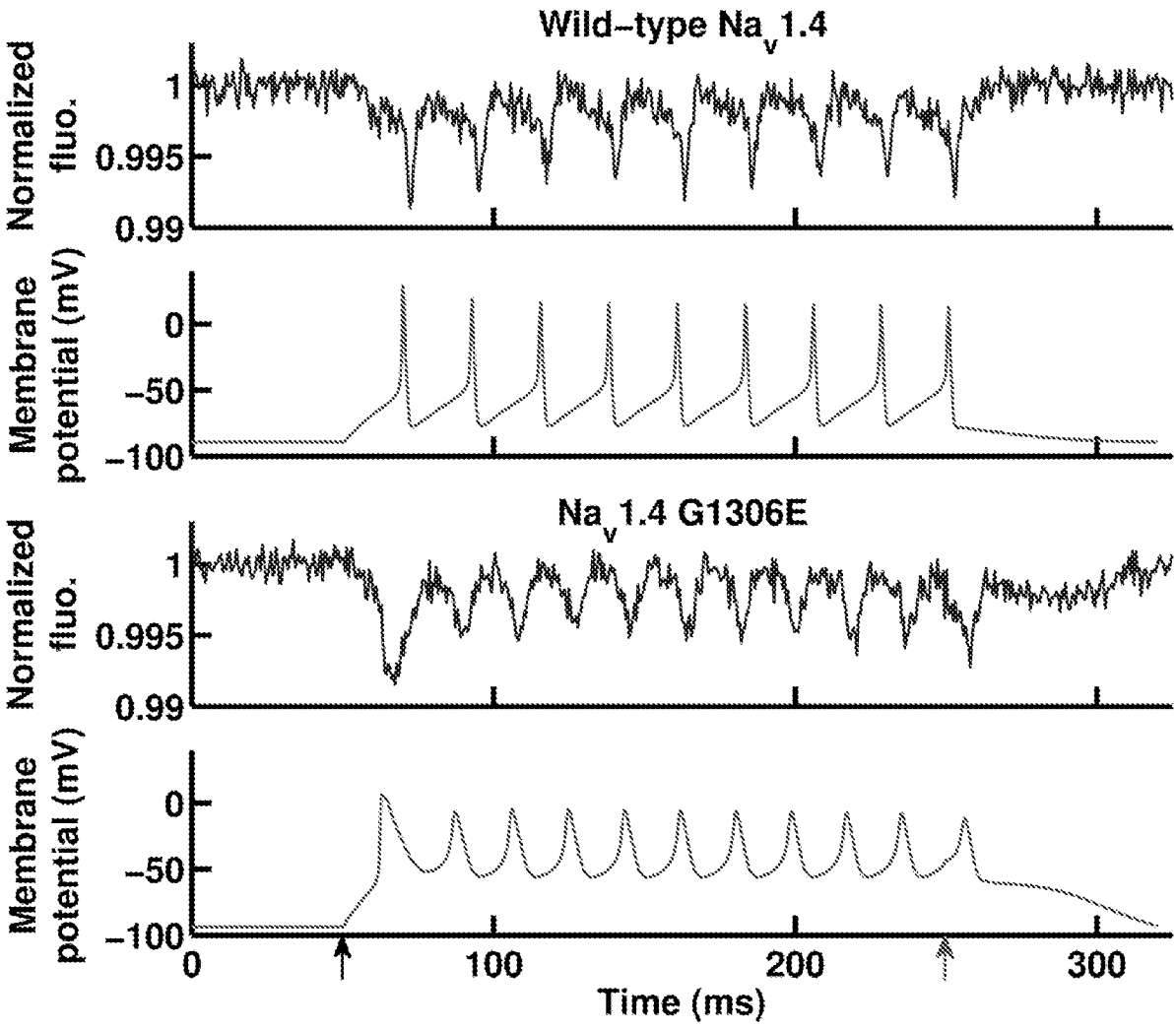


Figure 9

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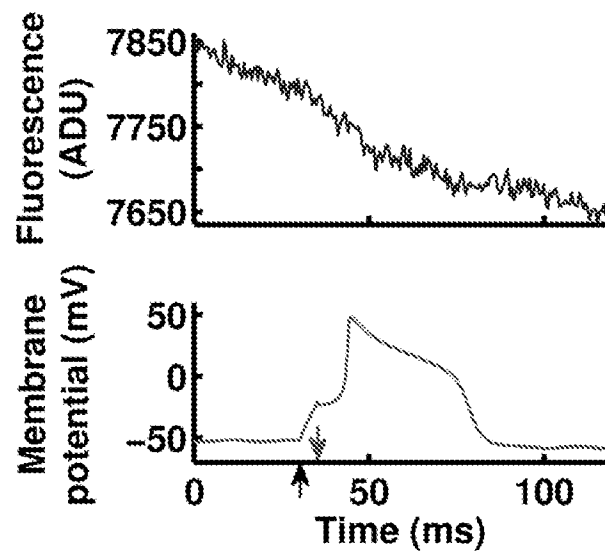


Figure 10

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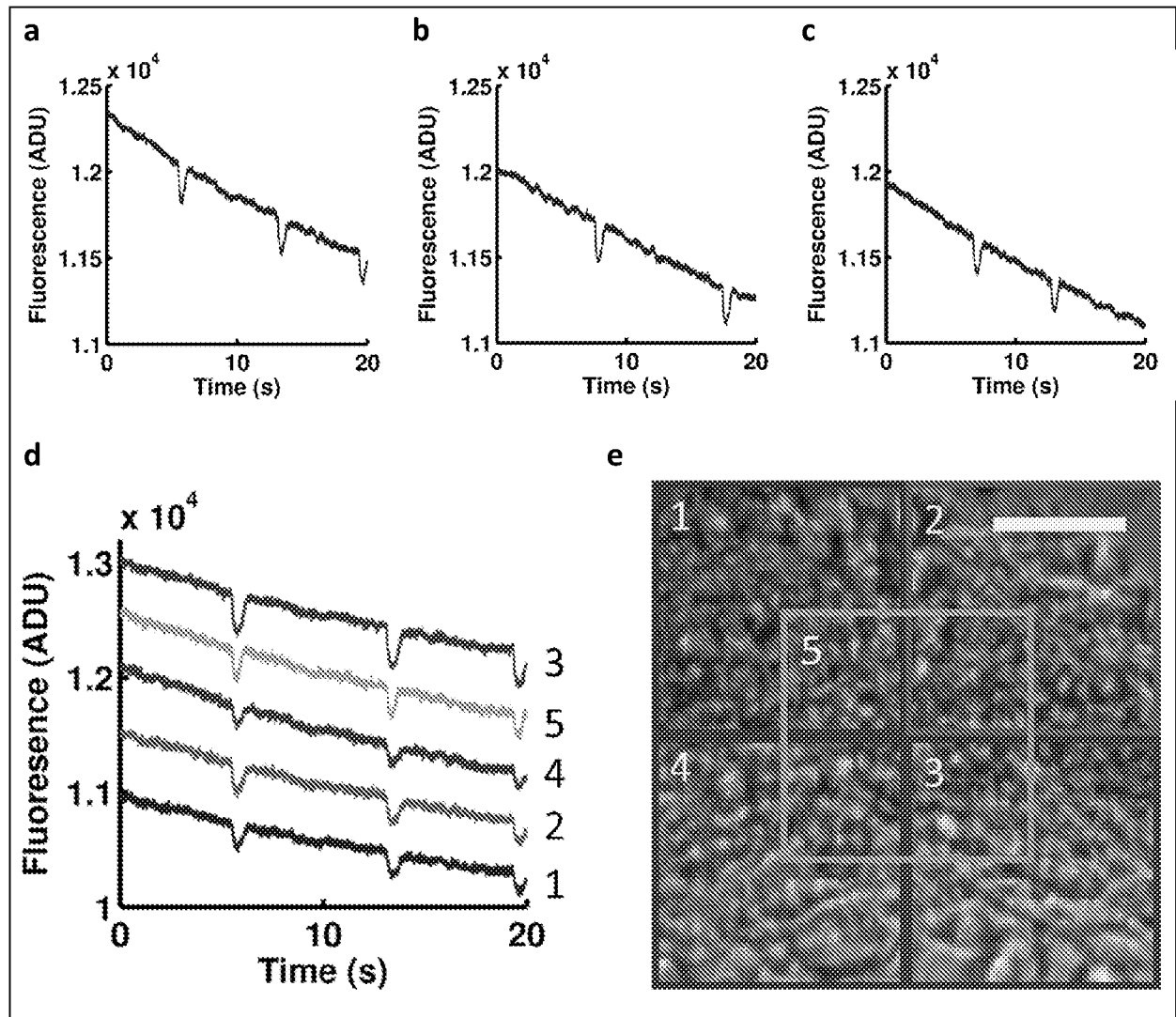


Figure 11

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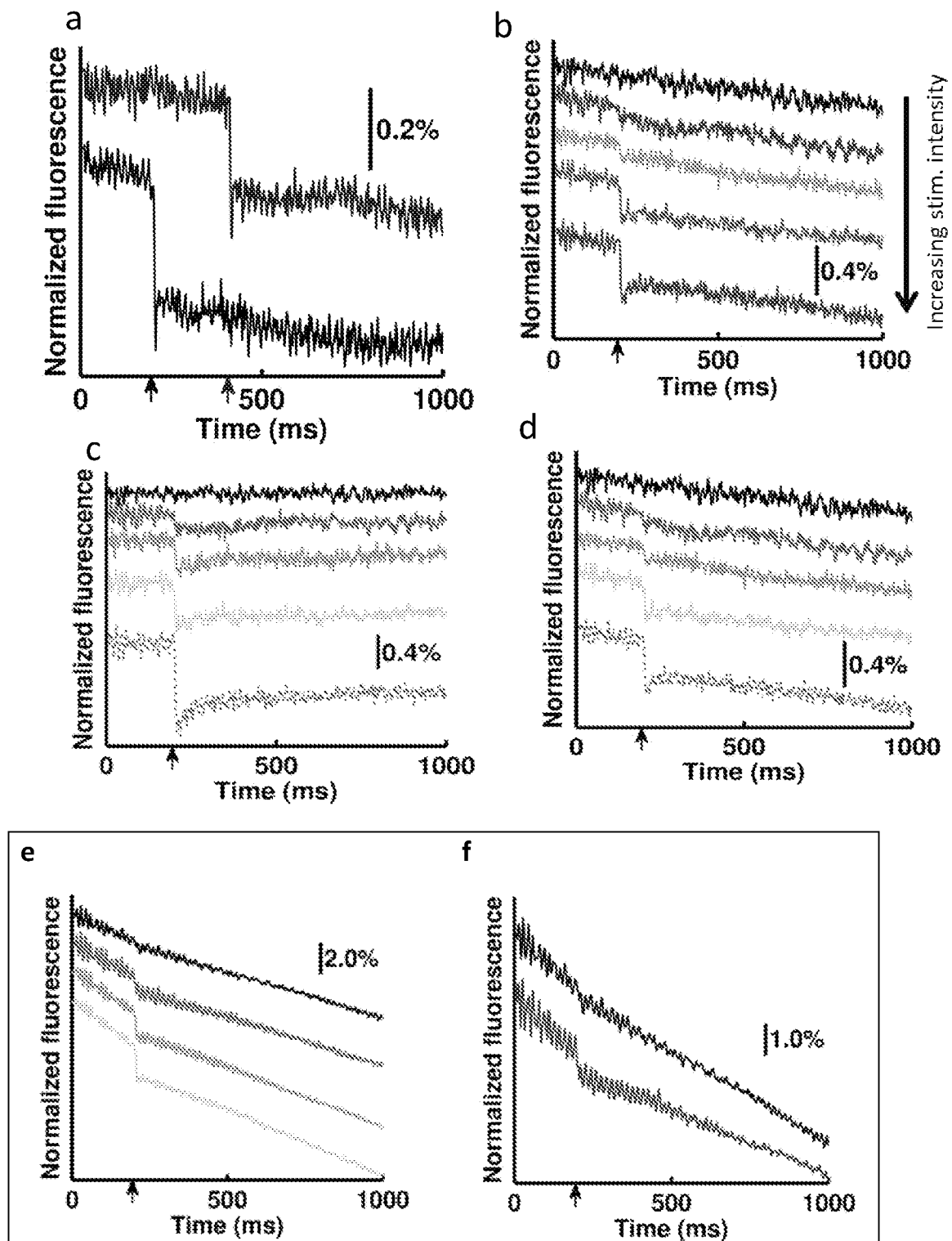


Figure 12

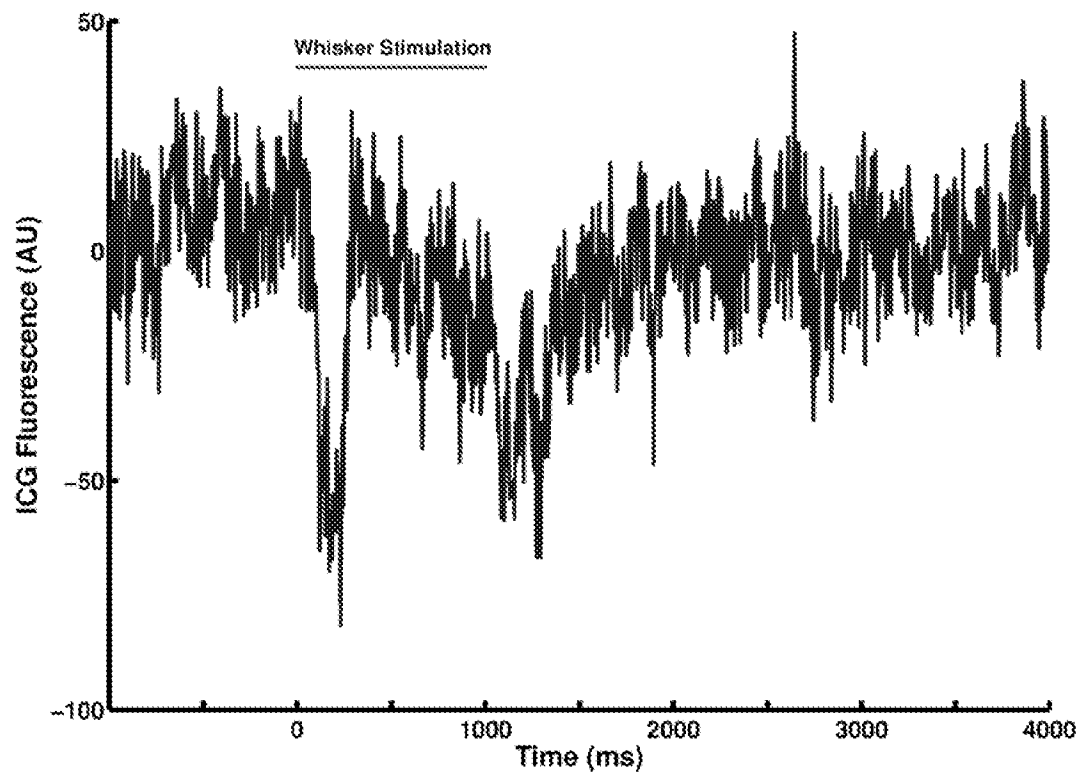


Figure 13

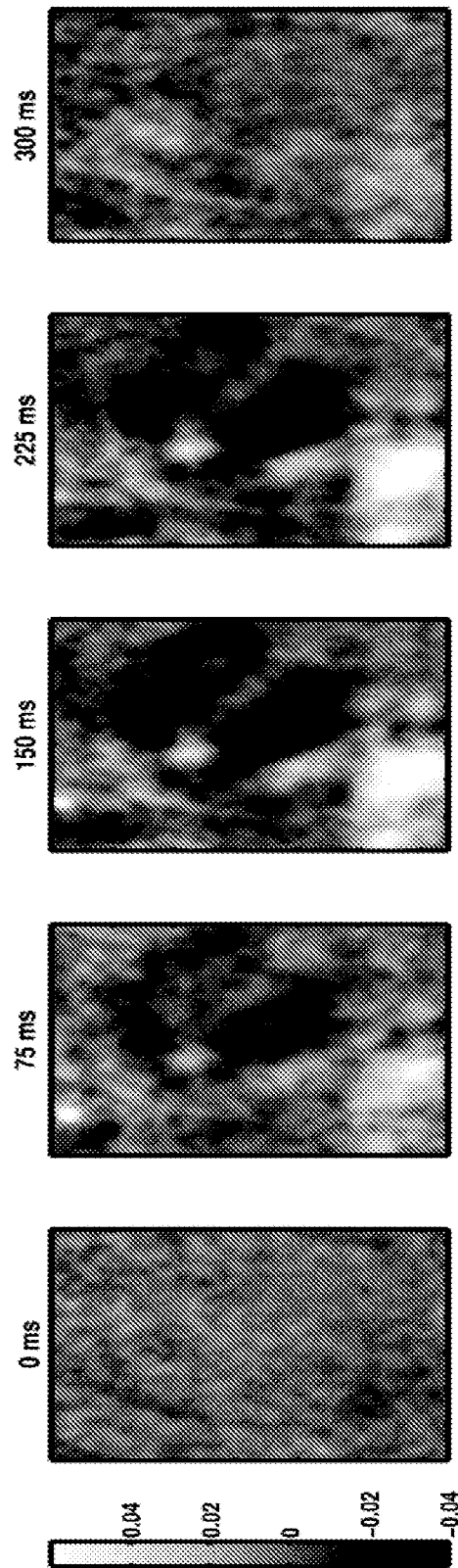


Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/070887

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61B 5/05 (2015.01)

CPC - A61B 5/05 (2015.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61B 5/04, 5/05 (2015.01)

CPC - A61B 5/04, 5/05 (2015.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 600/1, 308, 309, 312, 407, 420; IPC(8) - A61B 5/04, 5/05; CPC - A61B 5/04, 5/05 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit.com, Google Patents, Google Scholar, Public AppFT and PatFT.

Search terms used: indocyanine green retinal ganglion "electrical activity" "basal firing" spectra voltage sensitive dye

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0196893 A1 (PERTSOV et al) 02 August 2012 (02.08.2012) entire document	1-20
Y	WO 1994/023646 A1 (MALLINCKRODT MEDICAL, INC) 27 October 1994 (27.10.1994) entire document	1-20
Y	US 6,478,424 B1 (GRINVALD et al) 12 November 2002 (12.11.2002) entire document	5-7, 12-17, 19, 20
Y	US 2008/0305046 A1 (HAFEZI-MOGHADAM) 11 December 2008 (11.12.2008) entire document	15, 20

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 February 2015

Date of mailing of the international search report

10 APR 2015

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