

IF YOU BUILD IT, THEY WILL COME: ENGINEERING THE NEXT GENERATION
OF OPTICAL TOOLS TO IMAGE NEURAL ACTIVITY
DEEP WITHIN THE LIVING BRAIN

by

Lauren Marie Barnett

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Neuroscience

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2017

©COPYRIGHT

by

Lauren Marie Barnett

2017

All Rights Reserved

DEDICATION

To my incredible family and friends who have believed in me, supported me, sacrificed time spent with me, and given me all the unconditional love I needed to succeed.

And to my advisor, mentor, and friend, Dr. Thom Hughes, for introducing me to the world to science and changing my life forever.

ACKNOWLEDGEMENTS

This work was possible thanks to several important people in my life. First, thank you to my committee members--Dr. Thom Hughes, Dr. Mikhail Drobizhev, Dr. Frances Lefcort, and Dr. Vincent Pieribone—for their efforts, advice and guidance in helping advance my education and career. To Dr. Mikhail Drobizhev, thank you for teaching me everything I know about fluorescence spectroscopy and two-photon photophysics. Beyond the technical skills, you taught me the significance of careful measurements, attention to detail, and the importance of taking time to get it *exactly* right. Your methodical, deeply thoughtful approach to science will remain with me always.

Thank you to Dr. Susy Kohout for being an extraordinary female role model to look up to, a reliable and unbiased source of advice, and a sincere friend to lean on whenever I needed you.

To my advisor Dr. Thom Hughes, thank you for taking a chance on a non-traditional undergraduate student. For giving me an inspiring place to grow, explore, and learn. For teaching me everything I know about the world of science, expertly guiding me through it, and constantly pushing me beyond my limits. For being tough on me. For being patient with me. For always believing in me. Thank you for being my incredible mentor, and, more importantly, for being my friend. Meeting you changed my life, I will never be able to fully express how grateful I am for everything you have shared with me.

Finally, to my best friend Mr. Tyson Bradley, thank you for tirelessly supporting me throughout this work. You are my rock. Without your tough-love, grounding advice, and unwavering belief in my true potential, none of this would have been possible.

TABLE OF CONTENTS

1. AN INTRODUCTION TO GENETICALLY-ENCODED FLUORESCENT BIOSENSORS AND THEIR IMPORTANT ROLE IN NEUROSCIENCE	1
Fluorescent biosensors	2
Genetically-encoded fluorescent Ca^{2+} sensors.....	6
Genetically-encoded fluorescent voltage sensors	8
Imaging biosensors with two-photon microscopy	12
2. DECIPHERING THE MOLECULAR MECHANISM OF GCAMP6M'S Ca^{2+} DEPENDENT CHANGE IN FLUORESCENCE	15
Contribution of Authors and Co-Authors	15
Manuscript Information Page	16
Abstract	17
Introduction.....	17
Results.....	18
The mechanism of GCaMP6m is more complicated than a simple “dim” to “bright” transition of the chromophore	18
There are four different Ca^{2+} -dependent states of GCaMP6m	22
There is a Ca^{2+} -dependent change in pK_a for the GCaMP6m chromophore	24
To better understand GCaMP6m, we need to understand the molecular mechanisms of the Ca^{2+} -dependent change in pK_a	26
The two-photon absorption properties do not necessarily follow the same behavior as the one-photon absorption.....	29
Discussion	31
Materials and methods	33
Imaging intracellular Ca^{2+} response in HEK293 cells.....	34
Preparation of purified protein samples	34
Ca^{2+} Titration measurements	34
Fluorescence spectra, fluorescence excitation spectra, and quantum yield measurements.....	34
Extinction Coefficient measurements	35
pK_a measurements.....	35
Two-photon measurements	36
Supporting Information.....	36
Acknowledgements.....	37
Author Contributions	37
References.....	37
Supporting Figures.....	41

TABLE OF CONTENTS — CONTINUED

3. MAKING pK _a -ALTERING MUTATIONS IN GCAMP6M CHANGES THE Ca ²⁺ DEPENDENT FLUORESCENCE RESPONSE	44
Contribution of Authors and Co-Authors	44
Manuscript Information Page	45
Abstract	46
Introduction	46
Results	48
Mutations that alter the neutral – anionic equilibrium in EGFP affect the pK _a values and ΔpK _a in GCaMP6m.....	48
A single mutation in GCaMP6m, E60G, increases the Ca ²⁺ -dependent change in fluorescence in an unexpected way	53
The mutant two-photon excited fluorescence spectra highlight the importance of ΔpK _a across a physiological pH range.....	54
Discussion	55
Methods and Materials.....	55
Introducing mutations into GCaMP6m.....	55
Preparation of purified protein samples.....	56
Ca ²⁺ Titration measurements	56
Fluorescence spectra, fluorescence excitation spectra, and quantum yield measurements.....	57
Extinction Coefficient measurements	57
pK _a measurements.....	58
Two-photon measurements	58
References.....	59
Figures.....	61
4. A FLUORESCENT, GENETICALLY-ENCODED VOLTAGE PROBE CAPABLE OF RESOLVING ACTION POTENTIALS	67
Contribution of Authors and Co-Authors	67
Manuscript Information Page	68
Abstract	69
Introduction	69
Results	70
Discussion	70
Materials and methods	73
Molecular Biology	73
Cell culture.....	73
Electrophysiology	74
Imaging	74

TABLE OF CONTENTS — CONTINUED

Data Analysis	74
Supporting Information.....	74
Acknowledgements.....	75
Author Contributions	75
References.....	75
 5. TWO-PHOTON DIRECTED EVOLUTION OF GREEN FLUORESCENT PROTEINS	 76
Contribution of Authors and Co-Authors	76
Manuscript Information Page	77
Abstract.....	78
Introduction/Results/Discussion	78
Methods.....	84
Measurements of the 2PEF and 1PEF signals	84
Measuring the two-photon absorption cross section.....	84
References.....	85
Acknowledgements.....	85
Author Contributions	86
Supplemental Information	87
 6. FUTURE RESEARCH DIRECTIONS	 106
Voltage Sensor Optimization.....	106
Multi-Color Imaging.....	107
Two-Photon Biosensor Imaging	109
 REFERENCES CITED.....	 110

LIST OF TABLES

Table	Page
2.1. Photophysical parameters for GCaMP6m	25
2.2. Calculated changes of $\Delta\Delta G$ and ΔpK_a for the transition from GCaMP3 to GCaMP5A in a model that assumes different states of chromophore atoms interacting with D380 residue.....	29
2.3. The changes in ΔpK_a between recent GCaMP generations	29
2.4. Contributions to $\Delta\Delta G$ and ΔpK_a from important charged amino acids in GCaMP2 and GCaMP6m	30
3.1. Photophysical properties of GCaMP6m mutants.....	51
5.1. Photophysical properties and DNA analysis data of the 23 selected mutants of EGFP	82

LIST OF FIGURES

Figure	Page
2.1. Schematic of Ca^{2+} -saturated GCaMP6m structure	18
2.2. The Ca^{2+} -dependent change in GCaMP6m fluorescence is more complicated than a chromophore transition from dim to bright.....	19
2.3. The GCaMP6m Ca^{2+} -dependent spectra are consistent with four fluorescent states of the chromophore	21
2.4. A four state model requires the quantitative characterization of each of the four distinct Ca^{2+} -dependent states of GCaMP6m	22
2.5. Alkaline titration makes it possible to measure the extinction coefficient of two different forms in the same sample	24
2.6. GCaMP6m has a single pK_a for the Ca^{2+} -saturated state and two pK_a 's for the Ca^{2+} -free state	25
2.7. Atom numbering for the GCaMP chromophore	28
2.8. Two-photon excitation spectra for GCaMP6m.....	30
2.S1. Emission spectra for purified GCaMP6m protein	41
2.S2. There is an optimal excitation wavelength to measure $\Delta F/F_0$ for GCaMP6m.....	41
2.S3. Emission spectra used to resolve the fluorescence quantum yield for the Ca^{2+} -free neutral form emitting near 515 nm	42
2.S4. Two-photon absorption for Ca^{2+} -saturated GCaMP6m (black solid circles, left y-axis and bottom x-axis)	43
3.1. Mutations in the EGFP chromophore and surrounding protein β -barrel can alter the neutral – anionic equilibrium.....	61
3.2. EGFP pK_a -altering mutations affect the pK_a and ΔpK_a in GCaMP6m	62

LIST OF FIGURES — CONTINUED

Figure	Page
3.3. Ca^{2+} -binding has varying effects on GCaMP6m-mutant chromophore equilibriums	63
3.4. The E60G mutation in GCaMP6m most likely increases the Ca^{2+} -dependent ΔF via a change in quantum yield	64
3.5. ΔpK_a is the predominant mechanism for Mutant Ca^{2+} -dependent changes in 470 nm excited fluorescence, however other parameters can significantly contribute	65
3.6. The mutant two-photon excited spectra illustrate the importance of ΔpK_a	66
4.1. Probe design, expression level and $\Delta F/\Delta V$ sensitivity of CiVSP::cpEGFP constructs	70
4.2. Schematic diagram of CiVSP::cpEGFP based construct design	70
4.3. The protein sequence of the various fusion sites of cpEGFP with CiVSP described in this report.....	71
4.4. Examples of CiVSP::cpEGFP constructs expression in HEK293 cells and voltage sensitivity	72
4.5. Voltage sensitivity and response kinetics of ElectricPk	72
4.6. Linear $\Delta F/\Delta V$ response of ElectricPk	73
4.7. Detection of action potentials in hippocampal neurons in vitro using ElectricPk	74
5.1. Schematic of the 2PEF and 1PEF imaging setup.....	79
5.2. Fluorescence data of randomly mutagenized EGFP	81
5.3. Absorption spectra of selected mutants of EGFP	83
5.S1. Fluorescence images of a Petri plate with intermixed <i>E.coli</i> colonies.....	88

LIST OF FIGURES — CONTINUED

Figure	Page
5.S2. Cumulative distribution functions of fluorescence data of randomly mutagenized EGFP	100
5.S3. Absorption spectra	102
5.S4. Imaging rate estimation versus laser parameters	105

ABSTRACT

To see the activity of large, integrated neural circuits functioning in real-time inside of a living brain, neuroscientists will need multiple genetically-encoded fluorescent activity sensors that can be individually targeted to specific cell types, are fast enough to resolve multiple action potentials, can be distinguished from one another and imaged deep within the brain. The goal of this work is to better understand and improve upon the most recent generations of genetically-encoded Ca^{2+} and voltage sensors, and to expand biosensor utility in two-photon excitation, which will be necessary to image neural activity deep within the brain.

Genetically-encoded Ca^{2+} sensors measure the intracellular Ca^{2+} release that occurs downstream of an action potential. The GCaMP6 series are the best Ca^{2+} sensors available, however little is known about how they work. Measurements of four different states in GCaMP6m reveal that its large Ca^{2+} -dependent change in 470 nm excited fluorescence is due to a redistribution of the chromophore protonation state, from a neutral form excited at ~400 nm to an anionic form excited at ~470 nm, via a change in pK_a . Making pK_a -altering mutations in GCaMP6m changes the Ca^{2+} -dependent fluorescence response. This highlights the importance of ΔpK_a and identifies key amino acid positions that will be important for improving GCaMP6m and GCaMP-like biosensors.

A direct readout of an action potential would be ideal for capturing complex signal transduction in the brain. This will require a bright, fast voltage sensor. ElectricPk is the first genetically-encoded voltage sensor with a fluorescence response fast enough to resolve multiple action potentials in mammalian neurons. This design indicates it is possible to couple a fluorescence change with a very fast (~1 ms) voltage-dependent movement in the *Ciona intestinalis* voltage-sensitive phosphatase protein.

Whether imaging a downstream Ca^{2+} signal or a direct change in membrane potential, to image neuronal activity in deep brain tissue biosensors will need to be brightly fluorescent in two-photon excitation. The two-photon directed evolution of green fluorescent proteins presented here is a proof-of-principle design that shows a high-throughput screen focused on improving the two-photon properties of a fluorescent protein is possible.

CHAPTER ONE

AN INTRODUCTION TO GENETICALLY-ENCODED FLUORESCENT
BIOSENSORS AND THEIR IMPORTANT ROLE IN NEUROSCIENCE

A goal most neuroscientists share is to be able to see the activity of large, integrated neural circuits functioning in real-time, inside of a living brain (Kandel et al. 2013). The human brain is made up of thousands of different cell types, ~100 billion neurons, each containing on average ~1000 different synapses. This enormous, almost incomprehensible complexity, makes simply watching the mass activity of neurons as a whole uninformative (Sherrington 1941, p.178). Instead, it is necessary to be able to watch the activity of specific sets of cell types in relation to one another with clarity. To do this, neuroscientists will need to be able to image signal transduction in individual neurons as they occur in relation to one another, throughout an entire living, functioning brain. Visualizing neural activity on this scale will require multiple genetically-encoded fluorescent activity sensors that can be individually targeted to specific cell types, are fast enough to resolve individual action potentials, can be distinguished from one another, and imaged deep within the brain. The work presented here is an effort to advance the field of protein engineering to one day achieve genetically-encoded fluorescent tools that are capable of performing at this level.

Fluorescent Biosensors

Fluorescence is the process in which a molecule absorbs a photon of a particular wavelength and emits a photon of a longer wavelength of light (Lakowicz 2007). Altering the chemical structure or electrostatic environment of a fluorescent molecule changes its fluorescent properties. Fluorescent biosensors convert analyte binding or protein movement into changes in fluorescence by altering chemical structures or electrostatics surrounding a fluorescent molecule (Davila et al. 1973; Giepmans et al. 2006; Peterka et al. 2011; Grienberger & Konnerth 2012; Lin & Schnitzer 2016). The changes in fluorescence signal produced by this diverse family of biological tools are used to visualize otherwise invisible biochemical and biophysical events inside cells. Before the discovery of fluorescent proteins, biosensors used for optical imaging were based on fluorescent dyes (Davila et al. 1973; Peterka et al. 2011; Grienberger & Konnerth 2012) and aequorin (Shimomura et al. 1962). Chemical dyes and aequorin are still used today to monitor intracellular calcium release, membrane potential, and many other biologically important analytes. Fluorescent dye based biosensors are bright and easily administered to homogenous populations of cell cultures, however, there are drawbacks to their use. Dyes cannot be targeted to specific cell types, they often exhibit high levels of cytotoxicity, and are difficult to use for most *in vivo* application (Peterka et al. 2011; Grienberger & Konnerth 2012).

The discovery of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* revolutionized the field of fluorescent biosensors (Chalfie et al. 1994; Tsien 1998; Pieribone & Gruber 2005). GFP consists of an autocatalytically formed

chromophore, self-contained at the center of an 11 stranded β -barrel protein structure (Ormö et al. 1996). GFP is easily introduced and expressed in various cell types, making it possible to genetically target to specific cell types (Tsien 1998). Various mutations in the GFP chromophore and/or the β -barrel structure surrounding the chromophore has expanded the palette of fluorescent protein colors across the entire visible spectrum (Tsien 1998; Matz et al. 2002). Furthermore, many analogous fluorescent proteins have been discovered from other organisms (Shaner et al. 2005; M. Z. Lin 2011), making the fluorescent protein family quite diverse (Day & Davidson 2009). One of the most desirable traits of these fluorescent proteins, aside from genetic targeting, is that they are resilient to many structural manipulations, particularly fusions with other proteins.

Genetically-encoded fluorescent biosensors are specialized fusion proteins consisting of one or more fluorescent protein(s) with one or more biologically functional protein(s) that either binds and/or changes conformation in response to a biological event (i.e. a change in intracellular calcium or membrane potential) (Tsien 1998; Frommer et al. 2009; Chudakov et al. 2010; Okumoto et al. 2012). These proteins are made in the cell through transcription of an exogenous nucleic acid sequence, which can be targeted to a specific cell type. They are particularly useful for *in vivo* imaging and long-term optical experiments as most of them do not require the addition of any co-factors or chemicals to produce fluorescence and they do not present significant cytotoxic properties, compared to chemical dye biosensors.

There are two main families of genetically-encoded fluorescent biosensors. Förster resonance energy transfer (FRET) based biosensors consist of two fluorescent

proteins and change fluorescence via a change in the relative positions of the two fluorescence proteins. This occurs through non-radiative transfer of energy between two fluorophores in close proximity (Stryer & Haugland 1967). FRET-based biosensors require the use of two different excitation wavelengths and the collection of two separate emission bands. Single fluorescent proteins are constructed with a single fluorescent protein fused to one or more functional sensor proteins. For these biosensors, analyte binding / voltage-induced conformational changes in the sensor protein alters the environment of the chromophore in the fluorescent protein, resulting in a change in the fluorescent properties of fluorescent protein (Tsien 1998; Frommer et al. 2009; Chudakov et al. 2010; Okumoto et al. 2012). These sensors only require a single excitation wavelength and collection of one emission band. The work presented here focuses on single fluorescent protein based biosensors.

There are three ways the fluorescent properties can change in single fluorescent protein based biosensors: the extinction coefficient can change, the quantum efficiency can change, or the concentration of the chromophore species can change (i.e. altered protonation state or altered chemical bonds). These sensors are particularly desirable because they occupy a minimum range of the visible spectrum, corresponding to the absorption and emission of one fluorescent protein, whereas FRET-based biosensors involve absorption and emission of two different fluorescent proteins. Single fluorescent protein biosensors can be more easily combined with other optical tools (Akerboom et al. 2013; Dana et al. 2016), for example other fluorescent biosensors (Tewson et al. 2012; Li & Tsien 2012; Tewson et al. 2013) and/or optogenetic actuators (J. Y. Lin 2011; Fenno et

al. 2011; Bernstein & Boyden 2011; Reiner & Isacoff 2013; Chow et al. 2012). They can be constructed with a traditional fluorescent protein or a circularly-permuted fluorescent protein (Baird et al. 1999).

In a circularly-permuted fluorescent protein, the original N- and C-termini are linked with a short amino acid sequence and new termini are introduced into the side of the β -barrel structure (Baird et al. 1999). The key to this design is that the new N- and C-termini are located directly adjacent to the chromophore, creating a chromophore environment that is more sensitive to external factors. For use in biosensor designs, the fusion of the sensor protein(s) to the N- and/or C-termini at this location can exploit the increased sensitivity to produce large activity-dependent changes in fluorescence. Many types of biosensors apply this design approach, including biosensors for Ca^{2+} (Nagai et al. 2001; Nagai 2004; Ohkura et al. 2005; Tallini 2006; Tian et al. 2009; Muto et al. 2011; Zhao et al. 2011; Akerboom et al. 2012; Akerboom et al. 2013; Chen et al. 2013; Wu et al. 2014; Inoue et al. 2014; Dana et al. 2016), voltage (Barnett et al. 2012; St-Pierre et al. 2014; Abdelfattah et al. 2016), glutamate (Marvin et al. 2013), maltose (Marvin et al. 2011), cAMP (Tewson et al. 2012; Odaka et al. 2014), NADH-NAD⁺ (Hung et al. 2011), cGMP (Nikolaev et al. 2006; Bhargava et al. 2013), hydrogen peroxide (Belousov et al. 2006), ATP:ADP (Berg et al. 2009), and ammonium transport (De Michele et al. 2013; Ast et al. 2015).

Genetically-encoded Fluorescent Ca^{2+} Sensors

Ca^{2+} sensors are one of the most important imaging tools in neuroscience for imaging neural activity in living, functioning brains (Grienberger & Konnerth 2012; Bovetti et al. 2014). Ca^{2+} ions are involved in many different cellular processes and signaling pathways (Grienberger & Konnerth 2012). In neurons, the firing of action potentials signal rapid changes in intracellular concentrations of Ca^{2+} . Imaging the Ca^{2+} response of neurons has become an important, non-invasive way of monitoring activity in individual neurons and neuronal populations. This is because Ca^{2+} sensors have large signal-to-noise ratios, compared to current genetically-encoded voltage sensors or other biosensors, and the ease of expression in a wide range of animal models.

Genetically-encoded Ca^{2+} sensors are a family of fusion proteins based on one or more fluorescent protein(s) and a Ca^{2+} binding protein or protein complex (Whitaker 2010). Ca^{2+} is a ubiquitous signaling molecule and there are many proteins that interact with Ca^{2+} . Calmodulin (Chin & Means 2000) is an intermediate Ca^{2+} binding protein that is involved in many different second messenger pathways in eukaryotic cells. Two important proteins calmodulin interacts with are Myosin Light Chain (MLC) Kinase and Calmodulin Kinase II (CaMK-II). The M13 peptide is a synthetic peptide consisting of the same amino acid sequence as the calmodulin-binding domain in MLC. M13 is sufficient to interact with calmodulin in the presence of Ca^{2+} , the rest of the MLC protein is not necessary. Most genetically-encoded Ca^{2+} sensors are constructed with a Ca^{2+} -sensitive complex that includes a calmodulin domain and a target peptide from one of these two calmodulin associated proteins.

The first single fluorescent protein based Ca^{2+} sensor, GCaMP (Nagai et al. 2001), was based on a circularly-permuted GFP with calmodulin fused to the C-terminus and the M13 peptide fused to the N-terminus. In the presence of Ca^{2+} , calmodulin binds Ca^{2+} and then wraps around the M13 peptide. This Ca^{2+} -dependent interaction produces a significant change in the protein conformation at the side of the GFP β -barrel, directly adjacent to the chromophore. The GCaMP prototype has been extensively studied and iteratively improved upon (Ohkura et al. 2005; Nagai 2004; Tian et al. 2009; Muto et al. 2011; Nakai et al. 2001; Akerboom et al. 2012; Chen et al. 2013; Tallini 2006).

The most recent GCaMP6 generation (Chen et al. 2013) reports with such large changes in fluorescence that they have revolutionized what is possible with imaging neuronal activity in living, behaving animals (Boyd et al. 2015; Dana et al. 2014; Hinckley et al. 2015; Theis et al. 2016; Grover et al. 2016; Heckscher et al. 2015). Despite its wide use, there is still no quantitative biophysical model to explain the molecular mechanisms that drive the large change in fluorescence for the GCaMP sensors.

Understanding the molecular mechanisms of GCaMP6m will not only aid in development of future generations of Ca^{2+} sensors, but may also provide insight into how the field should be measuring and approaching biosensor design. Systematically measuring multiple fluorescence states in GCaMP6m or similarly designed biosensors is essential to understanding how they work and will reveal important application characteristics, such as potential pH sensitivity ranges and photostability properties.

Chapters 2 and 3 of this dissertation presents the results of a quantitative biophysical study for GCaMP6m and a series of GCaMP6m mutants.

Genetically-encoded Fluorescent Voltage Sensors

Measuring Ca^{2+} in a neuron during an action potential is not the same as measuring the membrane potential directly. First of all, the Ca^{2+} response happens on a much slower time scale than an action potential. As a result, there is little resolution of individual action potentials if they occur within rapid succession. Temporal information on fast-firing action potentials or sub-threshold neuronal activity is difficult to obtain with Ca^{2+} sensors. Secondly, because Ca^{2+} is involved in many cellular processes, this is not necessarily a direct readout of an action potential. The GCaMP sensors are regarded as the best optical tool neuroscientists have for observing neuronal activity in living animals, but a genetically-encoded fluorescent voltage sensor that can report changes in membrane potential directly is the ultimate goal.

Voltage-gated ion channels are complex, transmembrane proteins with a voltage sensitive domain, and a pore and gate domain. The voltage sensitive domain consists of a series of transmembrane α -helices, in which one α -helix includes a series of multiple charged amino acids. A change in the membrane potential induces a shift in the position of the charge carrying helix within the cell membrane, that causes a change in the conformation of the entire protein and gates an ion-conducting pore. Ion flux through these channels directly causes a change in the membrane potential and is the mechanism for action potential propagation (Bezanilla 1986; Bezanilla 2007; Bezanilla 2008).

Aside from ion channels, there are only two other transmembrane proteins with similar voltage-sensitive domains that gate other biological functions, voltage-sensitive GPCRs and phosphatases (Bezanilla 2008). The voltage-sensitive domain in voltage-sensitive phosphatases triggers protein rearrangements that are responsible for activating a phosphatase, which subsequently acts upon membrane associated proteins (Murata et al. 2005; Bezanilla 2008; Villalba-Galea et al. 2009; Okamura & Dixon 2011; Sakata et al. 2016).

Genetically-encoded fluorescent voltage sensors are fusion proteins that make it possible to see changes in the membrane potential of a cell (Bezanilla 2008; Baker et al. 2008; Storace et al. 2016) by coupling the movements of these voltage-sensitive proteins to a change in fluorescence. The ideal voltage sensor would traffic to cell membrane well in all types of cells, be brightly fluorescent, respond to changes in membrane potential with very fast, large changes in fluorescence, and would be highly resistant to photobleaching to withstand the increased sampling rate necessary to image the fast, millisecond time-scale event.

FlaSh, which stands for Fluorescent Shaker, was the first genetically-encoded voltage sensor (Siegel & Isacoff 1997). FlaSh is a fusion protein of a single, traditional green fluorescent protein inserted into the cytosolic C-terminal domain of a Shaker voltage-gated potassium channel subunit. The Shaker potassium channel is a homotetramer. To eliminate complications due to any additional ion conductance in the cell, FlaSh includes a point mutation in the pore of the channel which blocks the conductance of ions, but preserves the channel's gating rearrangements in response to a change in

membrane potential. When transiently expressed in oocytes, FlaSh labels the membrane with bright green fluorescence and reports changes in membrane potential with a voltage-dependent decrease in fluorescence (Siegel & Isacoff 1997). Unfortunately, FlaSh does not traffic to the membrane when transiently expressed in mammalian cells (Baker et al. 2008).

The fluorescence change in FlaSh is voltage-dependent, but how the protein movement of its voltage-sensitive domain affects the change in fluorescence of the GFP is unclear (Siegel & Isacoff 1997). What is clear, is that the kinetics of the voltage-dependent fluorescence change in FlaSh is slower than the voltage-dependent movement of the charge-carrying α -helix of the voltage sensing domain through the membrane, notably in the off-phase of the voltage response (Siegel & Isacoff 1997). The off-kinetics of the fluorescence is several milliseconds slower than the movement of charge through the membrane. This slow recovery of fluorescence in the Off-phase means that resolving individual action potentials in a neuron would not be possible with this voltage sensor.

SPARC was the first genetically-encoded fluorescent voltage sensor with a voltage-dependent fluorescence change fast enough to resolve individual events in a train of multiple action potentials (Ataka & Pieribone 2002). SPARC is a fusion protein of a single green fluorescent protein inserted between domain I and II of the four domain rat skeletal muscle sodium channel (rSkM1). SPARC captures a fast voltage dependent movement of the voltage sensing domain of rSkM1 (Ataka & Pieribone 2002). While SPARC traffics to the membrane in oocytes, it does not traffic to the membrane in

mammalian cells (Baker et al. 2008; Storace et al. 2016). This is a common issue with channel-based voltage sensors and has greatly limited their use in mammalian cell cultures and animal models.

The discovery of the voltage-sensitive phosphatase from *Ciona Intestinalis* (CiVSP) was a major advance in genetically-encoded fluorescent voltage sensors (Murata et al. 2005). The voltage sensing domain of CiVSP is similar to the voltage-gated channels, with four transmembrane α -helices including one charge carrying α -helix that is responsible for voltage-induced protein rearrangements. There are many benefits of CiVSP over voltage-gated channels: it is most likely a monomeric protein (Kohout et al. 2008), it is not an ion conducting channel (Murata et al. 2005), and it traffics well to the membrane when transiently expressed in mammalian cells (Halaszovich et al. 2009; Lundby et al. 2010). For all these reasons, CiVSP was quickly adopted as an important voltage sensing domain scaffold for voltage sensors (Lundby et al. 2008; Tsutsui et al. 2008; Jin et al. 2010; Mutoh et al. 2011).

The CiVSP-based voltage sensors before 2012 (Baker et al. 2008; Lundby et al. 2008; Storace et al. 2016) were only able to capture a slow movement in CiVSP, nothing had been able to capture a fast component like SPARC (Ataka & Pieribone 2002). If the goal of the voltage sensor tool is to be able to resolve individual action potentials within a mammalian neural circuit, which will likely involve multiple action potentials within a given time, the voltage sensor needs to be able to report the fast ($\sim 1 - 3$ ms) voltage-dependent movements in CiVSP. Chapter 4 presents the development of ElectricPk, the

first voltage sensor based on a circularly-permuted fluorescent protein that captures a fast voltage-dependent movement in CiVSP.

Imaging Biosensors with Two-photon Microscopy

To observe neural activity deep within the brain, biosensors will need to be imaged with two-photon excited fluorescence (Xu 1996; Zipfel et al. 2003; Bovetti et al. 2014). In one photon absorption, a molecule absorbs a single photon and emits a single photon. In two photon absorption, a molecule simultaneously absorbs two photons of identical wavelength and emits a single photon (Göppert-Mayer 1931). The probability of a molecule absorbing two photons simultaneously is extremely unlikely, therefore at low light intensities one-photon absorption processes will dominate. For instance, in bright sunlight, the dye rhodamine B will absorb a single photon once per second, while a two-photon absorption event will happen only once every 10,000,000 years (Denk & Svoboda 1997). One-photon absorption has a linear dependence on the excitation light intensity. Two-photon absorption is a non-linear process, meaning that it is dependent on the square of the excitation light intensity. To increase the probability of a two-photon absorption event, the excitation light must be focused in both space and time. A tightly focused, high-powered pulsed laser beam is necessary to deliver a large number of photons to the molecule in a small focused space, at a moment in time.

Confocal microscopy is a widely used three-dimensional imaging modality for living tissue samples. It uses one-photon absorption processes to excite the sample, collecting emitted photons only from the focal plane by use of a pinhole aperture at the

detection source. The pinhole eliminates all out-of-focus light and scattered photons coming from the out-of-focus excited tissue in the sample. In deep tissue, a majority of photons are scattered on their path out of the tissue which means they are blocked by the pinhole and are not detected. Higher light intensities are necessary to increase the emitted fluorescence from the focal plane, which increases tissue damage and photobleaching of the fluorescent molecules used in the sample. Absorption happens in the entire tissue sample within the path of excitation light, therefore tissue damage and photobleaching occurs throughout the entire path of light. While higher laser powers are mandatory, the nonlinear property of two-photon absorption is beneficial for imaging in deep tissue because only the molecules in the focal plane are excited, reducing background fluorescence and photo-bleaching of molecules in out-of-focus tissue, as well as eliminating tissue damage from surrounding area (Xu 1996; Denk & Svoboda 1997; Zipfel et al. 2003).

Two-photon absorption properties of a fluorescent protein do not necessarily follow the one-photon absorption properties (Drobizhev et al. 2011). Since the discovery of GFP, there have been extensive efforts to evolve GFP (Tsien 1998) and various other fluorescent proteins (Shaner et al. 2005) for improved one-photon excited properties. However, there is a paucity of effort given to improving the two-photon absorption properties of fluorescent proteins. This can be attributed to two important reasons. First, directed evolution is difficult and slow because of the complex and largely unknown relationship between two-photon cross-section of the chromophore and the protein structure. Second, random mutagenesis of fluorescent proteins requires high-throughput

screening of a high number ($\geq 10,000$) of mutants to be successful. The need for a focused, high-powered beam of light to successfully achieve two-photon excitation has limited the rate of screening and the number of variants that can be explored. Chapter 5 addresses these particular setbacks and presents a proof-of-principle design for the first high-throughput screen for optimizing the two-photon absorption properties of EGFP.

CHAPTER TWO

DECIPHERING THE MOLECULAR MECHANISM RESPONSIBLE FOR
GCAMP6M'S Ca^{2+} DEPENDENT CHANGE IN FLUORESCENCEContribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Lauren M. Barnett

Contributions: Conceived project; contributed to experimental design and performed critical data analysis; performed all molecular biology, protein purification and protein handling, and HEK293 cell culture and Ca^{2+} imaging experiments; performed all spectroscopic experiments; wrote main manuscript text and produced all figures; contributed to the review of the manuscript.

Co-Author: Thomas E. Hughes

Contributions: Conceived project; contributed to experimental design and performed critical data analysis; supervised project; contributed to the review of the manuscript.

Co-Author: Mikhail Drobizhev

Contributions: Conceived project; contributed to experimental design and performed critical data analysis; responsible for the conceptualization of the electrostatic interaction model and the mathematical derivations; contributed to the review of the manuscript; corresponding author.

Manuscript Information Page

Lauren M Barnett¹, Thomas E Hughes¹ and Mikhail Drobizhev¹

¹ Department of Cell Biology and Neuroscience, Montana State University, Bozeman, Montana, United States

Status of the manuscript:

☐ Prepared for submission to a peer-reviewed journal

☐ Officially submitted to a peer-reviewed journal

☐ Accepted by a peer-reviewed journal

☒ Published in a peer-reviewed journal

Published in PLoS ONE, doi:10.1371/journal.pone.0170934

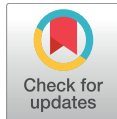
RESEARCH ARTICLE

Deciphering the molecular mechanism responsible for GCaMP6m's Ca^{2+} -dependent change in fluorescence

Lauren M. Barnett, Thomas E. Hughes, Mikhail Drobizhev*

Department of Cell Biology and Neuroscience, Montana State University, Bozeman, Montana, United States

* mikhail.drobizhev@montana.edu



Abstract

The goal of this work is to determine how GCaMP6m's fluorescence is altered in response to Ca^{2+} -binding. Our detailed spectroscopic study reveals the simplest explanation for how GCaMP6m changes fluorescence in response to Ca^{2+} is with a four-state model, in which a Ca^{2+} -dependent change of the chromophore protonation state, due to a shift in pK_a , is the predominant factor. The pK_a shift is quantitatively explained by a change in electrostatic potential around the chromophore due to the conformational changes that occur in the protein when calmodulin binds Ca^{2+} and interacts with the M13 peptide. The absolute pK_a values for the Ca^{2+} -free and Ca^{2+} -saturated states of GCaMP6m are critical to its high signal-to-noise ratio. This mechanism has important implications for further improvements to GCaMP6m and potentially for other similarly designed biosensors.

OPEN ACCESS

Citation: Barnett LM, Hughes TE, Drobizhev M (2017) Deciphering the molecular mechanism responsible for GCaMP6m's Ca^{2+} -dependent change in fluorescence. PLoS ONE 12(2): e0170934. doi:10.1371/journal.pone.0170934

Editor: Eugene A. Permyakov, Russian Academy of Medical Sciences, RUSSIAN FEDERATION

Received: September 28, 2016

Accepted: January 12, 2017

Published: February 9, 2017

Copyright: © 2017 Barnett et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the National Institute of Health / National Institute of Neurological Disease and Stroke grant R01 NS083875 and the BRAIN initiative grant U01 NS094246.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Genetically-encoded Ca^{2+} sensors based on a single fluorescent protein (i.e. non-FRET based) are important imaging tools in neuroscience. The newest generation GCaMP6 sensors (GCaMP6s, GCaMP6m, and GCaMP6f, [1]) are bright and respond to transient increases in Ca^{2+} within the cell with such large changes in fluorescence that they can be used to image neuronal activity in awake, behaving animals, throughout large regions of the brain [2–5] and in entire brains [6,7].

The GCaMP sensors are constructed with circularly-permuted GFPs [8] in which the original N- and C-termini of the enhanced green fluorescent protein (EGFP) are linked together with a short, 6 amino acid peptide, and new termini are created in the middle of the 7th β -strand of the 11-stranded β -barrel, creating an opening in the side of the barrel directly adjacent to the phenolate oxygen in the chromophore. A portion of the Ca^{2+} binding protein calmodulin, and its target peptide the M13 domain, are individually fused to the new C- and N-termini of the circularly-permuted GFP, respectively (Fig 1). The fusion of these proteins at this very sensitive location couples the Ca^{2+} -dependent calmodulin binding of M13 to large changes in the fluorescence of the circularly-permuted GFP.

The GCaMPs have been extensively studied and iteratively improved upon. More is known about the GCaMP sensors than any other biosensor, but there is still no quantitative molecular

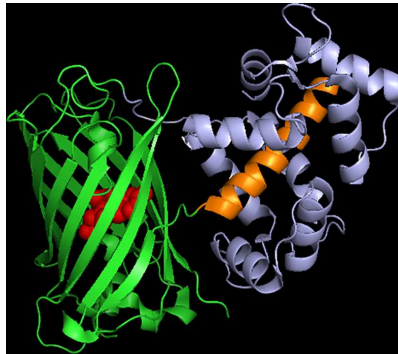


Fig 1. Schematic of Ca^{2+} -saturated GCaMP6m structure. M13 domain (orange); circularly-permuted GFP domain (green); calmodulin domain (purple). Made using 3WLD.pdb [9].

doi:10.1371/journal.pone.0170934.g001

model that explains how these Ca^{2+} sensors produce such large changes in fluorescence. There are, however, several hints in the literature. First, it is clear that the pK_a ($\text{pK}_a = -\lg K_a$, where K_a is the acid dissociation constant) of the chromophore in the Ca^{2+} sensor is different for the Ca^{2+} -free and Ca^{2+} -saturated states [8,10–15]. Second, amino acids at key positions close to the chromophore are critical to the function of the Ca^{2+} sensor [9,11,12,14–17]. Third, the protein conformation of the Ca^{2+} -free state allows increased solvent access directly to the chromophore, via the circularly-permuted GFP opening in the side of the protein barrel, compared to the Ca^{2+} -saturated state [9,11,14,18].

Ultimately, there are only three ways for GCaMP fluorescence to change as a function of Ca^{2+} -binding: the extinction coefficient can change, the quantum efficiency of emission can change, or there can be rapid changes in the concentration of the fluorescent chromophore. Our goal was to determine which of these mechanisms is most important.

Results

The mechanism of GCaMP6m is more complicated than a simple "dim" to "bright" transition of the chromophore

GCaMP fluorescence is typically imaged with ~ 480 nm excitation (for one-photon excitation), observing the total emission peaking at ~ 510 nm [19–22]. Fig 2A illustrates that when Ca^{2+} levels inside the cell are low, there is little fluorescence, and when Ca^{2+} levels rise, so does the 480 nm excited fluorescence intensity (green trace, Fig 2A). The GCaMP6m response can also be observed using 410 nm excitation, but the fluorescence is weaker (with comparable excitation power and the same detection filter) and it decreases during the response to Ca^{2+} (blue trace, Fig 2A). This phenomenon was also observed for the earlier, GCaMP-like ratiometric pericam Ca^{2+} sensor [12], and indicates that the mechanism of GCaMP6m is more complicated than a simple chromophore transition from a dim state to a bright state.

To understand this behavior, we need to characterize the photophysical properties of GCaMP6m. The absorption spectra for purified GCaMP6m protein are different in Ca^{2+} -free and Ca^{2+} -saturated states (black traces, Fig 2B and 2C). For the Ca^{2+} -free state, there is a dominant neutral form peak at 403 nm and a very small anionic form peak at 504 nm (Fig 2B). For Ca^{2+} -saturated state, the absorption intensities redistribute such that the anionic form peak at 497 nm is dominant and the neutral form peak at 397 nm is smaller (Fig 2C). There

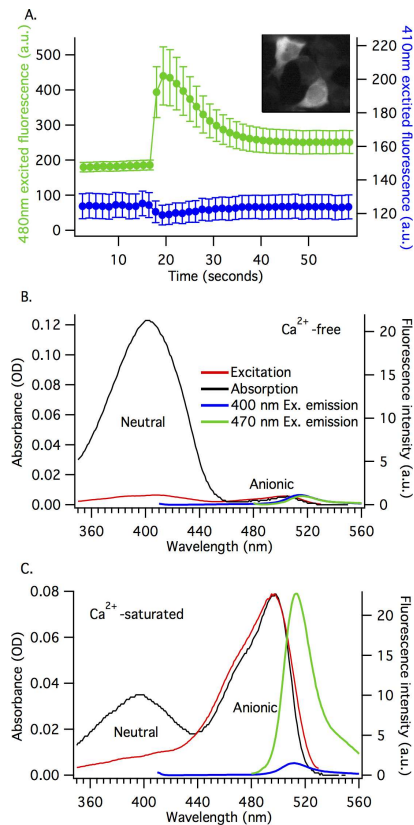


Fig 2. The Ca^{2+} -dependent change in GCaMP6m fluorescence is more complicated than a chromophore transition from dim to bright. A) Transient changes in Ca^{2+} produce different responses in the 410 nm and 480 nm excited fluorescence of GCaMP6m. This graph shows GCaMP6m response to the release of intracellular Ca^{2+} stores in HEK293 cells (average of 12 cells, mean $\pm$ standard error). Green trace represents 480 nm excited fluorescence (left axis), Blue trace represents 410 nm excited fluorescence (right axis), fluorescence emission was collected at ≥ 515 nm. Muscarinic receptor (M1) activation was used to trigger the Ca^{2+} transient. Inset image: two HEK293 cells at peak value of 480 nm excited fluorescence. B and C) show that GCaMP6m exists in an equilibrium between at least two forms of the chromophore. Absorption spectra (black trace, left axis) and excitation spectra (red trace, right axis), and emission spectra for 400 nm excited fluorescence (blue trace, right axis) and 470 nm excited fluorescence (green trace, right axis) for purified GCaMP6m protein in 0 μM free Ca^{2+} buffer (Ca^{2+} -free, B) and 39 μM free Ca^{2+} buffer (Ca^{2+} -saturated, C). Fluorescence emission collected at 550 nm for excitation spectra.

doi:10.1371/journal.pone.0170934.g002

is a notable blue-shift in the peak absorption of the anionic chromophore, from 504 nm in the Ca^{2+} -free state to 497 nm in the Ca^{2+} -saturated state (Fig 2B and 2C). This shift can be qualitatively explained by a change in the local electric field at the GCaMP6m chromophore due to Ca^{2+} -dependent rearrangements of the chromophore surrounding [23].

The intensity distribution in the fluorescence excitation spectra is different from the absorption spectra. Whether the neutral or anionic form of the chromophore is excited, almost all of the GCaMP6m fluorescence emission comes from the anionic form of the chromophore (~ 515 nm), due to the excited state proton transfer (ESPT) that occurs from the electronically

excited state of the neutral form of chromophore to a nearby proton accepting group [24–26]. The fluorescence excitation spectra (red traces, Fig 2B and 2C) are measured by detecting the fluorescence of the anionic form. By normalizing the excitation and absorption spectra with respect to the anionic form peak, it is clear that the excitation at the neutral form peak (~400 nm) produces a much weaker signal than expected from the neutral form absorption peak. This is true for both the Ca^{2+} -free and Ca^{2+} -saturated states of GCaMP6m.

If the quantum efficiency of the ESPT is much less than 1, the fluorescence efficiency of the indirectly-excited anionic form (via excitation of the neutral form) will be much smaller than that of the directly-excited anionic form and there will be a decrease of the excitation signal, compared to the corresponding absorption. The inefficient ESPT in GCaMP6m, compared to wild-type GFP, is most likely due to the T222 residue (T65 in EGFP) which breaks the essential proton transfer wire [24,27]. Without the proton transfer wire and with inefficient direct emission from the neutral form (blue trace, Fig 2), the relaxation of the neutral chromophore from the excited state to the ground state is dominated by a more efficient internal conversion [24]. By comparing the absorption and excitation spectra, the ratio of the quantum yields for the neutral and anionic forms in GCaMP6m can be roughly estimated: ~0.05:1 (neutral:anionic) for Ca^{2+} -free, and ~0.055:1 for Ca^{2+} -saturated states. These excitation spectra are consistent with previous measurements of GCaMP6m and other GCaMP genetically-encoded Ca^{2+} sensors [1,10,11,13,14,16,17,28].

The significant red-shift of the absorption peak of the anionic chromophore in both the Ca^{2+} -free and Ca^{2+} -saturated GCaMP6m (504 nm and 497 nm, respectively), relative to the parent EGFP (488 nm), can be explained by rearrangement of local hydrogen-bonding with the chromophore. Instead of three hydrogen bonds of the phenolate oxygen in EGFP (with T203, H148, and a water molecule) there are only two in GCaMP6m (with 2 water molecules) [9]. In GCaMP6m, the mutations T115V (T203V in EGFP) and H60E (H148E in EGFP) disrupt the hydrogen-bonding of these residues with the chromophore. Similar rearrangements in the hydrogen bond network of other GFP variants are known to result in the red shift of the anionic chromophore [23,24].

To examine the fluorescence behavior of the neutral and anionic forms of the chromophore upon binding Ca^{2+} , we collected Ca^{2+} -dependent fluorescence emission spectra with 470 nm (Fig 3A) and 400 nm (Fig 3B) excitation. The 470 nm excited fluorescence increases as a function of increasing Ca^{2+} concentration, whereas the 400 nm excited fluorescence decreases. The 400 nm excited fluorescence response is the opposite of the 470 nm excited response, but of qualitatively different magnitude. The Ca^{2+} concentration dependent behavior in the purified protein samples is similar to the fluorescent responses observed in our HEK293 cells experiments (Fig 2A).

Careful inspection reveals that there is a shift in the peak emission wavelength as a function of increasing Ca^{2+} concentrations in the spectra excited at 400 or 470 nm (Fig 3A and 3B). The peak emission of the anionic form blue-shifts 3 nm between the 0 and 39 μM buffered free Ca^{2+} samples when excited at 470 nm (from 516 to 513 nm, Fig 3A). The peak emission of the neutral form blue-shifts 2 nm between the 0 and 39 μM buffered free Ca^{2+} samples when excited at 400 nm (from 514 to 512 nm, Fig 3B). Normalized spectra in the expanded lower graphs highlight this shift for each Ca^{2+} titration. This reproducible shift (see S1 Fig for more data) is consistent with the shift in absorption of the anionic form, and inconsistent with a simple equilibrium between only two forms of the chromophore.

The peak fluorescence intensities for both the 470 and 400 nm excited emission spectra are plotted against buffered free Ca^{2+} concentration in Fig 3C and 3D, respectively. For the 470 nm excitation plot, the red line represents the best fit of the data to the Hill equation (Fig 3C), which illustrates the apparent Ca^{2+} affinity and cooperativity of GCaMP6m. The apparent K_d

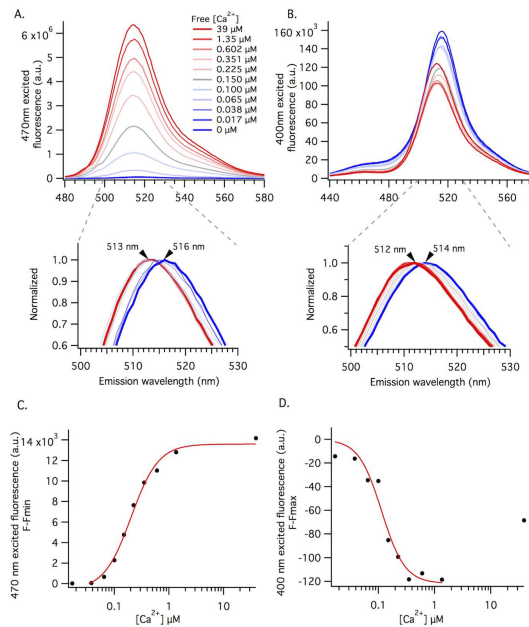


Fig 3. The GCaMP6m Ca^{2+} -dependent spectra are consistent with four fluorescent states of the chromophore. A and B illustrate the Ca^{2+} concentration dependent fluorescence of GCaMP6m. 470nm (top, A) and 400 nm (top, B) excited fluorescence spectra for purified protein at 11 different buffered free Ca^{2+} concentrations, ranging from 0 μM (dark blue trace) to 39 μM (dark red trace), at pH 7.2. The 400 nm excited spectra for 39 μM free Ca^{2+} sample is marked with black arrow (B). Extended lower graphs highlight Ca^{2+} concentration dependent λ_{max} with normalized 470 nm (A) and 400 nm (B) excited fluorescence (same spectra as top graphs). Arrows mark the apparent peak wavelength shift between 0 μM (dark blue trace) to 39 μM (dark red trace) buffered free Ca^{2+} concentrations. C and D) Peak fluorescence intensity values from spectra in 2A and 2B, respectively, plotted against buffered free Ca^{2+} concentration. F/F_{min} (y-axis, 2C) is used for 470 nm excited fluorescence because baseline fluorescence in the 0 μM free Ca^{2+} buffer is the minimum fluorescence at this excitation wavelength. Alternatively, F/F_{max} (y-axis, 2D) is used for 400 nm excited fluorescence because baseline fluorescence in the 0 μM free Ca^{2+} buffer is the maximum fluorescence at this excitation wavelength.

doi:10.1371/journal.pone.0170934.g003

and Hill coefficient values ($K_d = 164 \pm 31$ nM and $n = 2.43 \pm 0.44$) are consistent with the previously reported values [1].

For the 400 nm excitation (Fig 3D), the red line is only a qualitative illustration of the decreasing fluorescence trend. The 400 nm excited fluorescence signal cannot be accurately fit with the Hill equation because it includes the emission of more than one form of the chromophore. One is the neutral form, peaking near 400 nm, and the other is the anionic form, peaking near 500 nm but with a long absorption tail extending near 400 nm. The Ca^{2+} -saturated spectrum in Fig 3B (39 μM free Ca^{2+} buffer, red trace marked with black arrow) and the corresponding peak intensity in Fig 3D (final data point), illustrate this confounded measurement via an unexpected increase in 400 nm excited fluorescence. The relatively small fraction of the anionic form excited through the spectral tail overwhelms the response of the excited neutral form because it has a much larger fluorescence quantum yield.

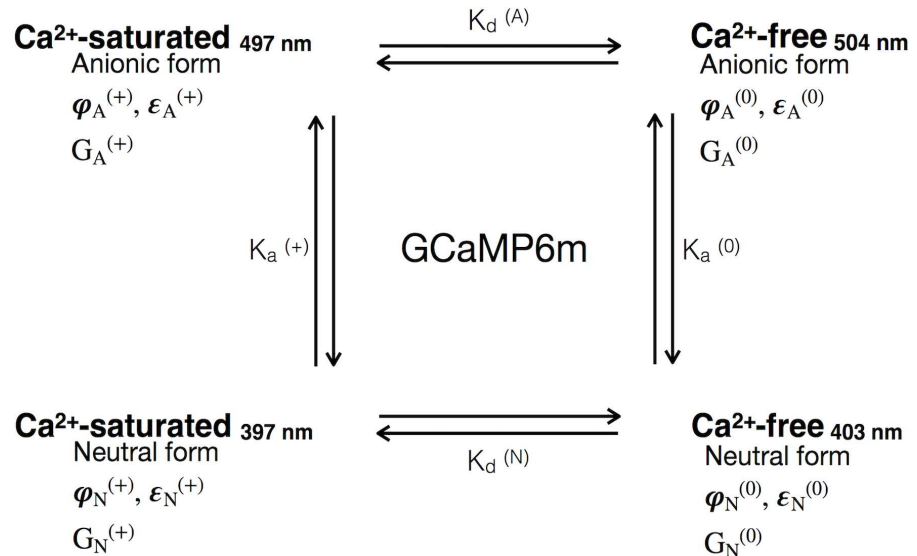


Fig 4. A four state model requires the quantitative characterization of each of the four distinct Ca^{2+} -dependent states of GCaMP6m. This model illustrates a simple four state equilibrium that includes all possible combinations of the Ca^{2+} -saturated and Ca^{2+} -free states of the sensor protein, as well as anionic and neutral forms of the chromophore. Indices: Anionic form (A); Neutral (N); Ca^{2+} -free (0); Ca^{2+} -saturated (+); Quantum yield (φ); extinction coefficient (ϵ); Gibbs free energy of the protein (G); dissociation constant for calmodulin in GCaMP6m with anionic chromophore ($K_d^{(A)}$); dissociation for calmodulin in GCaMP6m with neutral chromophore ($K_d^{(N)}$); Acid dissociation constant for chromophore in Ca^{2+} -saturated state of GCaMP6m ($K_a^{(+)}$); Acid dissociation constant for chromophore in Ca^{2+} -free state of GCaMP6m ($K_a^{(0)}$).

doi:10.1371/journal.pone.0170934.g004

There are four different Ca^{2+} -dependent states of GCaMP6m

A four-state model can explain the observed results. This model includes a Ca^{2+} -free neutral form, a Ca^{2+} -free anionic form, a Ca^{2+} -saturated neutral form, and a Ca^{2+} -saturated anionic form (Fig 4). Here, we assume that each state has a unique combination of photophysical parameters, including extinction coefficient, quantum yield and two-photon cross-section, and they are connected through equilibrium constants (K_d and K_a). Each GCaMP6m state is also characterized by a unique electrostatic interaction energy of the chromophore with the surrounding protein (part of Gibbs free energy, G).

With at least two forms of the chromophore in equilibria for a given sample, it is difficult to accurately measure the extinction coefficients for each form. The traditional 1-step alkaline denaturation of the entire sample is insufficient to resolve the concentrations of the individual chromophore species. Alkaline titration, however, makes it possible to measure both the ratio of the concentrations of two different forms of the chromophore and the total concentration of chromophore in the sample. Fig 5 illustrates the changes in absorption spectrum for the Ca^{2+} -free (5A) and Ca^{2+} -saturated (5B) purified protein samples as they are titrated from neutral to more alkaline pH.

For the Ca^{2+} -free state, with increasing pH, the neutral form absorption peak decreases and the anionic form peak increases (Fig 5A), as expected. At pH values larger than 10.5, the anionic form peak shifts to shorter wavelengths (485 nm) and another peak belonging to the anionic chromophore of the denatured protein (447 nm, [29]) starts to grow, becoming the only peak at pH > 11.

There appears to be two isosbestic points, or a "smearing" of the spectra, for Ca^{2+} -free state. The first point is at 450 nm and corresponds to absorption spectra between pH 7.15 and 7.90 (grey arrow, Fig 5A). The second isosbestic point is shifted to shorter wavelengths, at 440 nm, corresponding to absorption spectra between pH 8.29 and 9.32 (grey arrow, Fig 5A). The shift between the two isosbestic points is due to the emergence of an additional, shorter-wavelength absorbing anionic form (peaking at ~485 nm), that is not the denatured form (447 nm). This blue-shifted anionic form can be tentatively explained by the titration of a nearby acidic residue in GCaMP6m, E134 (E222 in EGFP), close to the deprotonated chromophore. Analogous spectral shifts are seen in the red fluorescent proteins, mCherry and mStrawberry, where the peaks also blue-shift because of the titration of the corresponding E215 residue at more alkaline pH [30].

The correlation between the optical densities of the two main absorption peaks in Ca^{2+} -free GCaMP6m (Fig 5A, inset) maintains a consistent linear relationship (see methods for more details) between pH 7.15 and 9.89, before the shorter-wavelength peak starts to dominate. The slope of the linear fit for the first four data points is used to obtain the ratio of the two extinction coefficients for the neutral and anionic chromophore species. These points correspond to the spectra in the titration range of the first isosbestic point, before the emergence of a new form. The individual extinction coefficients for the Ca^{2+} -free neutral and anionic chromophores are presented in Table 1.

For the Ca^{2+} -saturated state, with increasing pH, there is a gradual transition from the neutral form into the anionic form (Fig 5B). This transition is illustrated by a single, clean isosbestic point at 432 nm, corresponding to the absorbance spectra between pH 7.15 and 8.29. A clean isosbestic point indicates the presence of only two forms in this pH range. Similar to the Ca^{2+} -free state, at pH ≥ 8.7 , the anionic absorption peak significantly blue-shifts (~485 nm) most likely due to the titration of E134, before the protein is denatured (447 nm). Another isosbestic point at 455 nm corresponds to this second transition.

The inset graph (Fig 5B) illustrates the linear relationship between the optical densities of the neutral and anionic chromophore in Ca^{2+} -saturated GCaMP6m within the titration range of the first isosbestic point (pH 7.15 and 8.29), from which we extracted the ratio of the two individual extinction coefficients. The individual Ca^{2+} -saturated extinction coefficients and fluorescence quantum yields measured for each of the four distinct fluorescent forms in GCaMP6m are listed in Table 1.

The extinction coefficient of the neutral form of the chromophore is almost identical for the Ca^{2+} -free and Ca^{2+} -saturated states (Table 1). The extinction coefficient of the anionic form of the Ca^{2+} -saturated state is 14% larger than that of the Ca^{2+} -free state (Table 1). There are also very small changes in the quantum yields for the neutral form and the anionic form upon binding Ca^{2+} (Table 1).

The measurement of $\Delta F/F_0$ for GCaMP6m is wavelength dependent because the anionic chromophore absorption blue shifts when GCaMP6m binds Ca^{2+} (Fig 5A and 5B). The maximum relative change of fluorescence of anionic form between Ca^{2+} -free and Ca^{2+} -saturated states occurs when exciting near 470 nm, $\Delta F/F_0 = 30$ (see S2 Fig). At 470 nm, there is a 25% increase in extinction coefficient of the anionic form and 13% increase in quantum yield upon binding Ca^{2+} , which cannot explain $\Delta F/F_0 = 30$. The predominant factor responsible for the large Ca^{2+} -dependent change in 470 nm excited GCaMP6m fluorescence is most likely a dramatic change in the relative concentration of the neutral and anionic forms of the chromophore.

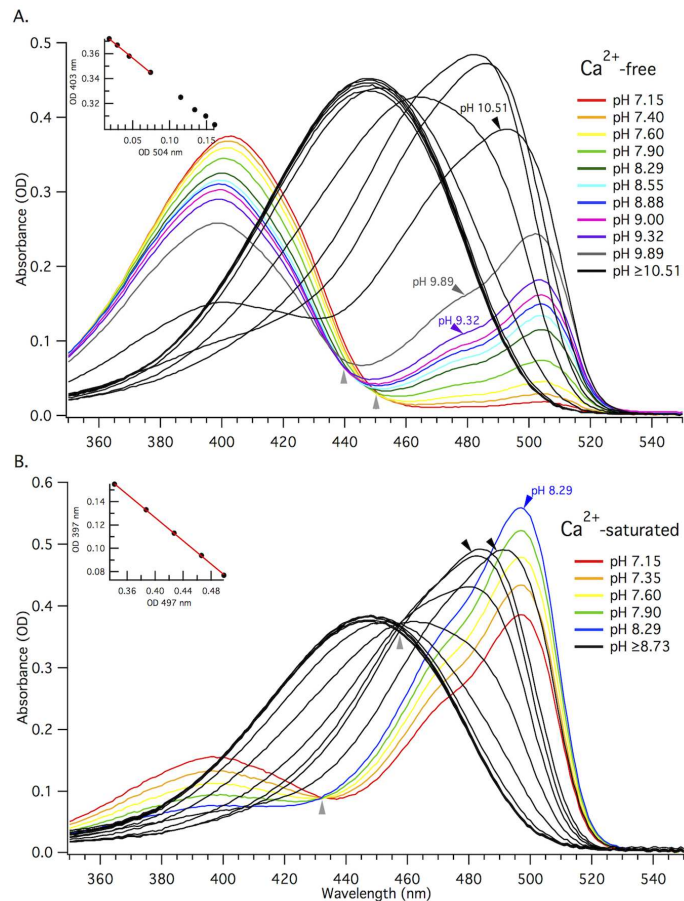


Fig 5. Alkaline titration makes it possible to measure the extinction coefficient of two different forms in the same sample. A and B) Absorption spectra for alkaline titration of purified GCaMP6m protein in 0 μM free Ca^{2+} buffer (Ca^{2+} -free, A) or 39 μM free Ca^{2+} buffer (Ca^{2+} -saturated, B). Individual traces represent absorption spectra at different pH values. The final two colored traces and the first black trace for Ca^{2+} -free sample (A) are marked with arrows. The last colored trace and first two black traces for Ca^{2+} -saturated sample (B) are marked with arrows. This boundary marks a significant pH dependent change in the shape of the absorption peak near 500 nm and the last spectrum where we see linear intercorrelation of the change in OD for the two peaks. Light grey arrows below traces mark the isosbestic points. The peak value at 447 nm (black trace), gives the total concentration of chromophore. Inset plots: Absorbance values for 403 nm versus 504 nm taken from the first 8 colored traces (from pH 7.15 to 9) of the absorption spectra for Ca^{2+} -free GCaMP6m (A), and for 397 nm versus 497 nm taken from all of the colored traces (from pH 7.15 to 8.29) for Ca^{2+} -saturated GCaMP6m (B). These plots illustrate the change in OD for the neutral form (397 nm or 403 nm) relative to the anionic (497 nm or 504 nm) form for the respective pH ranges. The red linear fit of the first 4 data points (from pH 7.15 to 7.90) for Ca^{2+} -free (A) and all 5 data points for Ca^{2+} -saturated (B) in the change in OD plots gives us the ratio of the extinction coefficients for the neutral and anionic forms in the given sample.

doi:10.1371/journal.pone.0170934.g005

There is a Ca^{2+} -dependent change in pK_a for the GCaMP6m chromophore

The shift of the neutral—anionic equilibrium upon binding Ca^{2+} is driven by a change of the pK_a value of the GCaMP6m chromophore. To examine this shift quantitatively, we collected

Table 1. Photophysical parameters for GCaMP6m.

	$\epsilon_{\text{max}} \text{ M}^{-1}\text{cm}^{-1}$		φ		$\sigma_2 \text{ GM}$		n		pK_a
	$(\lambda_{\text{abs}}, \text{nm})$		$(\lambda_{\text{fluor}}, \text{nm})$		$(\lambda_{\text{abs}}, \text{nm})$				
	Neutral	Anionic	Neutral	Anionic	Neutral	Anionic	Neutral	Anionic	
Ca²⁺-free	37,300	73,600	0.041	0.56	140	54	0.97	0.03	8.01,10.08
	(403)	(514)	(514)	(516)	(790)	(930)			8.68*
Ca²⁺-saturated	37,700	83,100	0.048	0.63	—	50	0.49	0.51	7.10
	(397)	(497)	(512)	(513)		(930)			6.90*
				0.61*					

Extinction coefficient (ϵ); quantum yield (ϕ); two-photon cross-section (σ_2); relative concentration of chromophore (n), previously reported values for GCaMP6m are in italics.

*[1]

doi:10.1371/journal.pone.0170934.t001

fluorescence emission from the anionic GCaMP6m chromophore excited at 470 nm as a function of pH, for the Ca^{2+} -saturated and Ca^{2+} -free states. Both the Ca^{2+} -saturated and Ca^{2+} -free states of GCaMP6m exhibit an increase in peak fluorescence as a function of increasing pH. For the Ca^{2+} -saturated state, the fluorescence signal increases up to pH ~8.9 (solid circles, Fig 6). The last two data points in the Ca^{2+} -saturated titration at pH 8.47 and 8.83, before fluorescence starts to decrease, do not correspond to the initial anionic form alone. The signals in this range include emission from the blue-shifted anionic form observed in the extinction coefficient absorption measurements (first two black traces, Fig 5B). To get the apparent pK_a for the chromophore, we only used the data for pH from 4 to 8.39 (colored traces, Fig 5B; green fit trace, Fig 6), giving us a pK_a of 7.10 ± 0.01 for Ca^{2+} -saturated GCaMP6m. This pK_a is slightly higher than previously reported value [1].

There are two distinct titration events that show up in the pH titration curve for the Ca^{2+} -free state (open circles, Fig 6), which requires a two-binding site model. After pH ~11.5, the protein begins to denature and the 470 nm excited fluorescence decreases because the chromophore in denatured state does not fluoresce. The apparent pK_a of the Ca^{2+} -free GCaMP6m chromophore in the first range is 8.01 ± 0.01 . The second titration at the larger pH values most likely corresponds

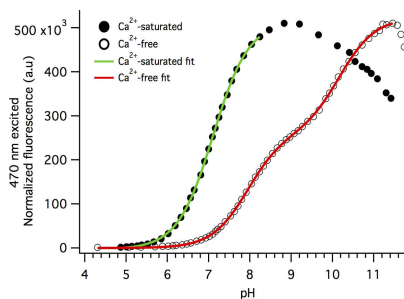


Fig 6. GCaMP6m has a single pK_a for the Ca^{2+} -saturated state and two pK_a 's for the Ca^{2+} -free state. Normalized 470 nm excited fluorescence intensity at 515 nm plotted against pH for Ca^{2+} -saturated (solid circles) and Ca^{2+} -free (open circles) states. A single-binding site model fit (green trace) was used for the Ca^{2+} -saturated state and includes data points for one titration event from pH 4.3 to 8.39 with a pK_a of 7.10 ± 0.01 . A two-binding site model fit (red trace) was used for the Ca^{2+} -free state and includes data points for two titration events from pH 4.3 to 10.5, the first with a pK_a of 8.01 ± 0.01 , and the second with a pK_a of 10.08 ± 0.02 .

doi:10.1371/journal.pone.0170934.g006

to the deprotonation of E134, and the apparent pK_a for this range is 10.08 ± 0.02 . If all the data are fitted with a one-binding site model curve, the pK_a will be close to 9.0, similar to what has been previously reported [1]. The large change in pK_a between the first titration curve of Ca^{2+} -free and Ca^{2+} -saturated states (8.01 to 7.10) results in an increase in the concentration of the anionic chromophore at neutral pH, and consequently, a significant increase of the fluorescence signal excited at 470 nm.

The pH titration fluorescence measurements are wavelength dependent. Using a more red shifted excitation wavelength for the pH-dependent fluorescence measurements (488 nm versus 470 nm, for example) limits the resolution of the multiple chromophore species that show up as pH increases, making it more difficult to determine individual pK_a values.

To better understand GCaMP6m, we need to understand the molecular mechanisms of the Ca^{2+} -dependent change in pK_a

The most significant factor in GCaMP6m's large change in fluorescence is a Ca^{2+} -dependent change of pK_a . In proteins, the pK_a depends on electrostatic potential energies of interactions between a titratable group, the GCaMP6m chromophore in this case, and the surrounding protein [31]. When the calmodulin domain in GCaMP6m binds Ca^{2+} , there is a significant conformational change in the protein and several charged amino acid residues shift position relative to the chromophore. The displacement of these charged residues results in a change of potential at the chromophore and, consequently, pK_a of the chromophore. Determining the main amino acids contributing to this change will help to better understand the GCaMP sensors and could prove useful in future improvement of GCaMP6m.

The pK_a is related to the change of the free energy (ΔG) that occurs in the process of proton transfer from a titrating group to the surrounding water molecules [31]:

$$2.3RT \cdot \text{pK}_a^{(p)} = G_s^{(p)}(A^-) - G_s^{(p)}(AH) + G_s^{(w)}(A^-) - G_s^{(w)}(AH) + 2.3RT \cdot \text{pK}_a^{(w)} \quad (1)$$

where $\text{pK}_a^{(w)}$ corresponds to pK_a of the chromophore in water (w), $\text{pK}_a^{(p)}$ corresponds to the pK_a of the chromophore surrounded by the protein barrel (p), and G_s is the solvation energy of either the anionic (A^-) or neutral chromophore (AH). We only consider the difference between the pK_a values for the Ca^{2+} -free (0) and Ca^{2+} -saturated (+) states. Applying Eq 1 to these two different states and subtracting one from another to get the ΔpK_a , we obtain,

$$\Delta \text{pK}_a = \text{pK}_a^{(p,0)} - \text{pK}_a^{(p,+)} = \frac{\Delta \Delta G_s}{2.3RT} \quad (2)$$

where,

$$\Delta \Delta G_s = \Delta G_s^{(p,0)} - \Delta G_s^{(p,+)} \quad (3)$$

and

$$\Delta G_s^{(p)} = G_s^{(p)}(A^-) - G_s^{(p)}(AH) \quad (4)$$

Eq 3 describes the difference between the changes of free energy of the proton transfer reaction upon binding Ca^{2+} .

It is essential to calculate the $\Delta \Delta G_s$ contributions from various amino acid residues in the chromophore environment to identify those that are most important for the Ca^{2+} -dependent change in pK_a . All of the free energy contributions from amino acids that do not move between the Ca^{2+} -free and Ca^{2+} -saturated states cancel in calculating $\Delta \Delta G_s$, therefore we only consider the $\Delta G_s^{(0)}$ and the $\Delta G_s^{(+)}$ contributions for amino acids with a Ca^{2+} -dependent change of position in proximity to the chromophore. We examined charged amino acid residues inside the

pocket close to the circularly-permuted GFP opening and occupying the surfaces of GFP-M13 linker in both the Ca^{2+} -free and Ca^{2+} -saturated states: the $\alpha 3$ helix in calmodulin for the Ca^{2+} -saturated state, $\alpha 4$ and $\alpha 5$ helices of calmodulin in the Ca^{2+} -free and Ca^{2+} -saturated states, the linker between $\alpha 4$ and $\alpha 5$ of calmodulin in the Ca^{2+} -free and Ca^{2+} -saturated states, and the linker between $\beta 6$ strand in GFP and $\alpha 1$ helix in calmodulin for the Ca^{2+} -free state. The selection of residues belonging to the pocket is somewhat subjective, but we picked those that interact with the chromophore directly. Direct interaction was defined as an unobstructed (by any other domain of the protein) line of connection between the residue and the center of chromophore (the CB2 atom). These residues represent the "first shell" of the chromophore surrounding. We assume other charged amino acids in more distant "shells" are screened by bulk water and therefore not contributing to the total electrostatic potential of the chromophore environment.

To calculate the electrostatic potential energy, we apply the point charge model for the amino acid residues and a set of Mulliken charges on the chromophore atoms for both anionic and neutral forms [32,33]:

$$G = 1390 \sum_i \frac{Qq_i}{R_i} \quad (5)$$

where G is calculated in kJ/mol, Q is the charge on the residue (in units of elementary charge), q_i is the charge on the i -th atom of chromophore, and R_i is the distance between Q and q_i (in Angstrom). For all potential energy calculations, we used the GCaMP2 protein as a template because both the Ca^{2+} -free and Ca^{2+} -saturated GCaMP2 crystal structures are available. The coordinates of the atoms for the Ca^{2+} -free monomeric form were taken from the 3EKJ pdf file [11] amended with a Ca^{2+} -free calmodulin structure (1CFD pdb file [34]) by overlaying the two proteins (to the best coincidence) using UCSF Chimera software. For the Ca^{2+} -saturated monomeric protein we used the 3EK4 pdb file [11]. The structures of circularly-permuted EGFP (3EVP.pdb [18]), and Ca^{2+} -saturated monomeric GCaMP2- Δ RSET (3EVR.pdb [18]), GCaMP3- Δ RSET (4IK5.pdb [35]) and GCaMP6m (3WLD.pdb [9]) proteins were also used to obtain additional information on the position of water molecules and amino acids in GCaMP6m that were missing in the GCaMP2 structure.

To check our model, we first considered the effect of point mutations on ΔpK_a . If a charged amino acid is removed from the structure, the $\Delta\Delta G$ and consequently ΔpK_a will change because of the eliminated electrostatic interaction. A single mutation D380Y in GCaMP3 protein was made to make the GCaMP5A protein [14]. This substitution results in a $\Delta pK_a = 1.93$ for GCaMP5A, compared to the $\Delta pK_a = 1.43$ for GCaMP3 [14]. In GCaMP3, the D380 residue is in the linker between the $\alpha 4$ and $\alpha 5$ helices of CaM, directly above the opening of GFP barrel. It is positioned approximately 31 Å from the center of the chromophore in the Ca^{2+} -free state and moves closer, ~15 Å from the center of the chromophore, in the Ca^{2+} -saturated state. The D380 residue belongs to the "first shell" of the chromophore surrounding. The calculated D380 $\Delta\Delta G$ contribution is $\Delta\Delta G = -73.0$ kJ/mol, which corresponds to a change of ΔpK_a by -12.6. This means that the removal of the D380 charge with the D380Y mutation should result in an increase of ΔpK_a by 12.6 units. Although there is an increase in ΔpK_a between the two proteins, magnitude of the calculated difference is much larger than what is observed experimentally, $\Delta\Delta pK_a = 0.5$ [14].

One explanation for this difference could be that we disregard the chromophore polarizability, i.e. the redistribution of electronic density that occurs when GCaMP3 binds Ca^{2+} and the negative charge of D380 moves closer to the chromophore. This charge-induced dipole interaction would reduce the interaction energy by, $(1/2)\alpha \cdot [e/R^2]^2$, where α is the polarizability of

the chromophore in the ground state, and R is the distance between the center of chromophore and D380 side chain in the corresponding Ca^{2+} -dependent state. Using the polarizability value for the GFP chromophore, $\alpha = 76 \times 10^{-24} \text{ cm}^3$ [36], and the distances described in the previous paragraph, we estimate the correction to $\Delta\Delta G < 1 \text{ kJ/mol}$ (absolute value), which cannot explain the observed difference between our calculated $\Delta\Delta G$ value (73.0 kJ/mol) and what is expected from the experimentally measured ΔpK_a ($\Delta\Delta G = 2.9 \text{ kJ/mol}$).

Large discrepancies between experimental and calculated values of electrostatic interaction energies have been observed for similar systems in which a charged amino acid moves relative to a fluorescent probe (tryptophan residue) at the protein surface, if only protein charges are considered [37]. The discrepancies are entirely resolved if point charges from all water molecules within $\sim 15 \text{ \AA}$ of the fluorescent probe are included, because of the opposing electrostatic field from the water molecules ordered by the protein charges (dielectric compensation) [37]. In circularly-permuted GFP, the opening in the β -barrel allows water molecules to access and hydrogen-bond with the chromophore, especially the phenolate moiety [11,18]. All of this considered, we make two important assumptions: one is that for part of the chromophore, the change of potential due to displacement of D380 is fully compensated by the dielectric response of nearby water molecules; and the second is that because the number of water molecules decreases towards the center of the protein barrel, the atoms with full compensation are most likely closer to the circularly-permuted opening. With these assumptions, we can make a series of calculations for the interaction energy (Eq 5) by sequentially setting the electric potential of each chromophore atom equal to zero, starting with a single atom, the phenolate oxygen closest to the circularly-permuted opening, and moving inward toward the imidazolinone ring, further from the circularly-permuted opening (Fig 7). The corresponding potential energies (Table 2) demonstrate that as more atoms experience an electric potential of zero, the calculated $\Delta\Delta pK_a$ value between GCaMP3 and GCaMP5A (due to the mutation D380Y) approaches the experimental value. The calculated $\Delta\Delta pK_a$ is consistent with the experimental $\Delta\Delta pK_a$ once the atom potentials from the phenolate oxygen to the CB2 atom are set to zero (i.e. considering only the electric potentials on all of the atoms of the imidazolinone ring).

We checked this approach for the following GCaMP transitions: GCaMP3 to GCaMP5D, GCaMP5D to GCaMP5G, and GCaMP5G to GCaMP6m. In all of these cases, the calculations are consistent with the reported experimental values of ΔpK_a (Table 3). We assumed that K379S (GCaMP5G to GCaMP6m transition) is in the pocket only for the Ca^{2+} -saturated state, R392G (GCaMP5G to GCaMP6m transition) is not in the pocket for both Ca^{2+} -free and Ca^{2+} -saturated

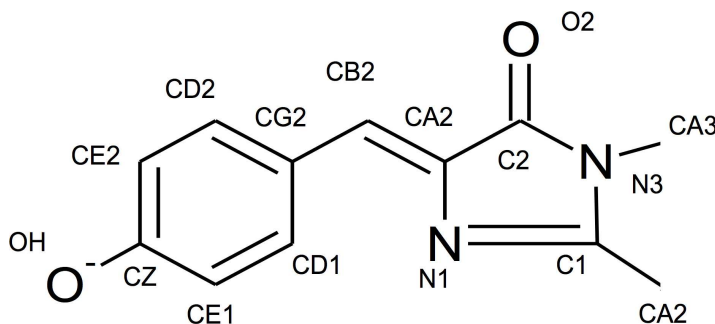


Fig 7. Atom numbering for the GCaMP chromophore.

doi:10.1371/journal.pone.0170934.g007

Table 2. Calculated changes of $\Delta\Delta G$ and ΔpK_a for the transition from GCaMP3 to GCaMP5A in a model that assumes different sets of chromophore atoms interacting with D380 residue.

	Atoms included in calculation with electric potential of non-zero							Experimental Value*
	All	CZ to CA3	CE2 to CA3	CD2 to CA3	CG2 to CA3	CB2 to CA3	CA2 to CA3	
$\Delta\Delta G$ (kJ/mol)	-73.0	-30.8	-42.2	-16.3	-8.5	-13.5	-4.6	—
$\Delta\Delta pK_a$	-12.8	-5.4	-7.4	-2.9	-1.5	-2.4	-0.79	-0.5 ± 0.03

Each step of included atoms for the sequential calculations in our model (excluded atoms have electric potential equal to zero). Refer to Fig 7 for labelled illustration of the GCaMP chromophore.

*[14]

doi:10.1371/journal.pone.0170934.t002

states, and all other mutated charged residues (for all other transitions in Table 3) are in the pocket for both Ca^{2+} -free and Ca^{2+} -saturated states.

This approach reveals critical charged amino acids that affect the $\Delta\Delta G$ and the corresponding Ca^{2+} -dependent ΔpK_a for GCaMP2 and GCaMP6m, individually (Table 4). As before, we consider only those residues that fill the pocket around the circularly-permuted GFP opening and parts of calmodulin, and disregard those that do not move significantly upon Ca^{2+} binding (residues with $\Delta\Delta G < 1$ kJ/mol). There are approximately 9–10 contributing charged amino acid residues for both proteins (Table 4). Their total effect on ΔpK_a agrees well with the experimental values (Table 4). The number of contributing residues and the magnitude of their contribution varies between the GCaMP generations. In GCaMP6m, E386 and E60 provide the largest effects (in absolute values), the rest contribute smaller amounts, in relatively equal proportions. In GCaMP2, the effect of E61 is very close to the total effect ($\Delta pK_a = 1.92$ vs. 1.6, respectively) because the interactions of the rest of the residues almost cancel each other. This can explain why the point mutations E61K and E61G (GCaMP2 numbering) can reduce or completely eliminate the Ca^{2+} -dependent change in fluorescence of GCaMP2 [18]. The important effects of amino acids E387, R377, D381, K380 (GCaMP2 numbering) on increasing or decreasing the Ca^{2+} -dependent fluorescence response have also been demonstrated by point mutagenesis [11,18].

The two-photon absorption properties do not necessarily follow the same behavior as the one-photon absorption

The GCaMP6 sensors are important tools for imaging activity in living brains, much of which is done with two-photon excitation [2–7]. The two-photon absorption properties do not necessarily follow the same behavior as the one-photon properties [38]. Fig 8 illustrates the two-photon excitation spectra of GCaMP6m Ca^{2+} -saturated and Ca^{2+} -free states at neutral pH. In both states, the chromophore exists as a mixture of neutral (N) and anionic (A) forms (concentrations n_N and n_A , respectively). Both forms of the chromophore are fluorescent within the same

Table 3. The changes in ΔpK_a between recent GCaMP generations.

GCaMP Transitions	Relevant Charge Changing Mutations	Experimental $\Delta\Delta pK_a$ *	Calculated $\Delta\Delta pK_a$
GCaMP3 to GCaMP5A	D380Y	0.50 ± 0.03	0.79
GCaMP3 to GCaMP5D	R303P	0.05 ± 0.06	0.02
GCaMP5D to GCaMP5G	D380Y	0.70 ± 0.08	0.79
GCaMP5G to GCaMP6m	K379S, T381R, R392G	-1.27 ± 0.06	-1.33

*[14]

doi:10.1371/journal.pone.0170934.t003

Table 4. Contributions to $\Delta\Delta G$ and ΔpK_a from important charged amino acids in GCaMP2 and GCaMP6m.

GCaMP2 amino acid	GCaMP6m amino acid	Position with respect to pocket		Contribution to $\Delta\Delta G$ (kJ/mol)		Contribution to ΔpK_a	
		Ca^{2+} -free	Ca^{2+} -saturated	GCaMP2	GCaMP6m	GCaMP2	GCaMP6m
E61	E60	in	in	11.0	11.0	1.92	1.92
E357	E356	out	in	-6.8	-6.8	-1.19	-1.19
E387	E386	out	in	-16.3	-16.3	-2.86	-2.86
E386	E385	in	out	4.8	4.8	0.84	0.84
D381	Y380	in	in	-4.6	—	-0.81	—
D383	D382	out	in	-6.1	-6.1	-1.07	-1.07
D305	D304	in	out	6.8	6.8	1.19	1.19
R377	R376	out	in	5.4	5.4	0.95	0.95
K378	K377	out	in	6.1	6.1	1.07	1.07
K380	S379	out	in	9.0	—	1.58	—
T382	R381	in	in	—	1.4	—	0.246
Total $\Delta\Delta G$ (kJ/mol) or ΔpK_a (calculated):				9.3	6.3	1.6	1.1
Total $\Delta\Delta G$ (kJ/mol) or ΔpK_a (experimental):				—	—	1.7*	0.91

*[11]

doi:10.1371/journal.pone.0170934.t004

spectral region (green), have unique two-photon absorption spectra, and they contribute to the observed spectrum based on their relative concentrations, $\rho_N = n_N/(n_N + n_A)$ and $\rho_A = n_A/(n_N + n_A)$. Considering all of the above, the y-axis of Fig 8 represents a linear combination of the two individual two-photon action cross-section spectra: $F_2(\lambda) = \sigma_{2,N}(\lambda) \phi_N \rho_N + \sigma_{2,A}(\lambda) \phi_A \rho_A$.

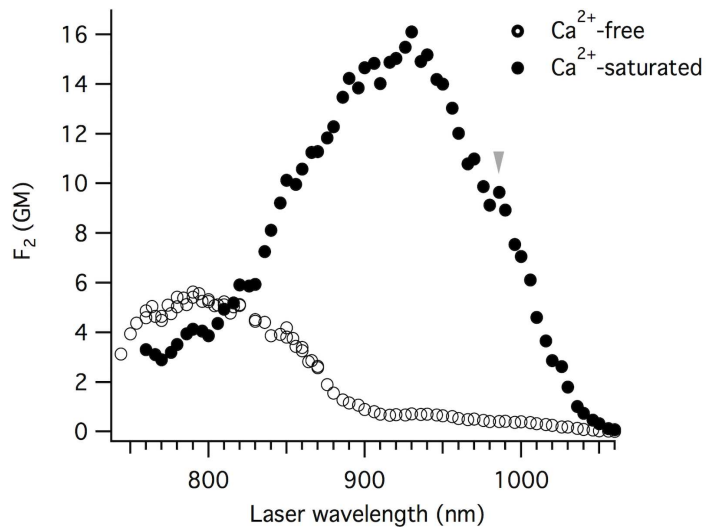


Fig 8. Two-photon excitation spectra for GCaMP6m. Spectra are presented as a linear combination of the two individual two-photon action cross-section spectra: $F_2(\lambda) = \sigma_{2,N}(\lambda) \phi_N \rho_N + \sigma_{2,A}(\lambda) \phi_A \rho_A$, where $\sigma_2(\lambda)$ is the wavelength-dependent two-photon excitation cross-section, ϕ is the fluorescence quantum yield, and ρ is the relative concentration of the chromophore species (neutral, N, or anionic, A). Ca^{2+} -saturated (solid circles) and Ca^{2+} -free (open circles).

doi:10.1371/journal.pone.0170934.g008

$\varphi_A \rho_A$, where $\sigma_2(\lambda)$ is the wavelength-dependent two-photon excitation cross-section and φ is the fluorescence quantum yield.

For the Ca^{2+} -saturated state, the spectrum is dominated by the anionic form, peaking at ~ 930 nm (black solid circles, Fig 8). This is because the neutral form has a significantly lower quantum yield (Table 1) and is in smaller relative concentration for the Ca^{2+} -saturated state. To obtain $F_2(\lambda)$, we normalized the whole spectrum to the value of $F_2 = \sigma_{2,A}(\lambda) \varphi_A \rho_A$ at 930 nm. Since the neutral form does not absorb at this wavelength, the latter was calculated as a product of independently measured $\sigma_{2,A}$ (930 nm) = 50 GM, $\varphi_A = 0.63$ (Table 1), and $\rho_A = 0.51$ (obtained from linear absorption spectrum in Fig 2, and extinction coefficients ϵ_A and ϵ_N in Table 1). The two-photon excitation spectral peak for the anionic form (930 nm) is blue-shifted with respect to twice the wavelength of the one-photon absorption peak, grey arrow (994 nm) which can be explained by the enhancement of vibronic transition in two-photon absorption versus one-photon absorption, as a result of the unique Herzberg-Teller coupling effect [39,40].

In the Ca^{2+} -free state, the neutral form dominates the two-photon excitation spectrum. The F_2 spectrum for this state was normalized to the peak value at 800 nm, which was obtained from independently measured $\sigma_{2,N}$ (800 nm) = 130 GM, $\varphi_N = 0.041$ (Table 1), and $\rho_N = 0.97$ (obtained similarly to ρ_A above). The neutral form two-photon excitation peak position (790 nm) is very close to twice the wavelength of the corresponding one-photon absorption peak, and the anionic form peak position is blue shifted with respect to twice the one-photon absorption peak.

We independently measured the two-photon absorption cross-section of the anionic form of the Ca^{2+} -free state to be $\sigma_2(930 \text{ nm}) = 54 \text{ GM}$. This value is very close to what would be expected from the $F_2(930 \text{ nm})$ value, obtained directly from the spectrum (Fig 8). This comparison is consistent with the fact that the equilibrium constant (ρ_A and ρ_N values) and quantum yields (φ_A and φ_N values) should be the same for one- and two-photon excited fluorescence. However, the two-photon cross-section for the neutral form ($\sigma_2(790 \text{ nm}) = 140 \text{ GM}$) is 2.6 times larger than the anionic form (54 GM) contrary to the ratio of extinction coefficients, where the neutral form is 2 times smaller than the anionic form (Table 1).

Even though the Ca^{2+} -free GCaMP6m two-photon fluorescence signal excited at 790 nm is much stronger compared to that of 930 nm (Fig 8), the excitation of the anionic form at 930 nm is still preferred for two-photon imaging. The anionic form produces a larger Ca^{2+} -dependent change in fluorescence due to a much larger fluorescence quantum yield of the anionic form: $\Delta F_{2,A}/\Delta F_{2,N} = 5.6$ (Fig 8).

The two-photon cross-sections for the neutral and anionic forms of GCaMP6m are significantly larger than the comparable non-circularly-permuted T203I EGFP mutant [41], even though they have similar one-photon absorption peak positions [23]. The $\sigma_{2,\text{max}}$ for the anionic form of the T203I EGFP mutant is 19 GM [41], compared to 50 and 54 GM for Ca^{2+} -saturated and Ca^{2+} -free GCaMP6m, respectively. The increase in anionic form $\sigma_{2,\text{max}}$ compared to T203I EGFP, is probably due to differences in the local surrounding of the chromophore, namely increased solvent access through the circularly-permuted opening and/or higher internal rotational freedom of the chromophore in both Ca^{2+} -free and Ca^{2+} -saturated states.

Discussion

There is a significant difference in the quantum yield and extinction coefficients between the neutral and anionic form of the chromophore, but the photophysical parameters of the anionic form do not change much upon Ca^{2+} -binding. When calmodulin binds Ca^{2+} , it is the redistribution of the GCaMP6m population, from the neutral to the anionic form of the chromophore, that is responsible for the large change in ~ 470 nm excited fluorescence. The pK_a value

of the chromophore strongly depends on specific amino acid positions in the chromophore environment. The change of the pK_a in our measurements, from 8.01 to 7.10, is consistent with the estimation of energies based on GCaMP2 and GCaMP6m crystal structures, as there are several essential charged amino acids that shift positions with respect to the chromophore when calmodulin binds Ca^{2+} . We predict that E386, E60, E356, D304, D382, K377, R376, and E385 are the most important. The change in pK_a is important, but the absolute values of pK_a are important as well. Large signals will only be produced when the pK_a values of the Ca^{2+} -free and Ca^{2+} -saturated states straddle physiological pH. This relationship is illustrated in the comparison of the $\Delta F/F_0$ and pK_a values reported for various GCaMP/GCaMP-like genetically-encoded Ca^{2+} sensors [9–17,19,42] and other similarly designed circularly-permuted fluorescent protein biosensors [42–46].

The pK_a of Ca^{2+} -saturated GCaMP6m ($\text{pK}_a = 7.10$) is very close to physiological pH. This means at pH 7.2, roughly 50% of the Ca^{2+} -saturated GCaMP6m remains in the non-fluorescent (with ~470 nm for one-photon excitation or ~930 nm for two-photon excitation) neutral form of the chromophore. It also means that any small fluctuations of intracellular pH will affect the fluorescence signal of the Ca^{2+} sensor. At pH 7.2, a 0.2 pH unit fluctuation would result in a 17% change (increase or decrease, for alkaline or acidic pH fluctuations, respectively) in the Ca^{2+} -saturated anionic chromophore fluorescence. If the pK_a of the Ca^{2+} -saturated state was shifted to a more acidic pH, it would reduce GCaMP6m pH sensitivity and increase $\Delta F/F_0$. Altering the GCaMP6m design to include positive residues that move closer to chromophore when calmodulin binds Ca^{2+} would produce this acidic pK_a shift, increasing the ΔpK_a and the ΔF signal. As shown in Fig 6B, there is room to improve GCaMP6m ΔF by about ~70%. Rational or random mutagenesis of GCaMP6m combined with screening for specific pK_a properties [47], such as shifted pK_a values (i.e. more acidic values for Ca^{2+} -saturated state) or increased ΔpK_a would provide more information regarding pK_a and ΔpK_a effects on Ca^{2+} sensor function, and could be beneficial for evolving GCaMP6m for increased ΔF without changing Ca^{2+} -binding dynamic.

The four-state model we have applied for GCaMP6m could also be applied to understand the Ca^{2+} -dependent fluorescence changes observed for other GCaMPs [9–17,19,28,42] and GCaMP-like biosensors [15,42–46,48–57]. iGluSnFR is a glutamate sensor based on a circularly-permuted GFP design similar to the GCaMPs [48]. The glutamate dependent pK_a shift reported for iGluSnFR is from 7.0 in the "free" state to 6.5 in the "bound" state, the relative concentrations of neutral and anionic forms change upon binding glutamate. FlinG2 and FlinG3 are cGMP sensors, also based on a GCaMP-like circularly-permuted GFP design, and report pK_a shifts of 7.48 (free) / 7.33 (bound) and 7.94 (free) / 7.53 (bound), respectively [43]. The FlinG biosensors also exhibit pH dependent changes in the ΔF signals, similar to what is seen for GCaMP6m. It is reasonable to consider that pK_a is important to how these sensors function, but to what extent is unclear. Applying a four-state model for these sensors, or any other circularly-permuted fluorescent protein biosensor [44–46,49–55,57], could reveal the predominant mechanism(s) responsible for their activity dependent changes in fluorescence and help improve future generations.

There are several clues in the literature that indicate a four-state model may also provide some insight into how ArcLight [58] and similar voltage sensors [59] function. ArcLight is constructed with a single, non-circularly-permuted, pH sensitive version of GFP, Super Ecliptic pHluorin [60] with an A227D mutation, fused to the intracellular C-terminal end of the voltage sensing domain of *Ciona intestinalis* voltage sensitive phosphatase (CiVSP) [58]. The Super Ecliptic pHluorin chromophore, like most GFP variants, exists in a protonation equilibrium. The neutral to anionic chromophore ratio is determined by its pK_a (~7.1) [60,61]. A change in the membrane potential of a cell expressing ArcLight triggers a conformational

change in the voltage sensing domain of CiVSP, which translates to a decrease in the Super Ecliptic pHluorin A227D fluorescence. It is clear that the pH sensitivity of the fluorescent protein in ArcLight is essential for a voltage-dependent change in fluorescence [58,62]. It is also clear that a change in pH near the cell membrane during depolarization is not responsible for the ArcLight signal: when a pH sensitive protein is anchored at the membrane, without a voltage-sensitive protein, it does not respond to voltage changes [59,62]. Pado is a genetically-encoded voltage sensor consisting of a single, non-circularly-permuted super ecliptic pHluorin A227D fused to the intracellular C-terminal end of the voltage sensitive domain in a functional proton channel [59]. Pado is capable of monitoring changes in membrane potential and intracellular pH [59]. In altered extracellular pH experiments, at voltage steps large enough to open the proton conducting channel, Pado exhibits an increase in the magnitude of its 470 nm excited fluorescence response with an increase in pH, and a decrease in the magnitude of the 470 nm excited fluorescence response with a decrease in pH [59], much like GCaMP6m. One explanation for this data could be that Pado and ArcLight work like GCaMP6m and the reporting mechanism involves an essential voltage-dependent change in the pK_a of the pH-sensitive fluorescent protein, triggered by conformational changes in the CiVSP voltage sensing domain. Remarkably, there are very few measurements of the neutral form in these single GFP-based voltage sensors [63], or biophysical studies on the hyperpolarized and depolarized states [64], that might help substantiate this idea. Applying a four-state model to this family of sensors and exploring the importance of a voltage-dependent change in pK_a is certainly worth exploring, and will be a topic of our future investigation.

The remarkable redistribution of the GCaMP chromophore from neutral to anionic forms has two important consequences. First, with 470 nm excitation, the Ca^{2+} sensor is dark at low Ca^{2+} concentrations, and only fluorescent in active cells, producing an excellent signal-to-noise ratio in complex tissues like the brain. Second, since the neutral form of the chromophore is not absorbing the 470 nm light, the Ca^{2+} sensor is only susceptible to bleaching during the brief periods that the cell is active. This is also true for two-photon excitation with 930 nm light. The Ca^{2+} dependent equilibrium of the GCaMP6m chromophore essentially helps the Ca^{2+} sensor "hide in the dark" when there is no activity to report, making these biosensors uniquely resistant to photobleaching. This will be a beneficial property to incorporate into any future biosensor designs, especially for applications involving high frame rates and increased excitation light intensity.

Materials and methods

Imaging intracellular Ca^{2+} response in HEK293 cells

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium, High Glucose (DMEM) and supplemented with 8% fetal bovine serum (FBS). Cells were plated in 24-well glass bottom plates coated with poly-D-lysine and kept in an incubator at 37°C with 5% CO_2 . Transient transfection were done with lipofectamine following the manufacturer's protocol (Invitrogen). Cells were co-transfected with pGP-CMV-GCaMP6m (Addgene, Plasmid #40754) and an M1 muscarinic receptor in the CMV expression vector pUB2.1 (Addgene, Plasmid #40728). For imaging, DMEM was replaced with warmed Dulbecco's Phosphate Buffered Saline (DPBS) with Ca^{2+} and Mg^{2+} . The wide-field imaging experiments were performed on an Olympus IX70 microscope fitted with Sutter filter wheels and a 20x dry lens, alternating excitation with 410/20 nm and 480/20 nm bandpass filters and collecting emission with a 515 nm long-pass filter and CCD camera (Hamamatsu). To elicit intracellular Ca^{2+} release, carbachol was added to the well to a final concentration of 50 μM .

Preparation of purified protein samples

The coding region of GCaMP6m was moved into a constitutive bacterial expression vector (pCP, generous gift from Nathan Shaner), using ligation-independent cloning [49]. *E. coli* colonies expressing GCaMP6m were picked for presence of 470 nm excited fluorescence and grown at 34°C for 48 hours in CircleGrow (MP Biomedicals) with Ampicillin. Bacterial pellets were lysed using BugBuster (Novagen) and Benzonase (Novagen). Cleared lysates were then His-tag purified using Protino Ni-TED 2000 packed columns (Macherey-Nagel). Purified fluorescent proteins were eluted in 1x PBS with imidazole pH 8 buffer solution. The protein was then concentrated and buffer exchanged into a pH 7.2 MOPS buffer using Vivaspin Turbo 15 (Sartorius) polyethersulfone ultrafiltration columns. The same buffer exchange columns were used to make all Ca^{2+} -free and Ca^{2+} -saturated samples for protein measurements, except for the Ca^{2+} titration experiments. All purified GCaMP6m Ca^{2+} -free protein measurements for this work were carried out in 10 mM EGTA / 100 mM KCl / 30 mM MOPS at pH 7.2. All purified GCaMP6m Ca^{2+} -saturated protein measurements for this work were carried out in 10 mM CaEGTA / 100 mM KCl / 30 mM MOPS at pH 7.2.

Ca^{2+} titration measurements

Ca^{2+} titration experiments for purified protein samples were done using Ca^{2+} Calibration Buffer Kit #1 (Life Technologies), following their protocol. We measured fluorescence intensity at the spectral peak (between 512 nm and 516 nm) as a function of Ca^{2+} concentration by varying CaEGTA concentrations of the 2 mL sample (0 to 10 mM CaEGTA). We used excitation at 470 nm to measure the Ca^{2+} dependent fluorescence of the anionic form, and 400 nm for the neutral form. The pH of the sample was measured at beginning and end of each titration series to ensure there was no shift in pH during titration. For the apparent K_d and Hill coefficient values from the 470 nm excited measurements, we averaged 3 separate Ca^{2+} titration measurements.

Fluorescence spectra, fluorescence excitation spectra, and quantum yield measurements

Fluorescence spectra and fluorescence excitation spectra of purified protein samples were collected using a PC1™ spectrofluorimeter (ISS). Fluorescence spectra were corrected for the detection spectral sensitivity using the quinine sulfate solution in 1M H_2SO_4 , whose corrected spectrum is published [65]. Fluorescence excitation spectra were corrected for excitation spectral variations using the styryl 9M (Aldrich) solution in ethanol, by comparing its excitation spectrum with its independently measured absorption spectrum. Fluorescence quantum yield (ϕ) of the GCaMP6m anionic chromophore (fluorescence peak for Ca^{2+} -saturated at 513 nm; for Ca^{2+} -free at 516 nm) upon its direct excitation at 470 nm was measured versus fluorescein in 1M NaOH ($\phi = 0.95$) as a reference standard [65]. Fluorescence quantum yield (ϕ) of the GCaMP6m neutral chromophore (fluorescence peak for Ca^{2+} -saturated at 512 nm, for Ca^{2+} -free at 514 nm) was measured using excitation at 380 nm versus quinine sulfate in 1M H_2SO_4 ($\phi = 0.53$) as a reference standard [65]. The fluorescence spectrum excited at 380 nm contains a minor short-wavelength contribution due to the direct emission from neutral chromophore near 450–470 nm. To get rid of this contribution, we used the fluorescence spectrum of Ca^{2+} -free form excited at 470 nm (which lacks this contribution) and normalized it to the maximum of the spectrum excited at 380 nm (see S3 Fig). This normalized spectrum was then integrated. The optical density of the solutions at each excitation wavelength and in the whole range of fluorescence was kept less than 0.07. Corrected integrated spectra were used to calculate quantum yields [65].

Extinction coefficient measurements

To measure extinction coefficients of the neutral and anionic forms of the chromophore in GCaMP6m (either in Ca^{2+} -free or Ca^{2+} -saturated states) separately, we measured the changes of the corresponding absorption spectrum upon alkaline pH titration of the purified protein solutions (cf. [66]). Absorption measurements were performed with a BioMate™ S3 spectrophotometer (ThermoFisher). To gradually change pH from 7.2 to >12, small volumes of 1M NaOH were added (1–5 μL) to the 2 mL sample. The pH of the sample was directly measured for each step, using an Orion™ PeropHecT™ ROSS™ combination pH microelectrode (ThermoFisher). In this titration, if the neutral form interconverts to the anionic form (i.e. before the third, denatured form appears), then at each step of the titration the changes of the concentration of the anionic and neutral forms, n_A and n_N , are directly related:

$$\Delta n_A = -\Delta n_N \quad (6)$$

The changes in optical densities of the anionic and neutral forms at each stage of titration are:

$$\Delta OD_A = \epsilon_A \cdot \Delta n_A \quad (7)$$

and,

$$\Delta OD_N = \epsilon_N \cdot \Delta n_N \quad (8)$$

Therefore, the dependence of OD_A on OD_N (in the region of pH where only two forms are present) will give the straight line with a slope equal to:

$$\frac{\Delta OD_A}{\Delta OD_N} = \frac{-\epsilon_A}{\epsilon_N} \quad (9)$$

An additional equation involving ϵ_A and ϵ_N is obtained from the fact that the total concentration of chromophore before the titration (index 0) $n^{(0)} = n_A^{(0)} + n_N^{(0)}$ is equal to the concentration of chromophore in alkaline denatured form $n^{(f)}_D$ (where index f corresponds to the final stage of denaturation before the onset of chemical degradation of the chromophore) which absorbs at 447 nm and has an extinction coefficient of 44,100 $\text{M}^{-1} \text{cm}^{-1}$ [29]. Therefore:

$$n^{(f)}_D = n_A^{(0)} + n_N^{(0)} \quad (10)$$

or,

$$\frac{OD^{(f)}(447 \text{ nm})}{44,100} = \frac{OD_A^{(0)}}{\epsilon_A} + \frac{OD_N^{(0)}}{\epsilon_N} \quad (11)$$

In this equation, everything is known except ϵ_A and ϵ_N . Combining Eqs (9) and (11) gives us ϵ_A and ϵ_N .

pK_a measurements

We independently measured the changes in fluorescence spectra for alkaline and acidic titration experiments with purified protein samples. To do this we used the same alkaline titration method as the extinction coefficient measurements, and for acidic titration method we gradually added small volumes of 1M HCl (1–3 μL) to the 2 mL sample, from pH 7.2 to 4.3. pH (measured with Orion™ PeropHecT™ ROSS™ combination pH microelectrode, ThermoFisher) and corresponding fluorescence spectra (measured with spectrofluorimeter, PC1 ISS) were collected upon excitation of anionic form at 470 nm for the Ca^{2+} -saturated and Ca^{2+} -free states. To calculate the pK_a, the peak fluorescence intensity near 515 nm was plotted against pH to

get a pH titration curve. The data were then fit using two-binding site model function in the range of pH 4.3 to 11.4 for Ca^{2+} -free GCaMP6m, and using a single-binding site model function for Ca^{2+} -saturated GCaMP6m in the range of pH 4.8 to 8.3.

Two-photon measurements

Ca^{2+} -free and Ca^{2+} -saturated purified GCaMP6m protein samples (5×10^{-5} M) were measured in 0.1 cm glass spectroscopy cuvettes (Starna cells). Two-photon excitation (TPE) spectra were measured using an MOM Sutter Instrument two-photon fluorescent microscope coupled with an Insight DeepSee (Newport) femtosecond laser tunable from 680 to 1300 nm in a similar approach as described in [67]. A Plan NeoFluar 2.5x/0.075 Zeiss objective was used to excite and collect fluorescence, which was passed through a Chroma ET 520/40m filter before reaching the PMT. To correct the TPE spectra for the wavelength-to-wavelength variations of laser properties (pulse duration and beam shape), fluorescein in a NaOH solution (pH 11) was used as a reference standard [68,69]. The TPE fluorescence had quadratic dependence on excitation power in the whole spectral range presented in our results section. Two-photon cross-section was measured at 790 nm for the neutral form of the chromophore in Ca^{2+} -free GCaMP6m and 930 nm for the anionic form in the Ca^{2+} -saturated and Ca^{2+} -free GCaMP6m, using coumarin 485 in methanol (for 790 nm excitation) and fluorescein in NaOH aqueous solution (pH 11) (for 930 nm excitation) as reference standards [69]. Fluorescence intensity, F , as a function of excitation power, P , was measured for both the sample and the reference in the same conditions through a 507/10 nm narrow-bandpass filter. From the fit of these dependences to a quadratic function, $F = \alpha P^2$, factors α values were obtained and then normalized to the concentrations (obtained spectrophotometrically, BioMate™ S3 spectrophotometer) and differential quantum efficiencies at 507 nm (obtained with spectrofluorimeter, PC1 ISS).

Supporting information

S1 Fig. Emission spectra for purified GCaMP6m protein. A and B) Emission spectra for purified GCaMP6m protein in the Ca^{2+} -free (blue, right axis) and Ca^{2+} -saturated (black, left axis) states, excited at 400 nm (A) and 470 nm (B). Inset graphs: zoomed in view of Ca^{2+} -dependent peak emission shift.
(TIF)

S2 Fig. There is an optimal excitation wavelength to measure $\Delta F/F_0$ for GCaMP6m. Excitation spectra for the anionic chromophore using purified GCaMP6m protein in the Ca^{2+} -free (dotted black trace) and Ca^{2+} -saturated (solid black trace) states, fluorescence emission of the anionic form collected at 550 nm. The ratio of the Ca^{2+} -saturated and Ca^{2+} -free spectra illustrates the $\Delta F/F_0$ wavelength dependence (red trace).
(TIF)

S3 Fig. Emission spectra used to resolve the fluorescence quantum yield for the Ca^{2+} -free neutral form emitting near 515 nm. Fluorescence spectra of Ca^{2+} -free GCaMP6m excited at 380 nm (black trace) and at 470 nm (red trace). The 470 nm excited spectrum was normalized to the maximum fluorescence intensity of the 380 nm excited spectrum.
(TIF)

S4 Fig. Two-photon absorption spectra for Ca^{2+} -saturated GCaMP6m (black solid circles, left x-axis and bottom y-axis). The cross-section of the Ca^{2+} -saturated anionic chromophore, 50 GM, is the absolute cross-section at 930 nm excitation. Light grey arrow (B) marks the weak shoulder at ~990 nm that corresponds to the pure electronic transition of the anionic form, which occurs at double the wavelength of the one-photon peak absorption. B) Two-photon

excitation spectra for Ca^{2+} -free GCaMP6m (black open circles, left x-axis and bottom y-axis). The cross-section of the Ca^{2+} -free neutral chromophore, 140 GM, is the absolute cross-section at 790 nm excitation, and the cross-section of the Ca^{2+} -free anionic chromophore, 54 GM, is the absolute cross-section at 930 nm excitation. For both Ca^{2+} -saturated (A) and Ca^{2+} -free (B), the one-photon absorption is illustrated by the red solid trace (right x-axis, top y-axis). (TIF)

Acknowledgments

This work was supported by NIH/NINDS grant R01 NS083875 and the BRAIN initiative grant U01 NS094246. We thank Pat Callis for his advice and thoughtful discussions regarding the physical models. We thank Vincent Pieribone and Jelena Platisa for thoughtful discussions about this work and ArcLight. We thank Nathan Shaner for the pCP constitutive bacterial expression vector. We thank Pat Callis, Larry Cohen, and Brad Baker for critical review of this manuscript. And a special thank you to Brad Baker for discussing Pado and sharing new unpublished data with us.

Author contributions

Conceptualization: LMB TEH MD.

Data curation: LMB.

Formal analysis: LMB TEH MD.

Funding acquisition: TEH MD.

Investigation: LMB MD.

Methodology: LMB TEH MD.

Project administration: LMB.

Resources: LMB TEH MD.

Supervision: TEH.

Validation: LMB TEH MD.

Visualization: LMB TEH MD.

Writing – original draft: LMB.

Writing – review & editing: LMB TEH MD.

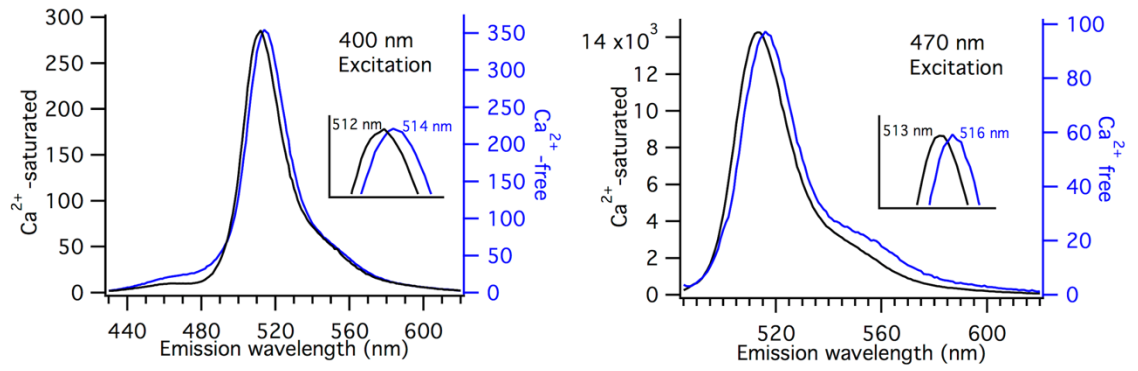
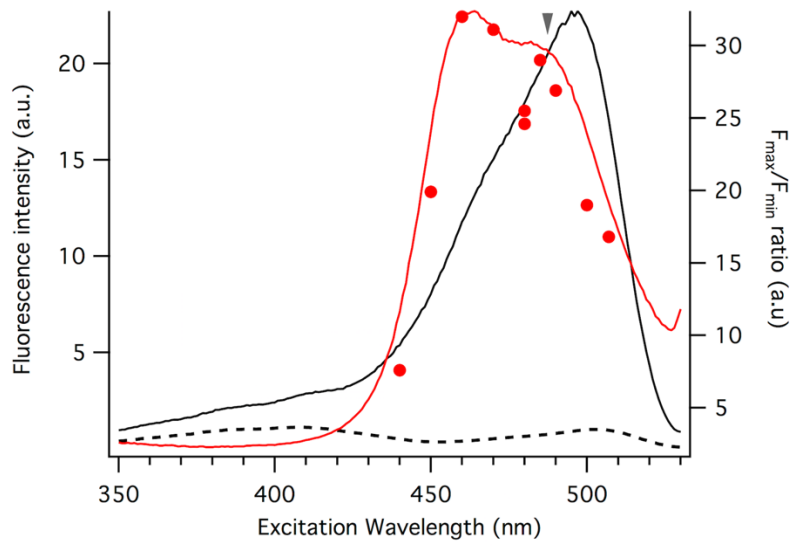
References

1. Chen T-W, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature*. 2013; 499: 295–300. doi: [10.1038/nature12354](https://doi.org/10.1038/nature12354) PMID: [23868258](https://pubmed.ncbi.nlm.nih.gov/23868258/)
2. Boyd AM, Kato HK, Komiyama T, Isaacson JS. Broadcasting of cortical activity to the olfactory bulb. *Cell Rep*. 2015; 10: 1032–1039. doi: [10.1016/j.celrep.2015.01.047](https://doi.org/10.1016/j.celrep.2015.01.047) PMID: [25704808](https://pubmed.ncbi.nlm.nih.gov/25704808/)
3. Dana H, Chen T-W, Hu A, Shields BC, Guo C, Looger LL, et al. Thy1-GCaMP6 transgenic mice for neuronal population imaging in vivo. *PLoS One*. 2014; 9: e108697. doi: [10.1371/journal.pone.0108697](https://doi.org/10.1371/journal.pone.0108697) PMID: [25250714](https://pubmed.ncbi.nlm.nih.gov/25250714/)
4. Hinckley CA, Alaynick WA, Gallarda BW, Hayashi M, Hilde KL, Driscoll SP, et al. Spinal Locomotor Circuits Develop Using Hierarchical Rules Based on Motoneuron Position and Identity. *Neuron*. 2015; 87: 1008–1021. doi: [10.1016/j.neuron.2015.08.005](https://doi.org/10.1016/j.neuron.2015.08.005) PMID: [26335645](https://pubmed.ncbi.nlm.nih.gov/26335645/)

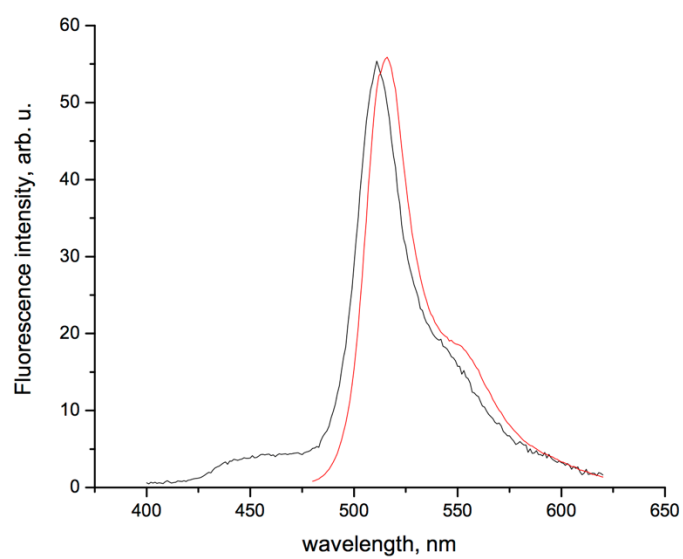
5. Theis L, Berens P, Froudarakis E, Reimer J, Román Rosón M, Baden T, et al. Benchmarking Spike Rate Inference in Population Calcium Imaging. *Neuron*. 2016; 90: 471–482. doi: [10.1016/j.neuron.2016.04.014](https://doi.org/10.1016/j.neuron.2016.04.014) PMID: [27151639](https://pubmed.ncbi.nlm.nih.gov/27151639/)
6. Grover D, Katsuki T, Greenspan RJ. Flyception: imaging brain activity in freely walking fruit flies. *Nat Methods*. 2016; 13: 569–572. doi: [10.1038/nmeth.3866](https://doi.org/10.1038/nmeth.3866) PMID: [27183441](https://pubmed.ncbi.nlm.nih.gov/27183441/)
7. Heckscher ES, Zarin AA, Faumont S, Clark MQ, Manning L, Fushiki A, et al. Even-Skipped+ Interneurons Are Core Components of a Sensorimotor Circuit that Maintains Left-Right Symmetric Muscle Contraction Amplitude. *Neuron*. 2015; 88: 314–329. doi: [10.1016/j.neuron.2015.09.009](https://doi.org/10.1016/j.neuron.2015.09.009) PMID: [26439528](https://pubmed.ncbi.nlm.nih.gov/26439528/)
8. Baird GS, Zacharias DA, Tsien RY. Circular permutation and receptor insertion within green fluorescent proteins. *Proc Natl Acad Sci U S A*. 1999; 96: 11241–11246. PMID: [10500161](https://pubmed.ncbi.nlm.nih.gov/10500161/)
9. Ding J, Luo AF, Hu L, Wang D, Shao F. Structural basis of the ultrasensitive calcium indicator GCaMP6. *Sci China Life Sci*. 2014; 57: 269–274. doi: [10.1007/s11427-013-4599-5](https://doi.org/10.1007/s11427-013-4599-5) PMID: [24390420](https://pubmed.ncbi.nlm.nih.gov/24390420/)
10. Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH, et al. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat Methods*. 2009; 6: 875–881. doi: [10.1038/nmeth.1398](https://doi.org/10.1038/nmeth.1398) PMID: [19898485](https://pubmed.ncbi.nlm.nih.gov/19898485/)
11. Akerboom J, Rivera JDV, Guilbe MMR, Malave ECA, Hernandez HH, Tian L, et al. Crystal Structures of the GCaMP Calcium Sensor Reveal the Mechanism of Fluorescence Signal Change and Aid Rational Design. *J Biol Chem*. 2008; 284: 6455–6464. doi: [10.1074/jbc.M807657200](https://doi.org/10.1074/jbc.M807657200) PMID: [19098007](https://pubmed.ncbi.nlm.nih.gov/19098007/)
12. Nagai T, Sawano A, Park ES, Miyawaki A. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+} . *Proc Natl Acad Sci U S A*. 2001; 98: 3197–3202. doi: [10.1073/pnas.051636098](https://doi.org/10.1073/pnas.051636098) PMID: [11248055](https://pubmed.ncbi.nlm.nih.gov/11248055/)
13. Zhao Y, Araki S, Wu J, Teramoto T, Chang YF, Nakano M, et al. An Expanded Palette of Genetically Encoded Ca^{2+} Indicators. *Science*. 2011; 333: 1888–1891. doi: [10.1126/science.1208592](https://doi.org/10.1126/science.1208592) PMID: [21903779](https://pubmed.ncbi.nlm.nih.gov/21903779/)
14. Akerboom J, Chen T-W, Wardill TJ, Tian L, Marvin JS, Mutlu S, et al. Optimization of a GCaMP calcium indicator for neural activity imaging. *J Neurosci*. 2012; 32: 13819–13840. doi: [10.1523/JNEUROSCI.2601-12.2012](https://doi.org/10.1523/JNEUROSCI.2601-12.2012) PMID: [23035093](https://pubmed.ncbi.nlm.nih.gov/23035093/)
15. Wu J, Abdelfattah AS, Miraucourt LS, Kutsarova E, Ruangkittisakul A, Zhou H, et al. A long Stokes shift red fluorescent Ca^{2+} indicator protein for two-photon and ratiometric imaging. *Nat Commun*. 2014; 5: 5262–5211. doi: [10.1038/ncomms6262](https://doi.org/10.1038/ncomms6262) PMID: [25358432](https://pubmed.ncbi.nlm.nih.gov/25358432/)
16. Ohkura M, Matsuzaki M, Kasai H, Imoto K, Nakai J. Genetically encoded bright Ca^{2+} probe applicable for dynamic Ca^{2+} imaging of dendritic spines. *Anal Chem*. 2005; 77: 5861–5869. doi: [10.1021/ac0506837](https://doi.org/10.1021/ac0506837) PMID: [16159115](https://pubmed.ncbi.nlm.nih.gov/16159115/)
17. Nakai J, Ohkura M, Imoto K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nat Biotechnol*. 2001; 19: 137–141. doi: [10.1038/84397](https://doi.org/10.1038/84397) PMID: [11175727](https://pubmed.ncbi.nlm.nih.gov/11175727/)
18. Wang Q, Shui B, Kotlikoff MJ, Sonderrmann H. Structural basis for calcium sensing by GCaMP2. *Structure*. 2008; 16: 1817–1827. doi: [10.1016/j.str.2008.10.008](https://doi.org/10.1016/j.str.2008.10.008) PMID: [19081058](https://pubmed.ncbi.nlm.nih.gov/19081058/)
19. Muto A, Ohkura M, Kotani T, Higashijima S-I, Nakai J, Kawakami K. Genetic visualization with an improved GCaMP calcium indicator reveals spatiotemporal activation of the spinal motor neurons in zebrafish. *Proc Natl Acad Sci U S A*. 2011; 108: 5425–5430. doi: [10.1073/pnas.1000887108](https://doi.org/10.1073/pnas.1000887108) PMID: [21383146](https://pubmed.ncbi.nlm.nih.gov/21383146/)
20. Cui G, Jun SB, Jin X, Pham MD, Vogel SS, Lovinger DM, et al. Concurrent activation of striatal direct and indirect pathways during action initiation. *Nature*. 2013; 494: 238–242. doi: [10.1038/nature11846](https://doi.org/10.1038/nature11846) PMID: [23354054](https://pubmed.ncbi.nlm.nih.gov/23354054/)
21. Warp E, Agarwal G, Wyart C, Friedmann D, Oldfield CS, Conner A, et al. Emergence of patterned activity in the developing zebrafish spinal cord. *Curr Biol*. 2012; 22: 93–102. doi: [10.1016/j.cub.2011.12.002](https://doi.org/10.1016/j.cub.2011.12.002) PMID: [22197243](https://pubmed.ncbi.nlm.nih.gov/22197243/)
22. Chen Q, Cichon J, Wang W, Qiu L, Lee S-JR, Campbell NR, et al. Imaging neural activity using Thy1-GCaMP transgenic mice. *Neuron*. 2012; 76: 297–308. doi: [10.1016/j.neuron.2012.07.011](https://doi.org/10.1016/j.neuron.2012.07.011) PMID: [23083733](https://pubmed.ncbi.nlm.nih.gov/23083733/)
23. Drobizhev M, Callis PR, Nifosi R, Wicks G, Stoltzfus C, Barnett L, et al. Long- and Short-Range Electrostatic Fields in GFP Mutants: Implications for Spectral Tuning. *Sci Rep*. 2015; 5: 13223. doi: [10.1038/srep13223](https://doi.org/10.1038/srep13223) PMID: [26286372](https://pubmed.ncbi.nlm.nih.gov/26286372/)
24. Jung G, Wiehler J, Zumbusch A. The photophysics of green fluorescent protein: influence of the key amino acids at positions 65, 203, and 222. *Biophys J*. 2005; 88: 1932–1947. doi: [10.1529/biophysj.104.044412](https://doi.org/10.1529/biophysj.104.044412) PMID: [15613627](https://pubmed.ncbi.nlm.nih.gov/15613627/)
25. Lossau H, Kummer A, Heinecke R, Pöllinger-Dammer F, Kompa C, Bieser G, et al. Time-resolved spectroscopy of wild-type and mutant Green Fluorescent Proteins reveals excited state deprotonation consistent with fluorophore-protein interactions. *Chem Phys*. 1996; 213: 1–16.

26. Chatteraj M, King BA, Bublit GU, Boxer SG. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. *Proc Natl Acad Sci U S A*. 1996; 93: 8362–8367. PMID: [8710876](#)
27. Brejc K, Sixma TK, Kitts PA, Kain SR, Tsien RY, Ormó M, et al. Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. *Proc Natl Acad Sci U S A*. 1997; 94: 2306–2311. PMID: [9122190](#)
28. Tallini YN, Ohkura M, Choi B-R, Ji G, Imoto K, Doran R, et al. Imaging cellular signals in the heart in vivo: Cardiac expression of the high-signal Ca^{2+} indicator GCaMP2. *Proc Natl Acad Sci U S A*. 2006; 103: 4753–4758. doi: [10.1073/pnas.0509378103](#) PMID: [16537386](#)
29. Ward WW. Biochemical and Physical Properties of Green Fluorescent Protein. In: Chalfie M, Kain SR, editors. *Green Fluorescent Protein*. Hoboken, NJ, USA: John Wiley & Sons; 2005. pp. 39–65.
30. Shu X, Shaner NC, Yarbrough CA, Tsien RY, Remington SJ. Novel chromophores and buried charges control color in mFruits. *Biochemistry*. 2006; 45: 9639–9647. doi: [10.1021/bi060773l](#) PMID: [16893165](#)
31. Warshel A. Calculations of enzymic reactions: calculations of pKa, proton transfer reactions, and general acid catalysis reactions in enzymes. *Biochemistry*. 1981; 20: 3167–3177. PMID: [7248277](#)
32. Martin ME, Negri F, Olivucci M. Origin, Nature, and Fate of the Fluorescent State of the Green Fluorescent Protein Chromophore at the CASPT2/CASSCF Resolution. *J Am Chem Soc*. 2004; 126: 5452–5464. doi: [10.1021/ja037278m](#) PMID: [15113217](#)
33. Zhang M-Y, Xu C, Lin C-S, Guan X, Cheng W-D. Theoretical study of the proton transfer wires influence on the one- and two-photon absorption properties of green fluorescent protein chromophore. *Org Biomol Chem*. 2013; 11: 1414–1422. doi: [10.1039/c2ob26914g](#) PMID: [23338242](#)
34. Kuboniwa H, Tjandra N, Grzesiek S, Ren H, Claude B, Bax A. Solution structure of calcium-free calmodulin. *Nat Struct Biol*. 1995; 2. Available: <http://spin.niddk.nih.gov/bax/lit/spdf/225.pdf>
35. Chen Y, Song X, Ye S, Miao L, Zhu Y, Zhang R-G, et al. Structural insight into enhanced calcium indicator GCaMP3 and GCaMPJ to promote further improvement. *Protein Cell*. 2013; 4: 299–309. doi: [10.1007/s13238-013-2103-4](#) PMID: [23549615](#)
36. Xie D, Zeng J. Electronic excitations of green fluorescent proteins: protonation states of chromophore model compound in solutions. *J Comput Chem*. 2005; 26: 1487–1496. doi: [10.1002/jcc.20273](#) PMID: [16092146](#)
37. Scott JN, Callis PR. Insensitivity of tryptophan fluorescence to local charge mutations. *J Phys Chem B*. 2013; 117: 9598–9605. doi: [10.1021/jp4041716](#) PMID: [23883101](#)
38. Drobizhev M, Makarov NS, Tillo SE, Hughes TE, Rebane A. Two-photon absorption properties of fluorescent proteins. *Nat Methods*. 2011; 8: 393–399. doi: [10.1038/nmeth.1596](#) PMID: [21527931](#)
39. Sarkisyan KS, Goryashchenko AS, Lidsky PV, Gorbachev DA, Bozhanova NG, Gorokhovatsky AY, et al. Green fluorescent protein with anionic tryptophan-based chromophore and long fluorescence lifetime. *Biophys J*. 2015; 109: 380–389. doi: [10.1016/j.bpj.2015.06.018](#) PMID: [26200874](#)
40. Drobizhev M, Makarov NS, Tillo SE, Hughes TE, Rebane A. Describing two-photon absorptivity of fluorescent proteins with a new vibronic coupling mechanism. *J Phys Chem B*. 2012; 116: 1736–1744. doi: [10.1021/p211020k](#) PMID: [22224830](#)
41. Stoltzfus CR, Barnett LM, Drobizhev M, Wicks G, Mikhaylov A, Hughes TE, et al. Two-photon directed evolution of green fluorescent proteins. *Sci Rep*. 2015; 5: 11968. doi: [10.1038/srep11968](#) PMID: [26145791](#)
42. Akerboom J, Carreras Calderón N, Tian L, Wabnitz S, Prigge M, Toló J, et al. Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. *Front Mol Neurosci*. 2013; 6: 1–29.
43. Bhargava Y, Hampden-Smith K, Chachlaki K, Wood KC, Vernon J, Allerston CK, et al. Improved genetically-encoded, FlnG-type fluorescent biosensors for neural cGMP imaging. *Front Mol Neurosci*. 2013; 6: 26. doi: [10.3389/fnmo.2013.00026](#) PMID: [24068983](#)
44. Odaka H, Arai S, Inoue T, Kitaguchi T. Genetically-Encoded Yellow Fluorescent cAMP Indicator with an Expanded Dynamic Range for Dual-Color Imaging. *PLoS One*. 2014; 9: e100252. doi: [10.1371/journal.pone.0100252](#) PMID: [24959857](#)
45. Hung YP, Albeck JG, Tantama M, Yellen G. Imaging Cytosolic NADH-NAD⁺ Redox State with a Genetically Encoded Fluorescent Biosensor. *Cell Metab*. 2011; 14: 545–554. doi: [10.1016/j.cmet.2011.08.012](#) PMID: [21982714](#)
46. Marvin JS, Schreiter ER, Echevarría IM, Looger LL. A genetically encoded, high-signal-to-noise maltose sensor. *Proteins: Struct Funct Bioinf*. 2011; 79: 3025–3036.
47. Shen Y, Rosendale M, Campbell RE, Perrais D. pHuji, a pH-sensitive red fluorescent protein for imaging of exo-and endocytosis. *J Cell Biol*. 2014; 207: 419–432. doi: [10.1083/jcb.201404107](#) PMID: [25385186](#)

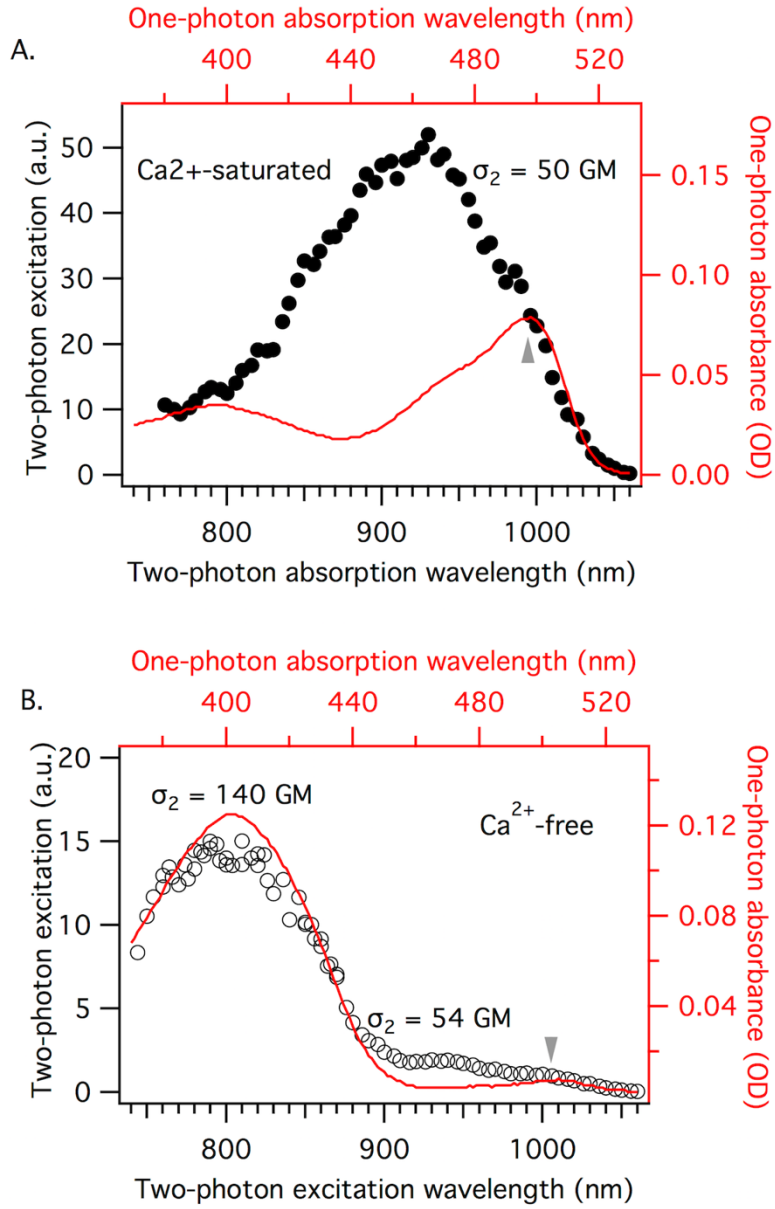
48. Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, et al. An optimized fluorescent probe for visualizing glutamate neurotransmission. *Nat Methods*. 2013; 10: 162–170. doi: [10.1038/nmeth.2333](https://doi.org/10.1038/nmeth.2333) PMID: [23314171](https://pubmed.ncbi.nlm.nih.gov/23314171/)
49. Barnett L, Platasa J, Popovic M, Pieribone VA, Hughes T. A fluorescent, genetically-encoded voltage probe capable of resolving action potentials. *PLoS One*. 2012; 7: e43454. doi: [10.1371/journal.pone.0043454](https://doi.org/10.1371/journal.pone.0043454) PMID: [22970127](https://pubmed.ncbi.nlm.nih.gov/22970127/)
50. Nikolaev VO, Gambaryan S, Lohse MJ. Fluorescent sensors for rapid monitoring of intracellular cGMP. *Nat Methods*. 2006; 3: 23–25. doi: [10.1038/nmeth816](https://doi.org/10.1038/nmeth816) PMID: [16369548](https://pubmed.ncbi.nlm.nih.gov/16369548/)
51. St-Pierre F, Marshall JD, Yang Y, Gong Y, Schnitzer MJ, Lin MZ. High-fidelity optical reporting of neuronal electrical activity with an ultrafast fluorescent voltage sensor. *Nat Neurosci*. 2014; 17: 884–889. doi: [10.1038/nn.3709](https://doi.org/10.1038/nn.3709) PMID: [24755780](https://pubmed.ncbi.nlm.nih.gov/24755780/)
52. De Michele R, Ast C, Loqué D, Ho C-H, Andrade SLA, Lanquar V, et al. Fluorescent sensors reporting the activity of ammonium transceptors in live cells. *Elife*. 2013; 2: e00800. doi: [10.7554/eLife.00800](https://doi.org/10.7554/eLife.00800) PMID: [23840931](https://pubmed.ncbi.nlm.nih.gov/23840931/)
53. Ast C, De Michele R, Kümke MU, Frommer WB. Single-fluorophore membrane transport activity sensors with dual-emission read-out. *Elife*. 2015; 4: e07113. doi: [10.7554/eLife.07113](https://doi.org/10.7554/eLife.07113) PMID: [26090909](https://pubmed.ncbi.nlm.nih.gov/26090909/)
54. Belousov VV, Fradkov AF, Lukyanov KA, Staroverov DB, Shakhbazov KS, Terskikh AV, et al. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat Methods*. 2006; 3: 281–286. doi: [10.1038/nmeth866](https://doi.org/10.1038/nmeth866) PMID: [16554833](https://pubmed.ncbi.nlm.nih.gov/16554833/)
55. Berg J, Hung YP, Yellen G. A genetically encoded fluorescent reporter of ATP:ADP ratio. *Nat Methods*. 2009; 6: 161–166. doi: [10.1038/nmeth.1288](https://doi.org/10.1038/nmeth.1288) PMID: [19122669](https://pubmed.ncbi.nlm.nih.gov/19122669/)
56. Inoue M, Takeuchi A, Horigane S, Ohkura M. Rational design of a high-affinity, fast, red calcium indicator R-CaMP2. *Nature*. 2015; Available: <http://www.nature.com/nmeth/journal/v12/n1/abs/nmeth.3185.html>
57. Tewson P, Westenberg M, Zhao Y, Campbell RE, Quinn AM, Hughes TE. Simultaneous detection of Ca^{2+} and diacylglycerol signaling in living cells. *PLoS One*. 2012; 7: e42791. doi: [10.1371/journal.pone.0042791](https://doi.org/10.1371/journal.pone.0042791) PMID: [22912738](https://pubmed.ncbi.nlm.nih.gov/22912738/)
58. Jin L, Han Z, Platasa J, Wooltorton JRA, Cohen LB, Pieribone VA. Single action potentials and sub-threshold electrical events imaged in neurons with a fluorescent protein voltage probe. *Neuron*. 2012; 75: 779–785. doi: [10.1016/j.neuron.2012.06.040](https://doi.org/10.1016/j.neuron.2012.06.040) PMID: [22958819](https://pubmed.ncbi.nlm.nih.gov/22958819/)
59. Kang BE, Baker BJ, Pado, a fluorescent protein with proton channel activity can optically monitor membrane potential, intracellular pH, and map gap junctions. *Sci Rep*. 2016; 6: 23865. doi: [10.1038/srep23865](https://doi.org/10.1038/srep23865) PMID: [27040905](https://pubmed.ncbi.nlm.nih.gov/27040905/)
60. Miesenböck G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*. 1998; 394: 192–195. doi: [10.1038/28190](https://doi.org/10.1038/28190) PMID: [9671304](https://pubmed.ncbi.nlm.nih.gov/9671304/)
61. Sankaranarayanan S, De Angelis D, Rothman JE, Ryan TA. The use of pHluorins for optical measurements of presynaptic activity. *Biophys J*. 2000; 79: 2199–2208. doi: [10.1016/S0006-3495\(00\)76468-X](https://doi.org/10.1016/S0006-3495(00)76468-X) PMID: [11023924](https://pubmed.ncbi.nlm.nih.gov/11023924/)
62. Han Z, Jin L, Chen F, Loturco JJ, Cohen LB, Bondar A, et al. Mechanistic studies of the genetically encoded fluorescent protein voltage probe ArcLight. *PLoS One*. 2014; 9: e113873. doi: [10.1371/journal.pone.0113873](https://doi.org/10.1371/journal.pone.0113873) PMID: [25419571](https://pubmed.ncbi.nlm.nih.gov/25419571/)
63. Ataka K, Pieribone VA. A Genetically Targetable Fluorescent Probe of Channel Gating with Rapid Kinetics. *Biophys J*. 2002; 82: 509–516. doi: [10.1016/S0006-3495\(02\)75415-5](https://doi.org/10.1016/S0006-3495(02)75415-5) PMID: [11751337](https://pubmed.ncbi.nlm.nih.gov/11751337/)
64. Treger JS, Priest MF, Bezaniilla F. Single-molecule fluorimetry and gating currents inspire an improved optical voltage indicator. *Elife*. 2015; Available: <http://elifesciences.org/content/4/e10482.abstract>
65. Lakowicz JR. Principles of Fluorescence Spectroscopy. 3rd ed. New York: Springer; 2006.
66. Hanson GT, McAnaney TB, Park ES, Rendell MEP, Yarbrough DK, Chu S, et al. Green fluorescent protein variants as ratiometric dual emission pH sensors. 1. Structural characterization and preliminary application. *Biochemistry*. 2002; 41: 15477–15488. PMID: [12501176](https://pubmed.ncbi.nlm.nih.gov/12501176/)
67. Tsai PS, Nishimura N, Yoder EJ, Dolnick EM, Allen White G, Kleinfeld D. Principles, Design, and Construction of a Two-Photon Laser-Scanning Microscope for In Vitro and In Vivo Brain Imaging. In: Frostig RD, editor. *In vivo optical imaging of brain function*. Boca Raton: CRC Press; 2002. pp. 113–171.
68. Xu C, Webb WW. Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. *J Opt Soc Am B*. 1996; 13: 481–491.
69. Makarov NS, Drobizhev M, Rebane A. Two-photon absorption standards in the 550–1600 nm excitation range: establishing a correction curve for accurate cross section calibration. *Integrated Optoelectronic Devices 2008*. International Society for Optics and Photonics; 2008. pp. 689105–689105–12.

S1 Fig. Emission spectra for purified GCaMP6m protein.**S2 Fig. There is an optimal excitation wavelength to measure $\Delta F/F_0$ for GCaMP6m.**

S3 Fig. Emission spectra used to resolve the fluorescence quantum yield for the Ca^{2+} -free neutral form emitting near 515 nm.



S4 Fig. Two-photon absorption spectra for Ca^{2+} -saturated GCaMP6m (black solid circles, left axis and bottom y-axis).



CHAPTER THREE

MAKING pK_a -ALTERING MUTATIONS IN GCAMP6M CHANGES
THE Ca^{2+} -DEPENDENT FLUORESCENCE RESPONSEContribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Lauren M. Barnett

Contributions: Conceived project; contributed to experimental design and performed critical data analysis; performed all molecular biology, protein purification and protein handling; performed all spectroscopic measurements; wrote main manuscript text and produced all figures; contributed to the review of the manuscript.

Co-Author: Mikhail Drobizhev

Contributions: Conceived project; contributed to experimental design and performed critical data analysis; performed spectroscopic measurements; contributed to the drafting of figures; review of the manuscript.

Co-Author: Thomas E. Hughes

Contributions: Conceived project; contributed to experimental design and performed critical data analysis; supervised project; contributed to the review of the manuscript; corresponding author.

Manuscript Information Page

Lauren M Barnett¹, Mikhail Drobizhev¹, and Thomas E Hughes¹

¹ Department of Cell Biology and Neuroscience, Montana State University, Bozeman, Montana, United States

Status of the manuscript:

- ☒ Prepared for submission to a peer-reviewed journal
- ☐ Officially submitted to a peer-reviewed journal
- ☐ Accepted by a peer-reviewed journal
- ☐ Published in a peer-reviewed journal

Prepared to be published in PLoS ONE

Making pK_a -altering mutations in GCaMP6m changes the Ca^{2+} -dependent fluorescence response

Lauren M Barnett¹, Mikhail Drobizhev¹, Thomas E Hughes¹

1. Department of Cell Biology and Neuroscience, Montana State University, Bozeman, MT, USA

Abstract

We previously described the molecular mechanism by which GCaMP6m changes fluorescence. When this remarkable biosensor binds Ca^{2+} in the cell, there is a change in the protonation state of the chromophore, shifting most of the protein population from the neutral to anionic form. This shift results in a large increase in the ~470 nm excited fluorescence, which is predominantly due to a change in the pK_a of the chromophore. Here we ask: what happens if we introduce mutations into GCaMP6m that are known to alter the pH sensitivity of the EGFP chromophore? The resulting mutants have shifted pK_a values in both the Ca^{2+} -free and Ca^{2+} -saturated states and varied Ca^{2+} -dependent fluorescence signals, yet the 470 nm excited fluorescence change mechanism is still predominantly a redistribution of the chromophore protonation state. The molecular mechanism for one mutant, GCaMP6m-E60G, also includes a significant Ca^{2+} -dependent increase in quantum yield of the anionic form of the chromophore.

Introduction

Genetically-encoded, fluorescent Ca^{2+} sensors are remarkable tools that enable neuroscientists to image neural activity in living brains [1–7]. Currently, the green fluorescent protein-based GCaMP6 series (-s, -m, and -f) are the best sensors, providing large changes in fluorescence when intracellular Ca^{2+} levels rise [2]. The GCaMP6 sensors are the product of many years of gradual improvement. Though structural studies of GCaMP have enabled rational design [8,9], much of the improvement in the GCaMP sensors has been the result of trial and error or random / semi-random mutagenesis [1,2,8,10].

The green fluorescent protein contains a chromophore that can exist in either a protonated neutral form or a deprotonated anionic form [11]. These two forms absorb at different wavelengths, the neutral form peaking at ~400

nm and the anionic form at ~480 nm, but the fluorescence (peaking at 500 – 520 nm) emits from the anionic form, independent of what form was excited. This is due to a fast (ps) excited state proton transfer [12,13] from the excited neutral form. Amino acid mutations in the chromophore, and in the surrounding protein β -barrel, can alter the balance of these two forms [14], by shifting it to the anionic (EGFP, [15]) or neutral form (Ametrine, [16]).

Many green fluorescent protein mutations have been described that alter the pK_a of the chromophore, which alters the balance the neutral and anionic forms at physiological pH [15,17,18]. Fig 1 illustrates the 1PA spectra of five EGFP-variants that include mutations that affect the pK_a of chromophore (Fig 1A), resulting in various ratios of neutral – anionic chromophore at a neutral pH [18]. For a given GFP, as the pK_a of the chromophore increases to more alkaline pH values, there will be less anionic form and more of the protonated neutral form at neutral pH (Fig 1B). This pH sensitive equilibrium in GFP has been used to create valuable sensors for vesicle exocytosis [19], in which the ratiometric excitation of the neutral and anionic forms are used to distinguish between protein within the vesicle lumen at acidic pH and protein in the extracellular space at neutral pH.

Fig 1. Mutations in the EGFP chromophore and surrounding protein β -barrel can alter the neutral – anionic equilibrium. A) One-photon absorption spectra illustrates the different ratios of neutral and anionic chromophore species in purified protein samples of EGFP (green) and various EGFP-mutants: EGFP-D117G,V163A (red), EGFP-N121S,V163A (dark blue), EGFP-S72G,V163A (black), EGFP-T65S,S202N (light blue), EGFP-V163A,T203I (yellow). Neutral (absorption peak at ~400 nm) and anionic (absorption peak at ~500 nm) forms of the chromophore are indicated by dotted lines. B) Schematic of pK_a effects on chromophore protonation state in EGFP.

Recently, we characterized the Ca^{2+} -dependent fluorescence behavior of GCaMP6m [2], which included measuring four different states of the chromophore [20]. This revealed that the large change in GCaMP6m's fluorescence is due to a change in the protonation state of the chromophore that occurs when calmodulin binds Ca^{2+} and interacts with the M13 domain. Only small changes in the extinction coefficient and quantum yield of the anionic form occur as a function of Ca^{2+} -binding. Instead, the GCaMP6m behavior is largely explained by a Ca^{2+} -dependent change in the pK_a (ΔpK_a) of the chromophore [20].

The goal of this work was to introduce mutations that alter the neutral – anionic equilibrium in EGFP into GCaMP6m to learn three things: 1) can these mutations change the GCaMP6m fluorescence response, 2) how these mutations might affect the GCaMP6m ΔpK_a , and 3) is there a combination of changed photophysical parameters that could produce a GCaMP6m-variant with improved characteristics?

Results

Mutations that alter the neutral – anionic equilibrium in EGFP affect the pK_a values and ΔpK_a in GCaMP6m.

GCaMP6m signals Ca^{2+} -binding through a large change in the relative concentrations of neutral and anionic forms, via ΔpK_a between the Ca^{2+} -free and Ca^{2+} -saturated states (Fig 2), producing a large change in ~480 nm excited fluorescence [20]. This shift in the neutral to anionic equilibrium is illustrated in Fig 2. In the Ca^{2+} -free state, the pK_a of the sensor (8.01 ± 0.01 , [20]) ensures that at physiological pH most of the sensor exists in the neutral form, absorbing at 400 nm (Fig 2B, left graph). In the Ca^{2+} -saturated state ($pK_a = 7.10 \pm 0.01$, [20]), more of the protein exists in the anionic form of the chromophore, absorbing at 504 nm (Fig 2B, right graph). If mutations that affect pK_a in EGFP were made in GCaMP6m, would they shift the pK_a of the sensor? And if so, would it shift the pK_a of the Ca^{2+} -free state, Ca^{2+} -saturated state, or both?

Fig 2. EGFP pK_a altering mutations affect the pK_a and ΔpK_a in GCaMP6m. A) Schematic of pK_a effects on the anionic to neutral chromophore ratio in a biosensor such as GCaMP6m. pK_a 1 and pK_a 2 represent the two different analyte or activity dependent states of the biosensor, for example: Ca^{2+} -free state versus Ca^{2+} -saturated state in GCaMP6m. B and C) Absorption, excitation, and emission spectra illustrate the redistribution of the neutral and anionic forms of the chromophore when calmodulin binds Ca^{2+} for GCaMP6m (B) and the GCaMP6m-A74V,S114N,T222S mutant (C). Absorption spectra (black trace, left axis), excitation spectra (red trace, right axis), and emission spectra for the 400 nm excited fluorescence (blue trace, right axis) and 470 nm excited fluorescence (green trace, right axis) for purified sensor protein in 0 μM free Ca^{2+} buffer (Ca^{2+} -free, left graph) and 39

μM free Ca^{2+} buffer (Ca^{2+} -saturated, right graph). All spectra are labeled (with arrows in the corresponding color) for the GCaMP6m graphs (B), for reference.

Introducing A74V, S114N, and T222S mutations into GCaMP6m (A163V, S202N, and T65S in EGFP numbering) changes the Ca^{2+} -dependent fluorescence response (Fig 2C). In the Ca^{2+} -free state, the GCaMP6m-A74V,S114N,T222S chromophore exists predominantly in the neutral form (Fig 2C, left graph), similar to Ca^{2+} -free GCaMP6m. Unlike GCaMP6m, the A74V,S114N,T222 mutant chromophore remains mostly neutral in the Ca^{2+} -saturated state as well (Fig 2C, right graph). The Ca^{2+} -saturated absorption spectrum for the A74V,S114N,T222S mutant closely resembles that of EGFP-T65S,S202N illustrated in Fig 1 (light blue trace, Fig 1A).

When GCaMP-A74V,S114N,T222 binds Ca^{2+} , there is a decrease in the 400 nm excited fluorescence and an increase in 470 nm excited fluorescence (Fig 2A and 2B). The general behavior is similar to GCaMP6m, however, GCaMP6m-A74V,S114N,T222S is brightly fluorescent when excited with 400 nm light, in both the Ca^{2+} -free and Ca^{2+} -saturated state, which is markedly different from GCaMP6m. This is due to the T222S mutation (T65S in EGFP numbering) which restores the excited state proton transfer chain in EGFP, significantly increasing the quantum efficiency of the neutral form of the chromophore [15,17]. The T222S mutation restores a brightly fluorescent neutral form [15,17] in of three of the mutants presented here: GCaMP6m-A74V,S114N,T222S (Fig 2C); GCaMP6m-E60C,T222S (Fig 3A); GCaMP6m-E60Q,T222S (Fig 3B). In the Ca^{2+} -free states of GCaMP6m-E60C,T222S (Fig 3A, left graph) and GCaMP6m-E60Q,T222S (Fig 3B, left graph), the absorption and excitation spectra match up when they are normalized with respect to the anionic form. This is not seen for GCaMP6m (Fig 2B). This match is consistent with a neutral and anionic form of the chromophore with equal, or near equal, quantum efficiencies (Table 1).

Fig 3. Ca^{2+} -binding has varying effects on GCaMP6m-mutant chromophore equilibria. Absorption, excitation, and emission spectra illustrate the different Ca^{2+} -dependent chromophore equilibria and the corresponding changes in the photophysical properties of the two forms of the chromophore. Absorption spectra (black trace, left axis), excitation spectra (red trace, right axis), and emission spectra for 400 nm excited fluorescence

(blue traces, right axis) and 470 nm excited fluorescence (green traces, right axis) for purified GCaMP6m-mutant protein in 0 μM free Ca^{2+} buffer (Ca^{2+} -free, left graph) and 39 μM free Ca^{2+} buffer (Ca^{2+} -saturated, right graph). Amino acid mutations are listed in bold (upper right-hand corner of Ca^{2+} -free graph) for each set of mutant spectra. Fluorescence emission was collected at 530 nm for excitation spectra.

The Ca^{2+} -dependent fluorescence change in GCaMP6m-E60C,T22S is similar to A74V,S114N,T22S. It has a decrease in 400 nm excited fluorescence and only a slight increase in 470 nm excited fluorescence (Fig 3A). Interestingly, GCaMP6m-E60Q,T22S has an increase in the 400 nm excited fluorescence and an increase in the 470 nm excited fluorescence, but both of these changes are small (Fig 3B). The T22S mutants' Ca^{2+} -dependent spectral changes are consistent with their Ca^{2+} -dependent change in the relative concentration of chromophore species driven by a change in pK_a (Table 1). All of them exhibit very small changes in the relative concentrations of the neutral and anionic chromophore species upon Ca^{2+} -binding, which is consistent with the alkaline shifted pK_a values for both the Ca^{2+} -free and Ca^{2+} -saturated states (Table 1). Although they have ΔpK_a values equal to or greater than parent GCaMP6m ($\Delta\text{pK}_a = \sim 1$ [20]), both Ca^{2+} -free and Ca^{2+} -saturated pK_a values are well above pH 7.2, limiting the Ca^{2+} -dependent shift in relative concentration of the neutral and anionic chromophore species and reducing the $F_{\text{max}}/F_{\text{min}}$ 470 nm excited fluorescence change at physiological pH relative to parent GCaMP6m (Table 1). This highlights the importance of pK_a values that straddle physiological pH.

Table 1: Photophysical properties of GCaMP6m mutants.

	EGFP amino acid #	Φ (λ_{fluo} , nm)				ϵ $\times 10^3$, M $^{-1}$ cm $^{-1}$ (λ_{abs} , nm)				ρ				$F_{\text{max}}/F_{\text{min}}$ (λ_{max} , nm)	pK_{a}	
		Neutral		Anionic		Neutral		Anionic		Neutral		Anionic				
		Free	Sat	Free	Sat	Free	Sat	Free	Sat	Free	Sat	Free	Sat		Free	Sat
GCaMP6m*	---	0.04 (514)	0.05 (512)	0.56 (516)	0.63 (513)	37.3 (403)	37.7 (397)	73.6 (514)	83.1 (497)	0.970	0.490	0.030	0.510	30 (470)	8.01 $\pm$ 0.01	7.10 $\pm$ 0.01
A74V S114N T222S	A163V S202N T65S	0.26 $\pm$ 0.01 (513)	0.24 $\pm$ 0.01 (507)	-- (511)	0.30 $\pm$ 0. .11 (504)	37.6 (395)	40 (394)	70.5	82.7 (481)	--	0.958	--	0.042	7 (473)	11.07 $\pm$ 0.02	10.94 $\pm$ 0.02
E60C T222S	E148C T65S	0.28 $\pm$ 0.01 (513)	0.20 $\pm$ 0.04 (507)	0.39 $\pm$ 0.02 (510)	0.39 $\pm$ 0.27 (504)	34.7 (396)	35.5 (393)	61.9 (492)	64.2 (482)	0.994	0.974	0.0062	0.026	5 (473)	11.90 $\pm$ 0.02	8.20 $\pm$ 0.03
E60Q T222S	E148Q T65S	0.28 $\pm$ 0.01 (513)	0.31 $\pm$ 0.02 (507)	0.38 $\pm$ 0.11 (511)	0.48 $\pm$ 0.15 (502)	37.2 (396)	34.9 (393)	67.5 (495)	70.9 (480)	0.995	0.970	0.0055	0.030	11 (475)	11.59 $\pm$ 0.03	9.55 $\pm$ 0.01
E60C	E148C	0.090 $\pm$ 0.006 (513)	0.088 $\pm$ 0.004 (511)	0.49 $\pm$ 0.02 (517)	0.54 $\pm$ 0.05 (514)	37 (401)	36.4 (398)	85.8 (507)	82.1 (497)	0.957	0.55	0.043	0.45	11 (465)	7.57 $\pm$ 0.03	7.12 $\pm$ 0.03
E60G	E148G	0.014 $\pm$ 0.001 (510)	0.032 $\pm$ 0.002 (510)	0.33 $\pm$ 0.11 (516)	0.60 $\pm$ 0.03 (514)	35.3 (400)	37.7 (398)	79.1 (501)	89.5 (496)	0.986	0.71	0.014	0.29	46 (462)	8.36 $\pm$ 0.02	7.37 $\pm$ 0.03
V115I	V203I	0.039 $\pm$ 0.004 (515)	0.044 $\pm$ 0.002 (513)	0.38 $\pm$ 0.14 (517)	0.43 $\pm$ 0.03 (515)	39 (405)	36 (400)	77.1 (504)	88 (497)	0.983	0.47	0.017	0.53	35 (485)	8.21 $\pm$ 0.04	7.26 $\pm$ 0.19

Data presented as mean $\pm$ standard error. If no error listed, mean $\pm$ 10% standard error. Indices: Ca^{2+} -free (Free); Ca^{2+} -saturated (Sat); Quantum yield (Φ); extinction coefficient (ϵ); relative concentration of the chromophore species (ρ), Ca^{2+} -induced change in fluorescence is presented as $F_{\text{max}}/F_{\text{min}}$.
*[20]

GCaMP6m-mutants without the T222S mutation, GCaMP6m-E60C, -V115I, and -E60G (Fig 3C, 3D, 4B, respectively), have Ca^{2+} -dependent spectral changes that are more similar to the parent GCaMP6m (Fig 2B). In the Ca^{2+} -free state, these mutants predominantly exist in the neutral form, and upon Ca^{2+} -binding, the equilibrium shifts to a greater concentration of the anionic form (Fig 3C, 3D, 4B, Table 1). As expected with these concentration changes, they have a Ca^{2+} -dependent decrease in the 400 nm excited fluorescence and an increase in the 470 nm excited fluorescence (Fig 3C, 3D, 4B), like GCaMP6m (Fig 2B).

Fig 4. The E60G mutation in GCaMP6m most likely increases the Ca^{2+} -dependent ΔF via a change in quantum yield. A) Schematic of GCaMP6m structure (created using 3WLD.pdb) with the E60G mutation site marked with pink spheres. GFP domain (green), chromophore (red), calmodulin (blue), and M13 peptide (light orange). B)

Absorption, excitation, and emission spectra for Ca^{2+} -free (left) and Ca^{2+} -saturated (right) states of GCaMP6m-E60G. Absorption spectra (black trace, left axis), excitation spectra (red trace, right axis), and emission spectra for 400 nm excited fluorescence (blue trace, right axis) and 470 nm excited fluorescence (green trace, right axis) for purified GCaMP6m-E60G protein in 0 μM free Ca^{2+} buffer (Ca^{2+} -free, left graph) and 39 μM free Ca^{2+} buffer (Ca^{2+} -saturated, right graph). Fluorescence emission was collected at 530 nm for excitation spectra. C) Peak 470 nm excited fluorescence emission as a function of pH, from pH 4 to pH ~12 for purified GCaMP6m-E60G (left graph) and GCaMP6m (right graph) in 0 μM free Ca^{2+} buffer (Ca^{2+} -free, open circles) or 39 μM free Ca^{2+} buffer (Ca^{2+} -saturated, solid circles). The apparent pK_a values for the chromophores are labeled for each state. D) Peak absorption of the anionic form of the chromophore during pH titration for purified GCaMP6m-E60G protein in 0 μM free Ca^{2+} buffer (Ca^{2+} -free, open circles) or 39 μM free Ca^{2+} buffer (Ca^{2+} -saturated, solid circles).

GCaMP6m-V115I and E60C have shifted Ca^{2+} -free and Ca^{2+} -saturated pK_a values compared to parent GCaMP6m (Table 1). Their Ca^{2+} -dependent fluorescence changes are also predominantly due to a shift the relative neutral – anionic chromophore concentration, like parent GCaMP6m (Table 1, Fig 5). The quantum yield and extinction coefficients of the neutral and anionic forms in GCaMP6m-E60C remain relatively constant across the Ca^{2+} -free and Ca^{2+} -saturated states (Table 1, Fig 5). GCaMP6m-V115I has slight increases in the quantum yield and extinction coefficient for the anionic form as a function of Ca^{2+} -binding, closely resembling parent GCaMP6m (Table 1, Fig 5). For GCaMP6m-E60G, while the fluorescence changes are predominantly due to a Ca^{2+} -dependent ΔpK_a like GCaMP6m and the rest of the mutants here, Ca^{2+} -binding also increases in the quantum yield of the anionic chromophore increases by two-times (Table 1, Fig 5). Combining a redistribution of the neutral to anionic form ratio with a change in quantum efficiency increases the Ca^{2+} -dependent $\Delta\text{F}/\text{F}$ response relative to GCaMP6m.

Fig 5. ΔpK_a is the predominant mechanism for Mutant Ca^{2+} -dependent changes in 470 nm excited fluorescence, however other parameters can significantly contribute. Bar graphs illustrate the contributions of quantum yield (ϕ , red), extinction coefficient (ϵ , blue), and the relative concentration of anionic chromophore (ρ , yellow) in the 470 nm excited fluorescence for Ca^{2+} -free (0, left bar plot) and Ca^{2+} -saturated (1, right bar plot) for each

protein. ϕ , ϵ , and ρ values are presented together on a logarithmic scale (y-axis) and represent the sum of the three parameters, or total 470 nm excited fluorescence, for the Ca^{2+} -free (0) or Ca^{2+} -saturated (1) state (x-axis) of each sensor protein.

A single mutation in GCaMP6m, E60G, increases the Ca^{2+} -dependent change in fluorescence in an unexpected way.

GCaMP6m-E60G has a ~53% larger Ca^{2+} -dependent change in 470 nm excited fluorescence compared to the parent GCaMP6m (Table 1). This increase in $\Delta F/F_0$ signal is due to an increase in all three essential photophysical parameters: quantum yield, extinction coefficient, and concentration of anionic chromophore.

The E60G mutant and GCaMP6m pH titration fluorescence curves for the Ca^{2+} -saturated states are dramatically different (solid circles, Fig 4C). From pH 5 to 7.2, the E60G mutant has a steep increase in 470 nm excited fluorescence (left graph, Fig 4C). Using a single-binding site fit for this curve, the apparent pK_a is 6.65 ± 0.03 (green trace, left graph, Fig 4C). This is slightly more acidic than the Ca^{2+} -saturated GCaMP6m pK_a , 7.10 ± 0.03 (green trace, right graph, Fig 4C). At pH 7.3, GCaMP6m-E60G's 470 nm excited fluorescence begins to drop, eventually matching the emission of the Ca^{2+} -free state (open circles, Fig 4C) at pH ~8.3, before beginning to increase again in a very similar titration curve. Using a two-binding site fit for the Ca^{2+} -free titration curve, the first site has a pK_a of 8.36 ± 0.02 (blue trace, Fig 4C). However, the apparent pK_a value that comes from fitting the Ca^{2+} -saturated 470 nm excited fluorescence is not consistent with the relative concentration numbers (ρ , Table 1). The pH titration absorption curve for Ca^{2+} -saturated GCaMP6m-E60G more closely resembles GCaMP6m behavior (solid circles, Fig 4D versus 4C). Fitting the Ca^{2+} -saturated absorption curve with a single-binding site fit gives an apparent pK_a value of 7.37 ± 0.03 (green trace, Fig 4D), which is consistent with the relative concentration numbers presented in Table 1. It may be there is a significant pH-dependent change in the Ca^{2+} -saturated GCaMP6m-E60G anionic form quantum yield that appears at pH ~7.3 in the pH titration, since the dramatic pH dependent changes are only seen when monitoring the fluorescence.

The mutant two-photon excited fluorescence spectra highlight the importance of ΔpK_a across a physiological pH range.

The mutants without the T222S mutation, GCaMP6m-E60C, -V115I, and -E60G, all exhibit Ca^{2+} -dependent two-photon excited fluorescence changes similar to parent GCaMP6m (Fig 6), which is consistent with their pK_a values (Table 1). The mutants that include the T222S mutation, GCaMP6m-A74V,S114N,T222S, -E60C,T222S, and -E60Q,T222S, have very small two-photon excited fluorescence changes because they don't have the dramatic neutral – anionic redistribution (Fig 6). This is also consistent with their pK_a values (Table 1). The T222S mutants have dominant neutral form peaks in the Ca^{2+} -free and Ca^{2+} -saturated two-photon excitation spectra and only exhibit small Ca^{2+} -dependent ΔF_2 signals (Fig 6E – 6G). GCaMP6m-A74V,S114N,T222S has the smallest ΔF_2 ($F_{2max}/F_{2min} = 1.3$) with a slight increase in at ~836 nm (Fig 6E). GCaMP6m-E60C,T222S has the most significant decrease in the F_2 signal, $F_{2max}/F_{2min} = 1.7$ at ~820 nm (Fig 6F). The E60Q,T222S mutant also has a decrease in F_2 signal ($F_{2max}/F_{2min} = 1.4$) at ~830 nm, which appears to be the result of a λ_{max} shift in the neutral form peak (Fig 6F).

Fig 6. The mutant two-photon excited spectra illustrate the importance of ΔpK_a . Two-photon excited spectra for GCaMP6m (A) and GCaMP6m-mutants (B - G). Spectra are presented as a linear combination of the two individual two-photon action cross-section (σ_2) spectra: $F_2(\lambda) = \sigma_{2,N}(\lambda) \phi_N \rho_N + \sigma_{2,A}(\lambda) \phi_A \rho_A$, where $\sigma_2(\lambda)$ is the wavelength-dependent two-photon excitation cross-section, ϕ is the fluorescence quantum yield, and ρ is the relative concentration of the chromophore species (neutral, N, or anionic, A). Ca^{2+} -saturated (solid circles) and Ca^{2+} -free (open circles). All Ca^{2+} -free F_2 spectra was normalized to the neutral form peak value at 800 nm, where σ_2 was independently measured. The Ca^{2+} -saturated spectra that are dominated by the neutral form (GCaMP6m-A74V,S114N,T222S, E; -E60C,T222S, F; and -E60Q,T222S, G) were also normalized to the neutral form peak at 800 nm in this manner. The Ca^{2+} -saturated spectra dominated by the anionic form (GCaMP6m, A; -E60G, B; -E60C, C; and -V115I, D) were normalized to peak value at 930 nm, where σ_2 was independently measured. Red arrows indicate the peak ΔF_2 signal wavelength. The independently measured σ_2 (GM) values at 800 nm (neutral, N) and/or 930 nm (anionic, A) are included on graphs.

One of the significant benefits of using an upward-going ΔF_2 sensor like GCaMP6m is that in the absence of Ca^{2+} , the chromophore does not absorb 930 nm light (Fig 6A). Therefore, GCaMP6m should not bleach while the cell is at rest [20]. The T222S mutants do not benefit from this unique molecular mechanism. Without the significant neutral – anionic redistribution of GCaMP6m, the ΔF_2 signals are small and in imaging applications the chromophore will absorb light at all times, regardless of Ca^{2+} . This would likely result in faster bleaching of sensor. The T222S mutants presented here reinforce importance of a ΔpK_a across physiological range and just how remarkable the GCaMP6m tool is.

Discussion

This mutagenesis effort highlights specific residues that could be useful to for future GCaMP optimization and potentially for other similarly designed biosensors. Recently, a study was published about directed evolution in ArcLight [21], in which they found similar pK_a altering mutations have effects on not only the magnitude of the $\Delta F/F_0$ but also the direction of the response (i.e. upward versus downward ΔF signal). This group of mutations, and certainly the locations of these targeted residues known to affect pK_a in EGFP should be explored in various different biosensor optimization efforts.

The mutants described here have unique $\Delta F/F_0$ signatures at ~515 nm when excited at two different wavelengths, for example, 400 nm and 470 nm excitation. This characteristic could be exploited to generate a suite of unique, distinguishable sensors based on ratiometric excitation that could be targeted to different cell types. There are currently blue, green, and red Ca^{2+} sensors, but using these three channels consumes most of the visible spectrum, leaving little room for actuators such as the channel rhodopsins or light activated enzymes [22–26]. Theoretically, a suite of sensors, including those described here, could potentially provide ~10 or more discernable signals while consuming only the blue/green portion of the spectrum.

Methods and Materials

Introducing mutations into GCaMP6m

Mutations discovered in our previous work [18], as well as ones reported in the literature on GCaMP improvements [1] were made in the FP and linker region of GCaMP6m (pGP-CMV-GCaMP6m, Addgene, Plasmid

#40754) using overlapping primers at each mutation site with ligation-independent cloning [27]. Template background was eliminated by cloning mutated GCaMP6m coding regions into different antibiotic resistant CMV expression plasmids, pUB2.1 (ampicillin resistance, Addgene Plasmid #40728) or pKM1 (kanamycin resistance).

Preparation of purified protein samples

The coding region of EGFP, EGFP-mutants, and GCaMP6m-mutants were moved into a constitutive bacterial expression vector (pCP, generous gift from Nathan Shaner), using ligation-independent cloning [27]. *E. coli* colonies expressing GCaMP6m were picked for presence of 400 nm or 470 nm excited fluorescence emission at ~515 nm and grown at 34°C for 48 hours in CircleGrow (MP Biomedicals) with Ampicillin. Bacterial pellets were lysed using BugBuster (Novagen) and Benzonase (Novagen). Cleared lysates were then His-tag purified using Protino Ni-TED 2000 packed columns (Macherey-Nagel). Purified fluorescent proteins were eluted in 1x PBS with imidazole pH 8 buffer solution. The protein was then concentrated and buffer exchanged into a pH 7.2 MOPS buffer using Vivaspin Turbo 15 (Sartorius) polyethersulfone ultrafiltration columns. The same buffer exchange columns were used to make all Ca^{2+} -free and Ca^{2+} -saturated samples for protein measurements, except for the Ca^{2+} titration experiments. All purified GCaMP6m Ca^{2+} -free protein measurements for this work were carried out in 10 mM EGTA /100 mM KCl / 30 mM MOPS at pH 7.2. All purified GCaMP6m Ca^{2+} -saturated protein measurements for this work were carried out in 10 mM CaEGTA /100 mM KCl /30 mM MOPS at pH 7.2.

Ca^{2+} Titration measurements

Ca^{2+} titration experiments for purified protein samples were carried out as previously described [20], using 10 mM EGTA /100 mM KCl / 30 mM MOPS at pH 7.2 and 10 mM CaEGTA /100 mM KCl /30 mM MOPS at pH 7.2. We measured fluorescence intensity at the spectral peak (between 512 nm and 516 nm) as a function of Ca^{2+} concentration by varying CaEGTA concentrations of the 2 mL sample (0 to 10 mM CaEGTA). We used excitation at 470 nm to measure the Ca^{2+} dependent fluorescence of the anionic form, and 400 nm for the neutral form. The pH of each sample was measured at beginning and end of each titration series to ensure there was no shift in pH during titration. For the purpose of this study, the Ca^{2+} titration experiments were for qualitative observation of fluorescence behavior and for quantitative measurement of the $\Delta F/F$ parameter for the purified protein sample without a change in

protein concentration. The apparent K_d and Hill coefficient values were not calculated from these Ca^{2+} titration measurements.

Fluorescence spectra, fluorescence excitation spectra, and quantum yield measurements

Fluorescence spectra and fluorescence excitation spectra of purified protein samples were collected using either a PC1™ spectrofluorimeter (ISS) or a LS55 spectrofluorimeter (Perkin Elmer). Fluorescence spectra were corrected for the detection spectral sensitivity using the quinine sulfate solution in 1M H_2SO_4 , whose corrected spectrum is published [28]. Fluorescence excitation spectra were corrected for excitation spectral variations using the styryl 9M (Aldrich) solution in ethanol, by comparing its excitation spectrum with its independently measured absorption spectrum. Fluorescence quantum yield (ϕ) of the GCaMP6m-mutant anionic chromophores were measured from the fluorescence peak (for Ca^{2+} -saturated at 504 nm (mutants: A74V,S114N,T222S; E60C,T222S), 502 nm (E60Q,T222S), 514 nm (E60G; E60C) and 515 nm for (V115I); for Ca^{2+} -free at 511 nm (E60Q,T222S), 510 nm (E60C,T222S), 516 nm (E60G), and 517 nm (E60C, V115I) upon its direct excitation at 470 nm was measured versus fluorescein in 1M NaOH ($\phi = 0.95$) as a reference standard [28]. The fluorescence quantum yield cannot be reported for the anionic form in Ca^{2+} -free A74V,S114N,T222S because the relative concentration of this form is too small. Fluorescence quantum yield (ϕ) of the GCaMP6m-mutant neutral chromophores were measured from the fluorescence peak (for Ca^{2+} -saturated at 507 nm (mutants: A74V,S114N,T222S; E60C,T222S; E60Q,T222S) 510 nm (E60G), 511 nm (E60C), 513 nm (V115I), for Ca^{2+} -free at 510 nm (E60G), 513 nm (mutants: A74V,S114N,T222S; E60C; E60C,T222S; E60Q,T222S), 515 nm (V115I) using excitation at 380 nm versus quinine sulfate in 1M H_2SO_4 ($\phi = 0.53$) as a reference standard [28]. The optical density of the solutions at each excitation wavelength and in the whole range of fluorescence was kept less than 0.07. Corrected integrated spectra were used to calculate quantum yields [28].

Extinction Coefficient measurements

To measure the extinction coefficients of the neutral and anionic forms of the chromophore in each GCaMP6m-mutant (either in Ca^{2+} -free or Ca^{2+} -saturated states) separately, we measured the changes of the

corresponding absorption spectrum upon alkaline pH titration of the purified protein solutions as recently described [20]. Absorption measurements were performed with a BioMate™ S3 spectrophotometer (ThermoFisher) or a Lambda950 spectrophotometer (Perkin Elmer). The pH of the sample was directly measured for each step, using an Orion™ PeropHecT™ ROSS™ combination pH microelectrode (ThermoFisher).

pK_a Measurements

We independently measured the changes in fluorescence spectra for alkaline and acidic titration experiments with purified protein samples. To do this we used the same alkaline titration method as the extinction coefficient measurements, and for acidic titration method we gradually added small volumes of 1M HCl (1 – 3 μ l) to the 2 mL sample, from pH 7.2 to 4.3. pH (measured with Orion™ PeropHecT™ ROSS™ combination pH microelectrode, ThermoFisher) and corresponding fluorescence spectra (measured with spectrofluorimeter) were collected upon excitation of anionic form at 470 nm for the Ca²⁺-saturated and Ca²⁺-free states. To calculate the pK_a, the peak fluorescence intensity near 515 nm was plotted against pH to get a pH titration curve. The data were then fit using two-binding site model function in the range of pH 4.3 to 11.4 for Ca²⁺-free GCaMP6m, and using a single-binding site model function for Ca²⁺-saturated GCaMP6m in the range of pH 4.8 to 8.3.

Two-photon measurements

Ca²⁺-free and Ca²⁺-saturated purified mutant protein samples (5 x 10⁻⁵ M) were measured in 0.1 cm glass spectroscopy cuvettes (Starna cells). Two-photon excitation (TPE) spectra were measured using an MOM Sutter Instrument two-photon fluorescent microscope coupled with an Insight DeepSee (Newport) femtosecond laser tunable from 680 to 1300 nm in a similar approach as described in [29]. A Plan NeoFluar 2.5x/0.075 Zeiss objective was used to excite and collect fluorescence, which was passed through a Chroma ET 520/40m filter before reaching the PMT. To correct the TPE spectra for the wavelength-to-wavelength variations of laser properties (pulse duration and beam shape), fluorescein in a NaOH solution (pH 11) was used as a reference standard [30,31]. The TPE fluorescence had quadratic dependence on excitation power in the whole spectral range presented in our results section. Two-photon cross-section was measured at 800 nm for the neutral form of the chromophore in Ca²⁺-free GCaMP6m-mutants and 930 nm for the anionic form in the Ca²⁺-saturated and Ca²⁺-free GCaMP6m-mutants, using coumarin 485 in methanol (for 800 nm excitation) and fluorescein in NaOH aqueous solution (pH 11) (for 930 nm excitation) as reference

standards [31]. Fluorescence intensity, F , as a function of excitation power, P , was measured for both the sample and the reference in the same conditions through either a 507/10 nm or 517/10 nm narrow-bandpass filter. From the fit of these dependences to a quadratic function, $F = \alpha P^2$, factors α values were obtained and then normalized to the concentrations (obtained spectrophotometrically, BioMate™ S3 spectrophotometer) and differential quantum efficiencies at 507 nm or 517 nm (obtained with spectrofluorimeter, PC1 ISS).

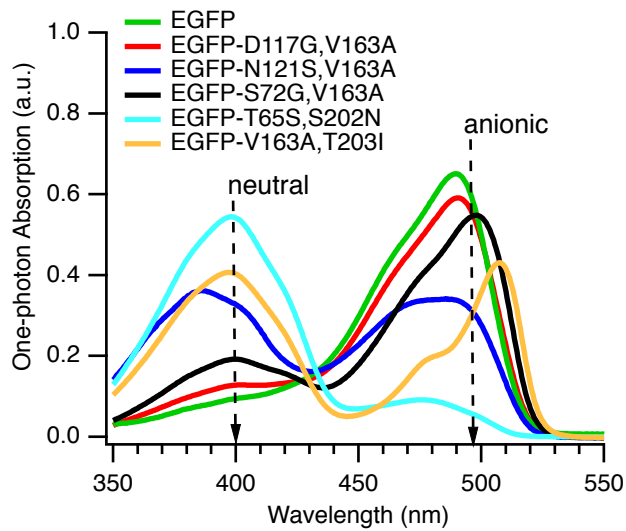
References

1. Akerboom J, Chen TW, Wardill TJ, Tian L, Marvin JS, Mutlu S, et al. Optimization of a GCaMP Calcium Indicator for Neural Activity Imaging. *Journal of Neuroscience*. 2012;32: 13819–13840.
2. Chen T-W, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature*. Nature Publishing Group; 2013;499: 295–300.
3. Dana H, Chen T-W, Hu A, Shields BC, Guo C, Looger LL, et al. Thy1-GCaMP6 Transgenic Mice for Neuronal Population Imaging In Vivo. *PLoS One*. Public Library of Science; 2014;9: e108697–9.
4. Boyd AM, Kato HK, Komiyama T, Isaacson JS. Broadcasting of Cortical Activity to the Olfactory Bulb. *CellReports*. The Authors; 2015;10: 1032–1039.
5. Hinckley CA, Alaynick WA, Gallarda BW, Hayashi M, Hilde KL, Driscoll SP, et al. Spinal Locomotor Circuits Develop Using Hierarchical Rules Based on Motorneuron Position and Identity. *Neuron*. Elsevier Ltd; 2015;87: 1008–1021.
6. Theis L, Berens P, Froudarakis E, Reimer J, Rosón MR, Baden T, et al. Benchmarking Spike Rate Inference in Population Calcium Imaging. *Neuron*. Elsevier Ltd; 2016;90: 471–482.
7. Grover D, Greenspan RJ, Katsuki T. Flyception: imaging brain activity in freely walking fruit flies. *Nat Methods*. Nature Publishing Group; 2016;13: 1–7.
8. Akerboom J, Rivera JDV, Guilbe MMR, Malave ECA, Hernandez HH, Tian L, et al. Crystal Structures of the GCaMP Calcium Sensor Reveal the Mechanism of Fluorescence Signal Change and Aid Rational Design. *J Biol Chem*. 2008;284: 6455–6464.
9. Ding J, Luo AF, Hu L, Wang D, Shao F. Structural basis of the ultrasensitive calcium indicator GCaMP6. *Sci China Life Sci*. 2014;57: 269–274.
10. Zhao Y, Araki S, Wu J, Teramoto T, Chang YF, Nakano M, et al. An Expanded Palette of Genetically Encoded Ca²⁺ Indicators. *Science*. American Association for the Advancement of Science; 2011;333: 1888–1891.
11. Brejc K, Sixma TK, Kitts PA, Kain SR, Tsien RY, Ormö M, et al. Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. *Proc Natl Acad Sci U S A*. 1997;94: 2306–2311.
12. Lossau H, Kummer A, Heinecke R, Pöllinger-Dammer F, Kompa C, Bieser G, et al. Time-resolved spectroscopy of wild-type and mutant Green Fluorescent Proteins reveals excited state deprotonation consistent with fluorophore-protein interactions. *Chem Phys*. 1996;213: 1–16.
13. Chatteraj M, King BA, Bublitz GU, Boxer SG. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. *Proc Natl Acad Sci U S A*. 1996;93: 8362–8367.
14. Warshel A. Calculations of enzymatic reactions: calculations of pKa, proton transfer reactions, and general acid

- catalysis reactions in enzymes. *Biochemistry*. American Chemical Society; 1981;20: 3167–3177.
15. Tsien RY. The green fluorescent protein. *Annu Rev Biochem*. 1998;67: 509–544.
 16. Ai H-W, Olenych SG, Wong P, Davidson MW, Campbell RE. Hue-shifted monomeric variants of *Clavularia* cyan fluorescent protein: identification of the molecular determinants of color and applications in fluorescence imaging. *BMC Biol*. 2008;6: 13.
 17. Jung G, Zumbusch A. The photophysics of green fluorescent protein: influence of the key amino acids at positions 65, 203, and 222. *Biophysj*. Biophysical Society; 2005;88: 1932–1947.
 18. Stoltzfus CR, Barnett LM, Drobizhev M, Wicks G, Mikhaylov A, Hughes TE, et al. Two-photon directed evolution of green fluorescent proteins. *Sci Rep*. Macmillan Publishers Limited SN -; 2015;5: 11968.
 19. Miesenböck G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*. nature.com; 1998;394: 192–195.
 20. Barnett LM, Hughes TE, Drobizhev M. Deciphering the molecular mechanism responsible for GCaMP6m's Ca²⁺-dependent change in fluorescence. *PLoS One*. Public Library of Science; 2017;12: e0170934.
 21. Platasa J, Vasan G, Yang A, Pieribone VA. Directed evolution of key residues in fluorescent protein inverses the polarity of voltage sensitivity in the genetically-encoded indicator ArcLight. *ACS Chem Neurosci*. ACS Publications; 2017; Available: <http://pubs.acs.org/doi/abs/10.1021/acscchemneuro.6b00234>
 22. Lin JY. A user's guide to channelrhodopsin variants: features, limitations and future developments. *Exp Physiol*. 2011;96: 19–25.
 23. Fenno L, Yizhar O, Deisseroth K. The development and application of optogenetics. *Annu Rev Neurosci*. 2011;34: 389–412.
 24. Bernstein JG, Boyden ES. Optogenetic tools for analyzing the neural circuits of behavior. *Trends Cogn Sci*. 2011;15: 592–600.
 25. Reiner A, Isacoff EY. The Brain Prize 2013: the optogenetics revolution. *Trends Neurosci*. 2013;36: 557–560.
 26. Chow BY, Han X, Boyden ES. Genetically encoded molecular tools for light-driven silencing of targeted neurons. *Prog Brain Res*. 2012;196: 49–61.
 27. Barnett L, Platasa J, Popovic M, Pieribone VA, Hughes T. A Fluorescent, Genetically-Encoded Voltage Probe Capable of Resolving Action Potentials. *PLoS One*. Public Library of Science; 2012;7: e43454.
 28. Lakowicz JR. *Principles of Fluorescence Spectroscopy*. 3rd ed. New York: Springer; 2006.
 29. Tsai PS, Nishimura N, Yoder EJ, Dolnick EM, Allen White G, Kleinfeld D. Principles, Design, and Construction of a Two-Photon Laser-Scanning Microscope for In Vitro and In Vivo Brain Imaging. In: Frostig RD, editor. *In vivo optical imaging of brain function*. Boca Raton: CRC Press; 2002. pp. 113–171.
 30. Xu C, Webb WW. Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. *J Opt Soc Am B, JOSAB*. Optical Society of America; 1996;13: 481–491.
 31. Makarov NS, Drobizhev M, Rebane A. Two-photon absorption standards in the 550–1600 nm excitation range: establishing a correction curve for accurate cross section calibration. *Integrated Optoelectronic Devices 2008*. International Society for Optics and Photonics; 2008. pp. 689105–689105–12.

Fig 1. Mutations in the EGFP chromophore and surrounding protein β -barrel can alter the neutral – anionic equilibrium.

A.



B.

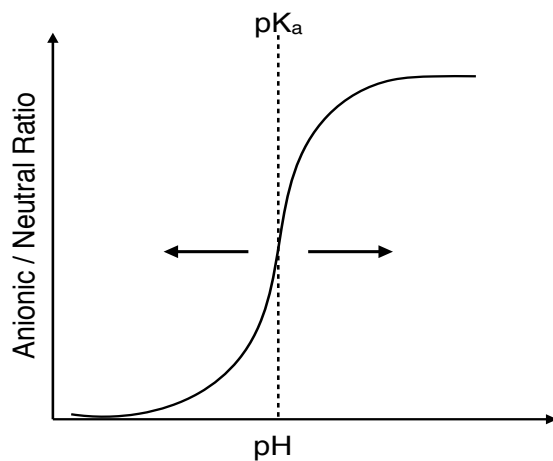


Fig 2. EGFP pK_a altering mutations affect the pK_a and Δ pK_a in GCaMP6m.

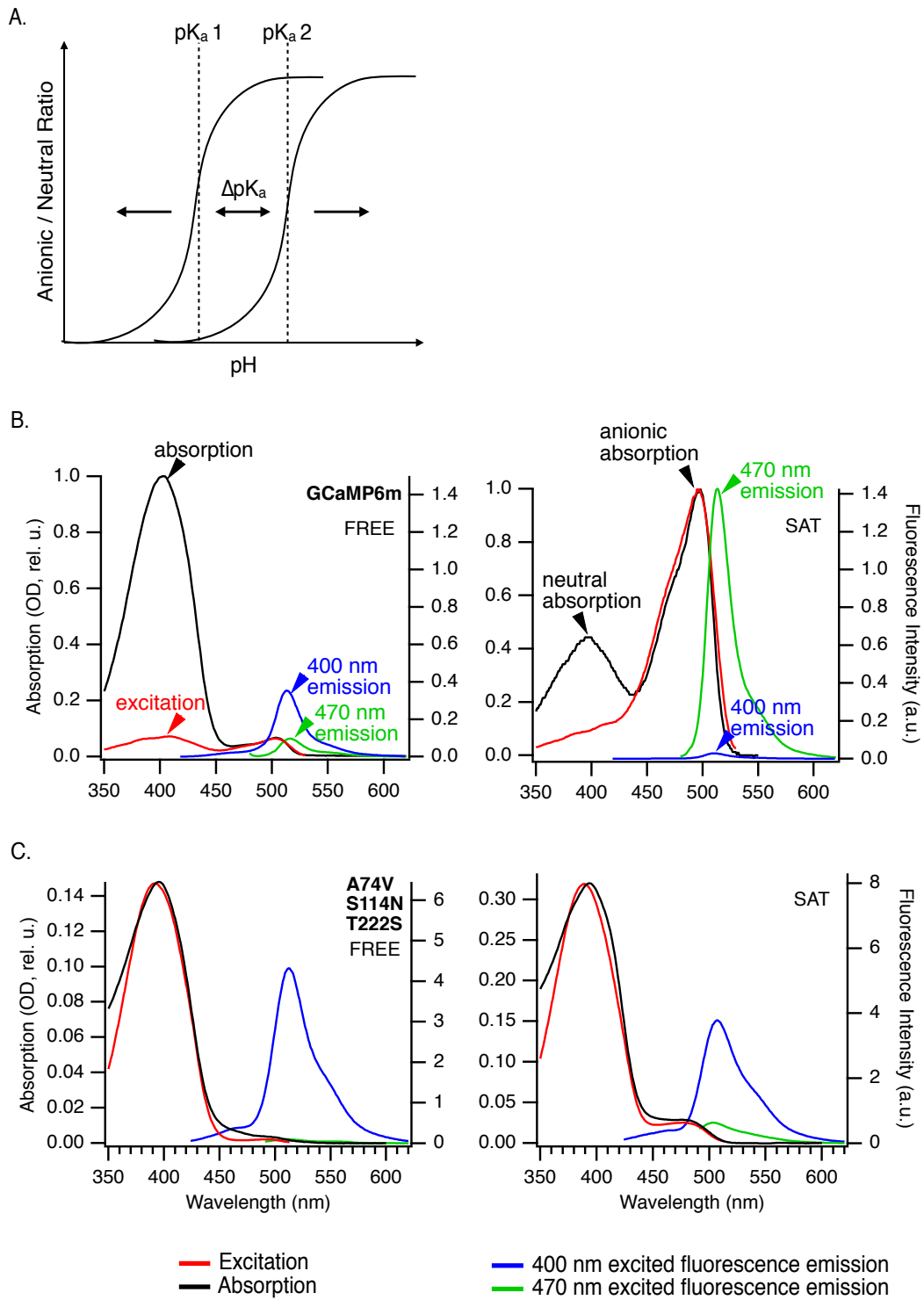


Fig 3. Ca^{2+} -binding has varying effects on GCaMP6m-mutant chromophore equilibria.

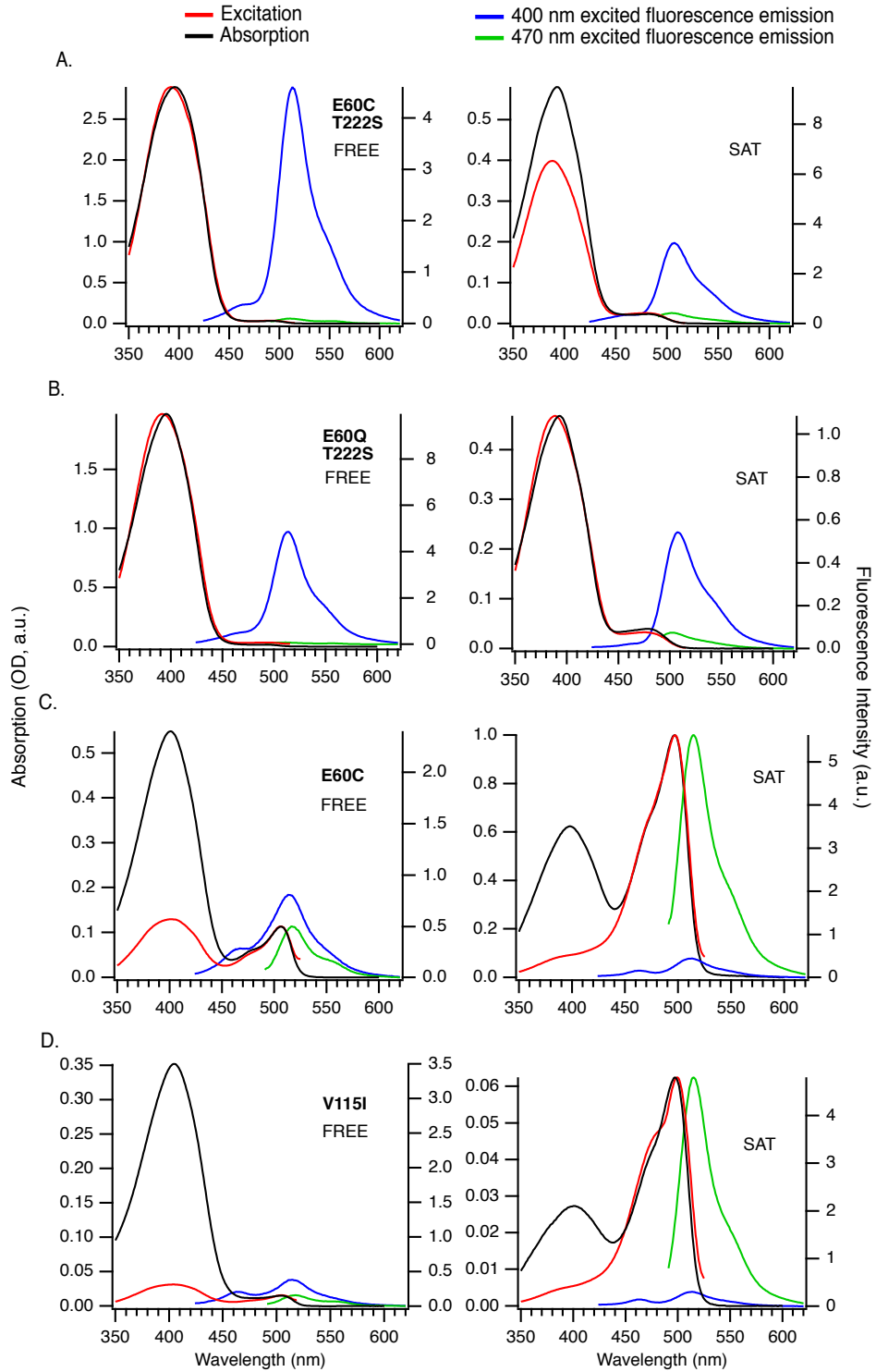


Fig 4. The E60G mutation in GCaMP6m most likely increases the Ca^{2+} -dependent ΔF via a change in quantum yield.

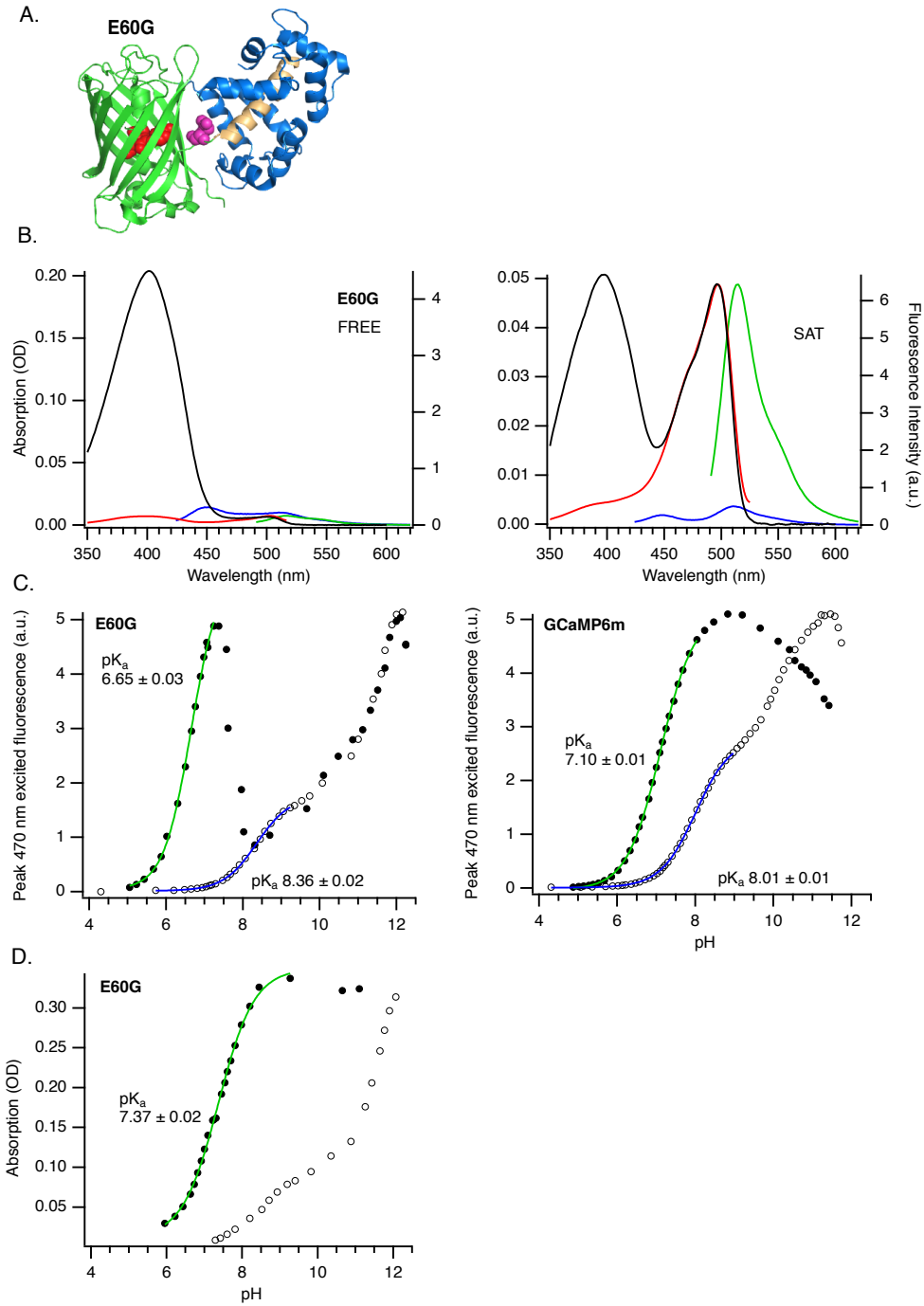


Fig 5. pK_a is the predominant mechanism for Mutant Ca^{2+} -dependent changes in 470 nm excited fluorescence, however other parameters can significantly contribute.

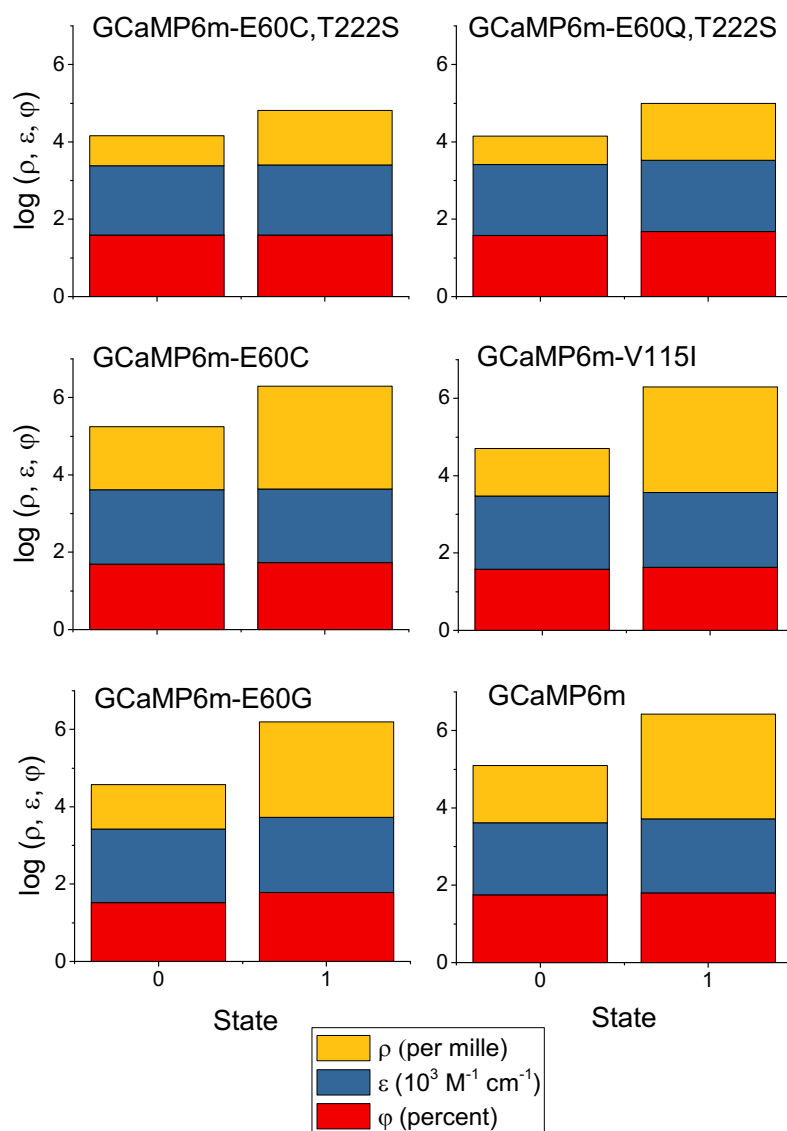
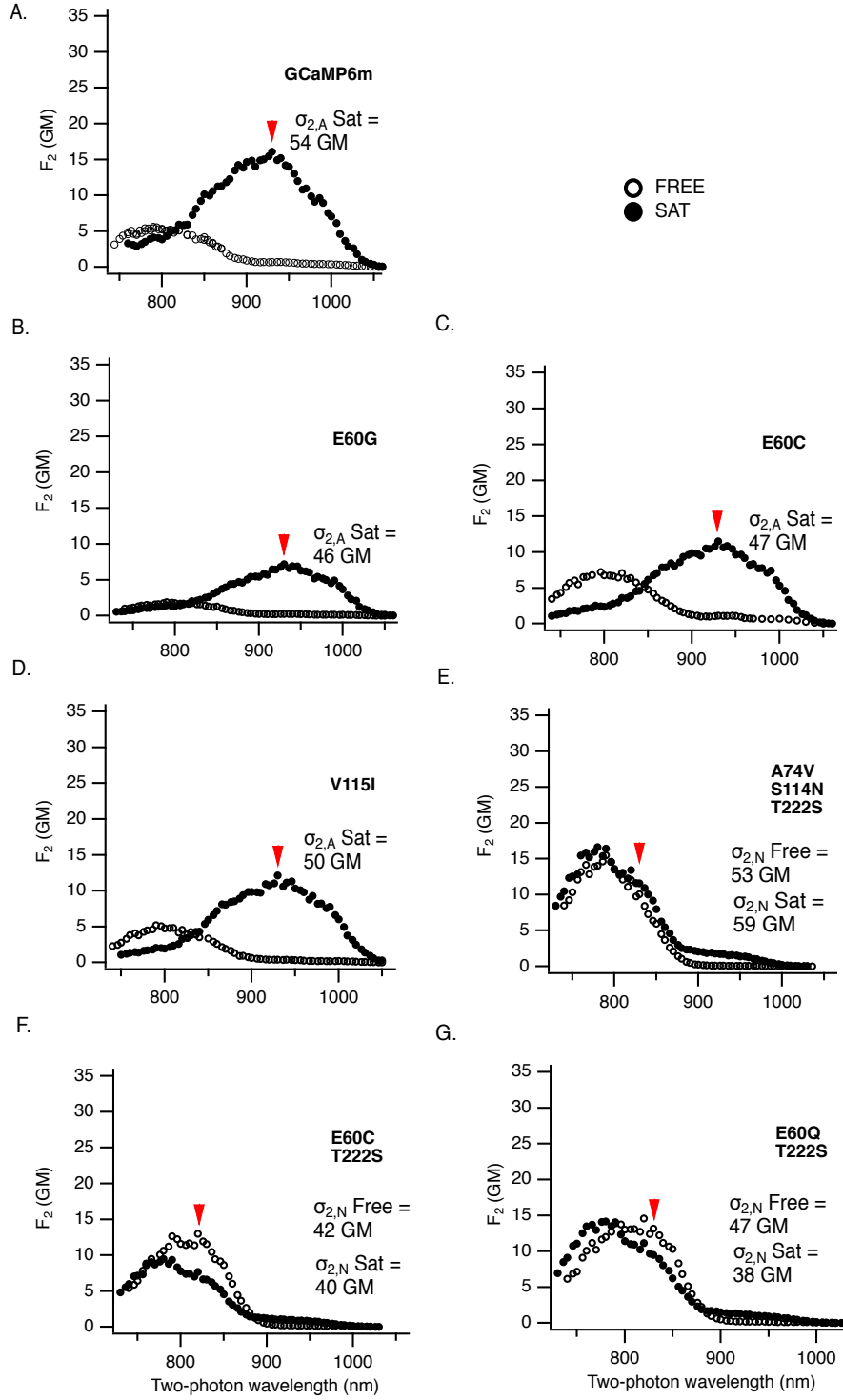


Fig 6. The mutant two-photon excited spectra illustrate the importance of ΔpK_a .



CHAPTER FOUR

A FLUORESCENT, GENETICALLY-ENCODED VOLTAGE PROBE CAPABLE OF
RESOLVING ACTION POTENTIALSContribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Lauren M. Barnett

Contributions: Conceived project; performed all design and construction of the novel voltage sensor proteins; performed all molecular biology; performed initial baseline fluorescence screens in HEK293 cells; contributed to critical data analysis, manuscript drafting, and final manuscript review.

Author: Jelena Platisa

Contributions: Conceived project; performed all electrophysiology and imaging in HEK293 cells and neurons; contributed to critical data analysis, manuscript drafting, and final manuscript review.

Co-Author: Marco Popovic

Contributions: helped performed electrophysiology and imaging in neurons; contributed to critical data analysis, manuscript drafting, and final manuscript review.

Co-Author: Vincent A. Pieribone

Contributions: Conceived project; supervised project; contributed to critical data analysis, manuscript drafting, and final manuscript review; corresponding author.

Co-Author: Thomas E. Hughes

Contributions: Conceived project; supervised project; contributed to critical data analysis, manuscript drafting, and final manuscript review.

Manuscript Information Page

Lauren Barnett ¹, Jelena Platisa ^{2,3}, Marko Popovic ⁴, Vincent A. Pieribone ^{2,4}, Thomas Hughes¹

¹ Department of Cell Biology and Neuroscience, Montana State University, Bozeman, Montana, United States of America

² The John B. Pierce Laboratory, Inc., New Haven, Connecticut, United States of America,

³ Faculty of Physical Chemistry, University of Belgrade, Belgrade, Serbia

⁴ Cellular and Molecular Physiology and Neurobiology, Yale University School of Medicine, New Haven, Connecticut, United States of America

Status of the manuscript:

☐ Prepared for submission to a peer-reviewed journal

☐ Officially submitted to a peer-reviewed journal

☐ Accepted by a peer-reviewed journal

☒ Published in a peer-reviewed journal

Published by PLoS ONE, doi:10.1371/journal.pone.0043454

A Fluorescent, Genetically-Encoded Voltage Probe Capable of Resolving Action Potentials

Lauren Barnett^{1*}, Jelena Platisa^{2,3*}, Marko Popovic⁴, Vincent A. Pieribone^{2,4*}, Thomas Hughes¹

¹ Department of Cell Biology and Neuroscience, Montana State University, Bozeman, Montana, United States of America, ² The John B. Pierce Laboratory, Inc., New Haven, Connecticut, United States of America, ³ Faculty of Physical Chemistry, University of Belgrade, Belgrade, Serbia, ⁴ Cellular and Molecular Physiology and Neurobiology, Yale University School of Medicine, New Haven, Connecticut, United States of America

Abstract

There is a pressing need in neuroscience for genetically-encoded, fluorescent voltage probes that can be targeted to specific neurons and circuits to allow study of neural activity using fluorescent imaging. We created 90 constructs in which the voltage sensing portion (S1–S4) of *Ciona intestinalis* voltage sensitive phosphatase (CiVSP) was fused to circularly permuted eGFP. This led to ElectricPk, a probe that is an order of magnitude faster ($\tau_{\text{aus}} \sim 1\text{--}2\text{ ms}$) than any currently published fluorescent protein-based voltage probe. ElectricPk can follow the rise and fall of neuronal action potentials with a modest decrease in fluorescence intensity ($\sim 0.7\% \Delta F/F$). The probe has a nearly linear fluorescence/membrane potential response to both hyperpolarizing and depolarizing steps. This is the first probe based on CiVSP that captures the rapid movements of the voltage sensor, suggesting that voltage probes designed with circularly permuted fluorescent proteins may have some advantages.

Citation: Barnett L, Platisa J, Popovic M, Pieribone VA, Hughes T (2012) A Fluorescent, Genetically-Encoded Voltage Probe Capable of Resolving Action Potentials. PLoS ONE 7(9): e43454. doi:10.1371/journal.pone.0043454

Editor: Huibert D. Mansvelder, Neuroscience Campus Amsterdam, VU University, The Netherlands

Received: April 17, 2012; **Accepted:** July 24, 2012; **Published:** September 6, 2012

Copyright: © 2012 Barnett et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported as an NINDS funded U24 consortium to produce voltage sensors for the neuroscience community (U24NS057631, ARRA U24NS057631-03S1). Consortium members include: Randall Stewart (National Institutes of Health [NIH] Program Officer) and the laboratories of Lawrence B. Cohen, Thomas Hughes, Ehud Isacoff, Thomas Knöpfel, Vincent A. Pieribone, and Brian Salzberg. This project was also supported by NIH grant ARRA-R01NS065110 and by The John B. Pierce Laboratory, Inc. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have the following conflicts: Funding for this study came in part from The John B. Pierce Laboratory, Inc. Authors Jelena Platisa and Vincent A. Pieribone are employees of The John B. Pierce Laboratory, Inc. There are no other relevant declarations relating to employment, consultancy, patents, products in development or marketed products. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

* E-mail: vpieribo@jbpierce.org

† These authors contributed equally to this work.

Introduction

The discovery of the green fluorescent protein (GFP) and its many orthologs (FP) rapidly led to the creation of genetically-encoded, fluorescent biosensors. These probes make it possible to optically record physiologically important signals such as transmembrane potential and intracellular calcium levels. The first voltage sensor was described in 1997 [1], and since then several additional FP-based probes have been described [2–6]. The majority of these probes were designed to convert conformational changes in the voltage sensing domains from either an ion channel, or the *Ciona intestinalis* voltage sensitive phosphatase (CiVSP), into fluorescence changes in a single FP or pairs of FPs. However, to date these probes have lacked the speed to accurately reproduce action potentials with temporal fidelity. While there are detectable, fast components in the response of some of these probes ($\tau = 8\text{--}16\text{ ms}$), the responses are largely dominated by slow components ($\tau = >30\text{ ms}$).

The process of creating better genetically-encoded voltage sensors has lagged behind calcium sensor development [7]. The GCaMP probes [8,9] are genetically-encoded sensors of calcium ions based on circularly permuted eGFP (cpEGFP) [10,11]. The crystal structure of GCaMP2 [12] revealed that when the interacting Calmodulin and M13 domains are unbound, a hole

appears in the side of the fluorescent protein barrel, likely quenching the fluorophore. Calcium-mediated Calmodulin/M13 interactions in turn occlude this hole and the fluorescence returns. This mechanistic understanding of how GCaMP2 works led directly to the improved GCaMP3 [12], GECCO and R-GECCO probes [13] with signals that are so robust that the sensors can report changes in intracellular calcium levels from cells deep in living tissues [14,15].

We sought to leverage recent Ca^{++} probe design principles in our pursuit of improved voltage probes that capture the fastest movements of CiVSP. The design principles used in a previous study that explored circularly permuted FPs as possible reporters in voltage sensors produced no viable probes [16]. However, the development of both Ca^{++} and voltage probes has shown that very small adjustments in the linker between the sensing domain and the fluorescent protein are critical and that a large number of variants need to be rigorously explored. Accordingly, we created and tested 90 different CiVSP::cpEGFP fusions in transiently transfected mammalian cells for the production of fluorescence at the cell membrane and a subset were tested for voltage dependent changes in this fluorescence.

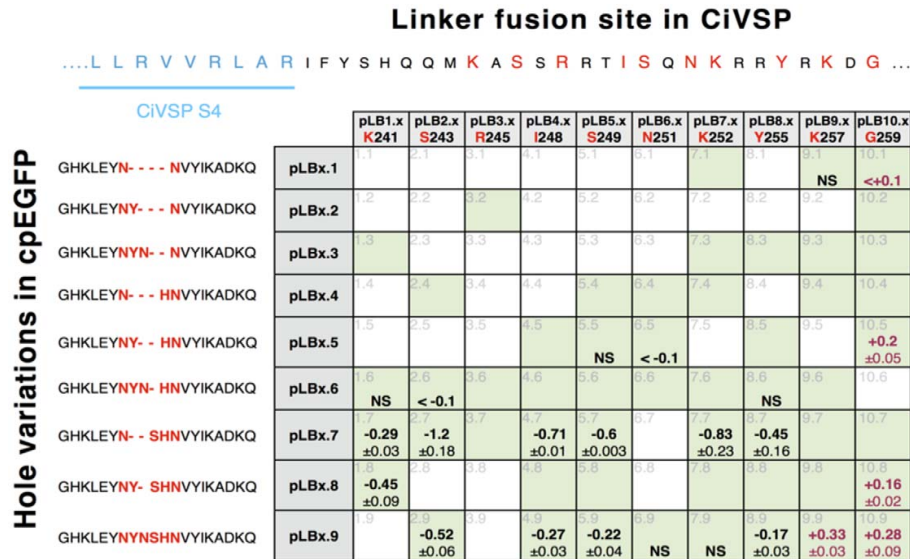


Figure 1. Probe design, expression level and $\Delta F/\Delta V$ sensitivity of CIVSP::cpEGFP constructs. Two variables were systematically explored: 1) the amino acid position within CIVSP in which the cpEGFP was fused (horizontal axis, e.g. 1.x), and 2) the size and position (vertical axis, e.g. x.1) of the hole in the cpEGFP. Gray numbers are construct number. The green shaded boxes within the plot indicate probes that produced fluorescence in HEK 293 cells. Bold numbers in boxes represent average values of peak $\Delta F/F \pm \text{SEM}$ for +100 mV/200 ms steps from a holding potential of -70 mV; NS = no detectable $\Delta F/F$ signal; Red numbers in boxes indicate reversed responses (increase in fluorescence) to depolarizing steps. The response kinetics of constructs 9.1,10.1,5.5,6.5,1.6,2.6,8.6,6.9,7.9 was either non existent or too small to accurately measure. Constructs 7.7 and 8.7 exhibited relatively slow ($\tau \sim 69$ ms) on and off kinetics. Constructs 10.5,1.7,2.7, 4.7,5.7,1.8,10.8,2.9,4.9,5.9,8.9,9.9,10.9 had on and off rates that were dominated by extremely fast components ($\tau \sim 2$ ms).

doi:10.1371/journal.pone.0043454.g001

Results

In designing the CIVSP::cpEGFP fusions, it was impossible to predict which nearby surface might be available to occlude the hole in cpEGFP, a process that occurs in the calcium-dependent structural changes in GCaMP2 [12]. Most successful CIVSP-based voltage probes involve the fusion of the FP to CIVSP, immediately C-terminal to the S1–4 domains. These fusions truncate the CIVSP, removing the phosphatase domain. The exact fusion site between the CIVSP and the FP plays an important role in voltage sensitivity of the probe. We selected 10 different fusion sites for our probes (Figure 1, 2, 3) all at - or adjacent to - sites used in previous probes. The cpEGFP in GCaMP3 removes amino acids 145 through 148 [13] in the eGFP, but it was unclear whether this would be the right hole size for these constructs, so nine different cpEGFPs were created and fused to each of the CIVSP truncations to produce 90 different constructs for testing (Figure 1, 2).

All constructs were transiently expressed in HEK293 cells and examined for expression level. After 48 hours of expression, 55 of 90 constructs produced fluorescent cells (Figure 1) that were generally of weaker fluorescence and poorer localization (Figure 4) to the plasma membrane when compared to other CIVSP voltage probes [4,15–18]. Comparative analysis of the cellular fluorescence done with confocal microscopy showed variations in the intensity and sub cellular distribution between constructs (Figure 4).

Out of 24 fluorescent constructs that were tested in patch clamp experiments for voltage sensitivity, nine showed negligible ($\sim 0.1\%$ $\Delta F/F$) or no change in fluorescence in response to test voltage steps (+100 mV from a -70 mV holding potential). The

remaining constructs exhibited from +0.33% to -1.2% $\Delta F/F$ (Figure 1). The largest fluorescence changes were detected in constructs with smaller holes in the FP. In most of the constructs, depolarizing steps provoked a decrease in fluorescence (Figure 4A–C) while five constructs (four at G259 and one at K257; Figure 4D)

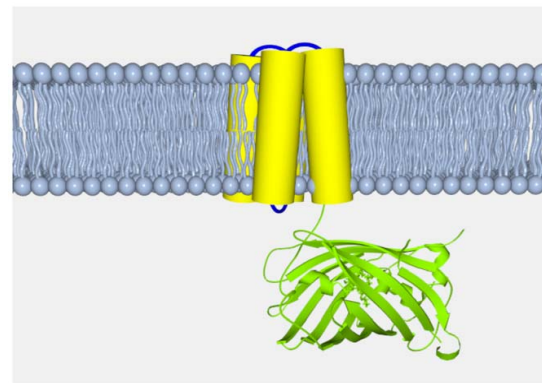


Figure 2. Schematic diagram of CIVSP::cpEGFP based construct design. The S1–4 domain of Ciona intestinalis voltage sensitive phosphatase (yellow) is fused to circularly permuted EGFP (green).

doi:10.1371/journal.pone.0043454.g002

```

...larllrvvrlarifyshqqmkassrrtisqnkrryrkdgfdldltyvtdh.....CionaVSP

...larllrvvrlarifyshqqmkassrrtisqnkrrcgrCFP.....VSFP2.1
...larllrvvrlarifyshqqcgrRFP.....VSFP3.1
...larllrvvrlarifyshqqmkassrrtisgdpmUKG.....Mermaid

...larllrvvrlarifyshqqmksvikGFP.....1.X
...larllrvvrlarifyshqqkamsvikGFP.....2.X
...larllrvvrlarifyshqqmkassrmsvikGFP.....3.x
...larllrvvrlarifyshqqmkassrrtimsvikGFP.....4.x
...larllrvvrlarifyshqqmkassrrtissmsvikGFP.....5.x
...larllrvvrlarifyshqqmkassrrtisqnmsvikGFP.....6.x
...larllrvvrlarifyshqqmkassrrtisqnkmsvikGFP.....7.X
...larllrvvrlarifyshqqmkassrrtisqnrrymsvikGFP.....8.X
...larllrvvrlarifyshqqmkassrrtisqnrryrkmsvikGFP.....9.X
...larllrvvrlarifyshqqmkassrrtisqnrryrkdgmsvikGFP.....10.X

...larllrvvrlarifyshqqkamsvikshnv.....ElectricPK

```

Figure 3. The protein sequence of the various fusion sites of cpEGFP with CiVSP described in this report. From top: CiVSP sequence includes S4 domain (bold cyan), linker domain (cyan and black) and phosphatase domain (red). The fusion sites and linker sequences of VSFP 2.1, 3.1 (17, 18) and Mermaid (4) probes. The ten different fusion sites and linkers described in this report (pLB1.x-10.x; see Figure 1). The fusion site is identified by the last CiVSP amino acid present in the probe (bold red). In all cases, this residue is followed by a five amino acid linker (purple) and the cpEGFP (green). The amino acid at the beginning of the cpEGFP depends on the hole size and position of the cpEGFP (See Figure 1). doi:10.1371/journal.pone.0043454.g003

exhibited increases in fluorescence in response to the same test potentials.

While two (pLB 7.7 and pLB 7.8) constructs exhibited a predominate slow on and off rate ($\tau \sim 69$ ms; Figure 4B), 13 constructs had on and off rates that were dominated by extremely fast components ($\tau \sim 2$ ms; Figure 4A and 4C). Our estimate of the maximum response kinetics of these constructs is likely limited by the speed of our voltage clamp (~ 1 ms) and the optical sampling rate (2,000 fps) used. The probe with the greatest response magnitude in this series pLB2.7 ($-1.2 \pm 0.18\%$ $\Delta F/F$; Figure 4A), named ElectricPk, was studied in detail.

ElectricPk exhibited a fast on rate ($\tau_{on} = 2.24 \pm 0.58$ ms, $n = 8$) and off rate ($\tau_{off} = 2.09 \pm 0.74$ ms, $\tau_{2off} = 69.07 \pm 20.29$ ms; Figure 5A). The on rate of ElectricPk is 5 times faster (Figure 5B) than the CiVSP-based probe Mermaid ($\tau_{on} = 2.24$ ms vs 12 ms, respectively; ref 4) and the off rate is 11 times faster ($\tau_{off} = 2.09$ ms vs. 23 ms, respectively). The speed and response magnitude of this probe make it possible to resolve multiple, high frequency short (1–3 ms) depolarization steps, each of which produce a discrete fluorescence response without a significant accumulating baseline drift (Figure 5C,D).

The fluorescence of ElectricPk exhibits linear relationship to voltage (Figure 6) over a broad range of hyperpolarizing and depolarizing steps (-100 - $+200$ mV). The voltage/fluorescence relationship became saturated only with large, unphysiologic depolarizing steps ($+310$ mV; data not shown).

We next expressed ElectricPk in cultured mouse hippocampal neurons. In addition to good membrane localization evident in the cell soma and processes, there were intracellular deposits in some cells, though this was variable (Figure 7A). This may be caused by poorly folded protein, or it could be an artifact caused by abnormal expression levels or the lipofectamine transfection reagents. These intracellular deposits can clearly raise the

background fluorescence and lower the signal to noise ratio, so improving on the localization would be beneficial. Fluorescent cells were patch clamped and action potentials generated with brief (2 ms) current injections in current clamp mode. ElectricPk was capable of resolving action potentials with extraordinary temporal fidelity (Figure 7B and Movie S1). The rising and falling phases of the action potential were evident. The signal size was modest ($\sim 0.7\%$ $\Delta F/F$), and varied with expression intensity, yet action potential trains were detectable without trial averaging (Figure 7C).

Discussion

Early versions of genetically-encoded voltage probes constructed with voltage-gated ion channels [1–3] failed to traffic adequately to the plasma membranes of mammalian cells [5]. CiVSP-based probes traffic to the cell membrane more completely and probes created with the CiVSP voltage sensing domain have been more successful in mammalian cells [4,6,17–19]. The fluorescence changes, however, have been generally slow. The rapid response properties of the probes described here (2 ms) are in striking contrast to the dominant response kinetics (>12 ms) of all other currently published CiVSP-based probes [4,6,17–19]. The only probes with similar characteristics are the sodium channel-based probe (SPARC; 2) and a probe based on the zebrafish voltage sensitive protein [20]. The SPARC probe was able to capture rapid movements of the VSPs but is less useful due to low signal size and poor membrane targeting [5]. The mechanism by which ElectricPk changes its fluorescence intensity is not clear, but it depends on the circular permutation of eGFP since the fusion of normal eGFP at the same site does not produce viable probes (data not shown).

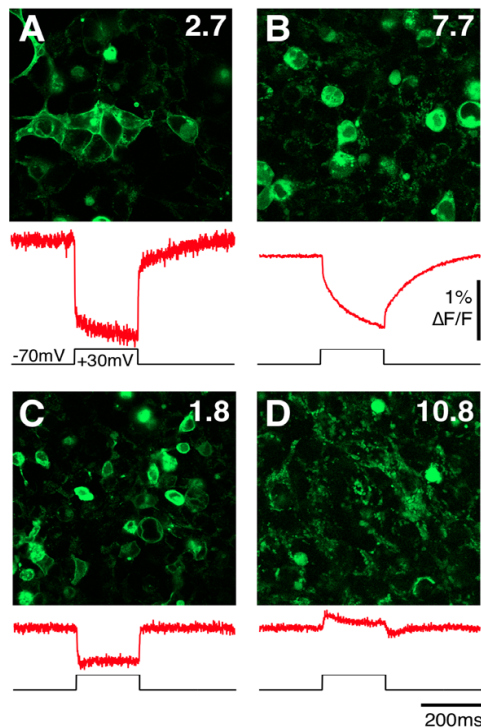


Figure 4. Examples of CivSP::cpEGFP constructs expression in HEK293 cells and voltage sensitivity. In all cases the upper panel is a confocal image of HEK293 cells transiently expressing the construct and the lower panel is the average fluorescence response (red trace) to ten +100 mV/200 ms voltage steps applied under whole-cell patch clamp configuration. Correction for FP photobleaching has been removed by division of a double exponential fit to the portions of the trace outside the effects of the voltage step. A) The ElectricPk (pLB 2.7) probe is localized both in the membrane and intracellularly. The probe exhibits a rapid decrease in fluorescence with a relatively low signal to noise ratio due to relatively weak basal fluorescence. B) The high expression levels of construct pLB7.7 produces a relatively high signal to noise ratio, however much of the fluorescent protein is localized intracellularly, and its fluorescence response is dominated by a slow component. C) Construct pLB1.8 has mixed membrane and intracellular distribution that produces a small and rapid negative response with a moderate signal to noise ratio. D) pLB10.8 has predominantly intracellular localization and a small positive fluorescence response with a moderate signal to noise ratio. doi:10.1371/journal.pone.0043454.g004

There appears to be several voltage driven rearrangements in the CivSP protein. One occurs quite quickly and can be measured as gating charge movement. Others occur more slowly [21], and it is these slow movements that likely drive the changes in fluorescence in many fluorescent voltage probes. The changes in ElectricPk, as well as in the majority of constructs described here, captures only the fast rearrangements in the CivSP protein as changes in fluorescence. ElectricPk demonstrates that it is possible to capture a voltage dependent movement in CivSP that previous sensor designs have missed, and to do this in neurons. It appears that the cpEGFP is capturing a fast, voltage-driven movement of CivSP that both FRET pairs of FPs and single FPs have missed.

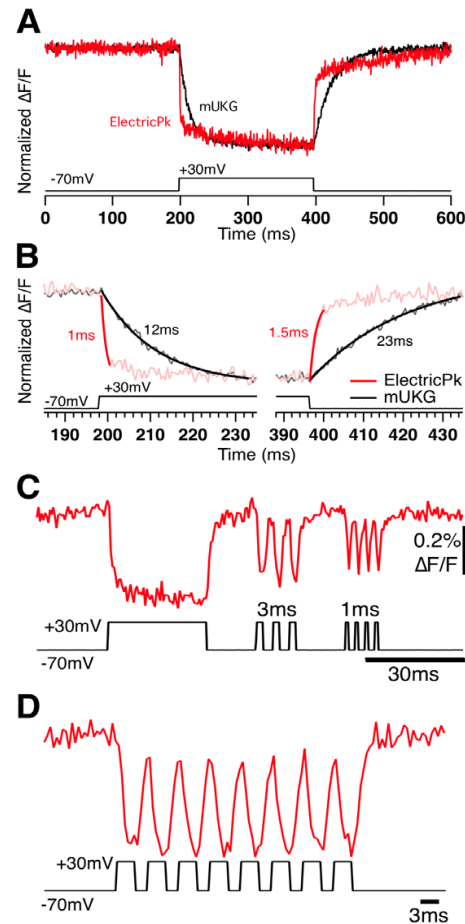


Figure 5. Voltage sensitivity and response kinetics of ElectricPk. A) Voltage-dependent fluorescence sensitivity of an HEK293 cell when transiently expressing ElectricPk (red trace) versus mUKG in Mermaid (upper black trace) tested with voltage steps of +100 mV/200 ms from a -70 mV holding potential (lower black trace). Fluorescence, in all cases, is normalized to initial and peak level. B) Single exponential fits to the dominant on and off response rates of ElectricPk and mUKG of Mermaid in cells tested as in A. C) and D) Response of HEK 293 cell expressing ElectricPk (red trace) to a trains of voltage steps of +100 mV from a -70 mV holding potential with a variable duration; 100 ms, 3 ms and 1 ms (black trace). doi:10.1371/journal.pone.0043454.g005

In addition, the linear F/V response is quite different from all previously published CivSP-based probes [4,6,17–19]. This indicates that this probe is likely capturing a different protein rearrangement, one associated with gating charge movements of the S4, which may help to explain its speed.

Previously the Knöpfel laboratory used cpEGFP derived from GCaMP2 to create a few fusions analogous to our constructs [16]. Their VSFP (B) cpEGFP is similar to our pLB1.1 or pLB2.1, and VSFP (C) cpEGFP as well as VSFP (D) cpEGFP are quite like pLB4.1 and pLB7.1, respectively. All of these constructs exhibited very low fluorescence in the initial screen and were not tested for

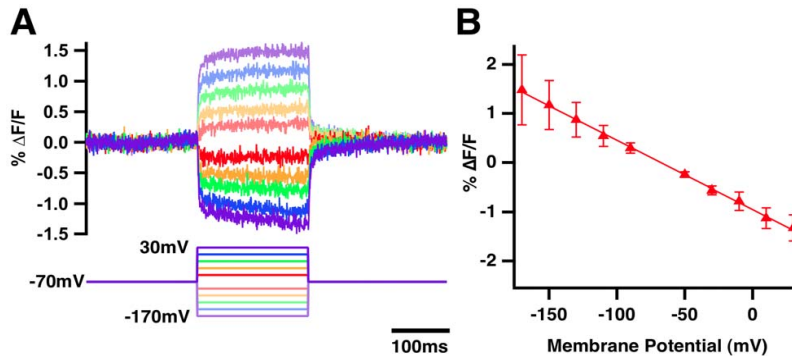


Figure 6. Linear $\Delta F/\Delta V$ response of ElectricPk. A) Fluorescence response (upper traces) of HEK293 cells transiently expressing ElectricPk to depolarizing and hyperpolarizing voltage steps (-170 to $+30$ mV from a -70 mV holding potential, lower traces). B) $\Delta F/\Delta V$ curve of ElectricPk derived from data presented in A.
doi:10.1371/journal.pone.0043454.g006

voltage driven changes in fluorescence. Their results, and ours, demonstrate that small changes in the size of the hole in the fluorescent protein and linkage to CiVSP, have profound effects on a probe's response speed and size.

As hyperpolarizing steps cause a linear increase in fluorescence in ElectricPk, some of the constructs that showed no fluorescence during the initial fluorescence screen may have increased fluorescence upon changes in membrane potential. Therefore, we may have missed some probes which exhibited increases in fluorescence in response to voltage steps, as only visibly fluorescent probes were tested for voltage dependent changes in fluorescence using patch clamp. To date, most CiVSP-based voltage sensors have been too slow for following fast neuronal activity. Recently a probe has been developed that makes it possible to detect action potentials, but it too is too slow to clearly resolve the waveform [6]. Tested in cultured neurons, ElectricPk detects action potentials with remarkable time resolution, comparable to what is seen with synthetic, small molecule voltage dyes. This is the first FP-based voltage sensor that could be, with additional adjustments of signal size, used for studies of the origin and propagation action potentials within single cells.

The weak fluorescence of a bacterial rhodopsin has recently been shown to be modulated by transmembrane potential [22]. This approach to genetically-encoded voltage probe development has several potential strengths: large, linear fractional change in fluorescence with voltage; extremely red-shifted excitation and emission spectra and resistance to bleaching. However, the quantum efficiency of fluorescence in these rhodopsins is so low (QY of 0.001 vs 0.65 for GFP) that it is unlikely they will be of much use in optical recordings in vivo. While in vitro conditions are artificially optimized for detecting fluorescence, the low fluorescence of these probes require high intensity (200 mW optical power) laser illumination combined with an EMCCD camera for detection in vitro. These limitations make in vivo use highly improbable with lower levels of expression, more light scattering, and greater sensitivity to phototoxicity. In addition, attempts to produce non-conducting probes have [22], to date, significantly slowed the response kinetics of the probe (<1 ms for wild type vs 45 ms for the non-conducting Arch variant, ArchD95N).

ElectricPk demonstrates that the design principles that have evolved for the creation of better calcium probes can be applied to

voltage sensors as well. The present study reconfirms [23] that identifying a viable probe requires a systematic analysis of numerous different fusion location and FP types. While FRET-based sensors offer the theoretical advantage of ratiometric recordings, and should be exquisitely sensitive to small changes in the distance/orientation of the two fluorophores, recent history has shown that sensors designed around a single, circularly permuted FP can produce more robust signals. In this case, ElectricPk captures only the fast movement in the voltage sensing domain of CiVSP that has eluded other sensor designs. While remarkably fast, the signal generated by ElectricPk is relatively small. It is likely that continued rounds of evolution will improve the signal size, much as it has done for calcium sensors.

Materials and Methods

Molecular Biology

The region encoding CiVSP (R217Q) was amplified with primers complementary to the translation initiation and a portion of the sequence 3' of the predicted fourth transmembrane domain. Each reverse primer truncated the CiVSP coding sequence at a different amino acid and appended 15 bp (5 codons) of linker sequence. In turn, the cpEGFP portion of GCaMP3 was amplified with 9 different combinations of 6 primers to create the initial set of fusion components with 15 bp ends identical to the linker sequence at the 5' end and plasmid at the 3' end. In-Fusion cloning reactions (Clontech) were used to combine the amplified portions of CiVSP and the fluorescent protein in a CMV expression plasmid adapted for robotic cloning. This plasmid carries a ccdB negative selection cassette flanked by Asc I and Pme I sites such that double digestion removes the selection cassette. In all constructs, the phosphatase domain is not present. ElectricPk is available at Addgene or at the site www.fluorogenetic-voltage-sensors.org.

Cell culture

The HEK 293 cell line was maintained in the Dulbecco's Modified Eagle Medium (High Glucose) (DMEM) (Invitrogen, NY) supplemented with 8% fetal bovine serum (FBS) (Sigma-Aldrich, MO). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The

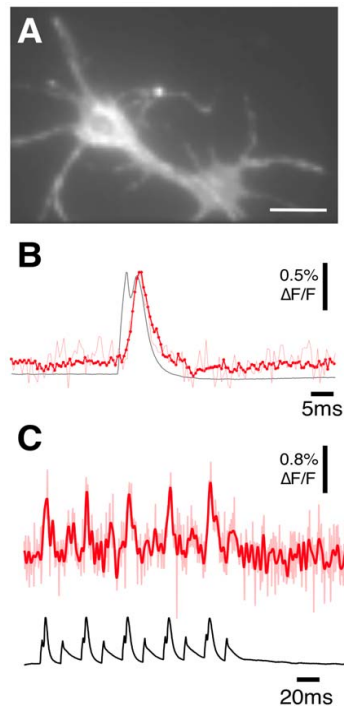


Figure 7. Detection of action potentials in hippocampal neurons in vitro using ElectricPK. A) Wide field image of an in vitro hippocampal neuron expressing ElectricPk. Bar = 15 μm. B) Single (light red trace) and averaged (red trace) optical response to action potentials evoked in the neuron seen in (A) taken using wide field microscopy and a RedShirtImaging NeuroCCD camera. The red trace is an average of 32 action potentials. All responses captured at 2000 fps. C) Fluorescence change (light red trace-unfiltered, red trace-filtered) to a single train of evoked action potentials recorded from an in vitro hippocampal neuron expressing ElectricPk. Lower black trace is the voltage recording made from the patch electrode. All fluorescence traces are bleach subtracted and where indicated, low pass filtered (Bessel) at 350 Hz.
doi:10.1371/journal.pone.0043454.g007

protocol was approved by the Pierce Animal Care and Use Committee. Animals were anesthetized with isoflurane prior to removal of embryos. Hippocampal neurons were isolated from E18 mouse embryos and maintained in culture medium containing Neurobasal medium (Invitrogen, NY), Glutamax-I 0.5 mM (Invitrogen, NY) and B-27 supplement (Invitrogen, NY). Cells were plated on No. 1 coverslips coated with poly-D-lysine (Sigma-Aldrich, MO) and kept in an incubator at 37°C with 5% CO₂. Transient transfection was accomplished using half of the manufacturer's recommended amount of DNA (2 μg per 35 mm dish) and Lipofectamine 2000 (5 μl) (Invitrogen, NY).

Electrophysiology

Recordings were performed in a perfused chamber with the bath temperature maintained at 35–37°C and a bath solution containing 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM D-glucose, and 5 mM HEPES pH 7.4. We used a 3–5 MΩ glass patch pipettes (capillary tubing with 1.5/0.75 mm

OD/ID) that were pulled on a P-97 Flaming/Brown type micropipette puller (Sutter Instrument Company, CA). Pipette solution contained 120 mM K-aspartate, 4 mM NaCl, 4 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA, 3 mM Na₂ATP and 5 mM HEPES, pH 7.2. Voltage-clamp recordings in the whole-cell configuration were performed using a Patch Clamp PC-505B amplifier (Warner Instruments, CT). A holding potential of −70 mV was kept at all cases.

For hippocampal neuron recordings, action potentials were initiated by 2 ms constant current injection in whole cell patch clamp mode. The pipette solution for neuron recordings contained (in mM): 120 K-gluconate, 3 KCl, 7 NaCl, 4 Mg²⁺-ATP, 0.3 Na-GTP, 20 HEPES and 14 Tris-phosphocreatin, pH adjusted with KOH to pH 7.3 [24].

Imaging

The whole cell patch-clamped cells were imaged using a Nikon Eclipse TE300 inverted microscope with a 60×1.40 N.A. oil immersion objective lens (Nikon, NY). For HEK 293 cells recordings, we used a 150W Xenon arc lamp (Opti Quip, NY) for episcopic fluorescent illumination using a GFP-3035B filter cube (Semrock Inc., NY) containing a 472/30 nm excitation filter, a 495 nm dichroic mirror and a 520/35 nm emission filter for cpEGFP probes and 445/20 nm excitation filter, a 458 nm dichroic mirror and a 510/84 nm emission filter for Mermaid. For fluorescence recordings in neurons we used 488 nm 50 mW laser illumination (DL488-050, CrystaLaser, NV) that was transmitted to the microscope by a multi-mode fiber coupler (Siskiyou, OR), a quartz light guide and an Achromatic EPI-Fluorescence Condenser (Till Photonics, NY). The fluorescence image was demagnified by an Optem® zoom system A45699 (Qioptiq LINOS, Inc, NY) and projected onto the 80×80 pixel chip of a NeuroCCD-SM camera (RedShirtImaging, LLC, GA) controlled by NeuroPlex software (RedShirtImaging, LLC, GA). The images were recorded at a frame rate of 2 kHz. The measured fluorescence was the average intensity across the entire cell.

Confocal images were obtained with a Olympus Fluoview FV1000 (Olympus, Japan) confocal laser scanning microscope using an Olympus LUMFL 60×/1.10 W objective. For chromophore excitation 488 nm wavelength Ar-laser was used. We used EGFP filter set with excitation filter 488BP (480–495 nm), beam splitter 505 nm and for emission filter BP515 (505–525 nm). For image acquisition and processing FV10-ASV confocal software was used.

Data Analysis

Data were analyzed with custom programs written in LabView (National Instruments Inc., TX) and Igor (Wavemetrics, OR). Bleaching was corrected by dividing the signal by a double exponential curve fitted to the portion of the trace that does not contain a voltage step. Data are presented as percent change in ΔF/F.

Supporting Information

Movie S1 The movie illustrates the optical response of an action potential (AP) in a pyramidal neuron from a hippocampal cell culture expressing the ElectricPK probe. The value of each pixel is the spike-triggered average value (over 43 action potentials) aligned to the peak of the action potential in the somatic whole cell recording. First, a low pass temporal filter (Gaussian at 200 Hz) is applied to the averaged data. Next, five iterations of spatial filtering in which each data pixel is averaged within a window of the 3×3 array of pixels

surrounding it is applied. The color code in each pixel is scaled so that blue is the most hyperpolarized membrane potential, red is the most depolarized membrane potential (e.g. AP peak) and yellow is the half-way point of the spike. All data processing (averaging, bleach correction, filtering) was done according to the procedure described in Popovic et al. [19]. (MOV)

Acknowledgments

We thank the technical support staff of The John B. Pierce Laboratory, Richard Rascati, John Buckley, Ron Goodman, Andrew Wilkens, Tom

D'Alessandro, Angelo DiRubba and Michael Fritz for development of instruments for these studies.

Author Contributions

Conceived and designed the experiments: LB JP VP TH. Performed the experiments: LB JP MP TH. Analyzed the data: LB JP MP VP TH. Contributed reagents/materials/analysis tools: LB JP VP TH. Wrote the paper: LB JP MP VP TH.

References

1. Siegel MS, Isacoff EY (1997) A genetically encoded optical probe of membrane voltage. *Neuron* 19: 735–741.
2. Sakai R, Repunte-Canonigo V, Raj CD, Knöpfel T (2001) Design and characterization of a DNA-encoded, voltage-sensitive fluorescent protein. *Eur J Neurosci* 13: 2314–2318.
3. Ataka K, Pieribone VA (2002) A genetically targetable fluorescent probe of channel gating with rapid kinetics. *Biophys J* 82: 509–516.
4. Tsutsui H, Karasawa S, Okamura Y, Miyawaki A (2008) Improving membrane voltage measurements using FRET with new fluorescent proteins. *Nat Methods* 5: 663–665.
5. Baker BJ, Mutoh H, Dimitrov D, Akemann W, Perron A, et al. (2008) Genetically encoded fluorescent sensors of membrane potential. *Brain Cell Biol* 36: 53–67.
6. Jin L, Han Z, Platisa J, Wooltorton JRA, Cohen LB et al. (2012) Single action potentials and subthreshold electrical events visualized in neurons using a novel fluorescent protein voltage sensor. *Neuron* In press.
7. Looger LL, Griesbeck O (2011) Genetically encoded neural activity indicators. *Curr Opin Neurobiol* 22:18–23.
8. Nakai J, Masamichi O, Kciji I (2001) A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nature Biotech* 19:137–141.
9. Nagai T, Sawano A, Park ES, Miyawaki A (2001) Circularly permuted green fluorescent proteins engineered to sense Ca^{2+} . *PNAS* 98:6: 3197–3202.
10. Baird GS, Zacharias DA, Tsien RY (1999) Circular permutation and receptor insertion within green fluorescent proteins. *PNAS* 96:11241–11246.
11. Topell S, Henneke J, Glockshuber R (1999) Circularly permuted variants of the green fluorescent protein. *FEBS Letters* 457: 283–289.
12. Akerboom J, Rivera JDV, Guilbe MMR, Malavé ECA, Hernandez HH et al. (2009). Crystal structures of the GCaMP calcium sensor reveal the mechanism of fluorescence signal change and aid rational design. *JBC* 284: 6455–6464.
13. Zhao Y, Araki S, Wu J, Teramoto T, Chang YF et al. (2011) An expanded palette of genetically encoded Ca^{2+} indicators. *Science* 333: 1888–1891.
14. Tian L, Hiers SA, Mao T, Huber D, Chiappe ME et al. (2009) Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat Methods* 6: 875–881.
15. Dombeck DA, Khabbazi AN, Collman F, Adelman TL, Tank DW (2007) Imaging large-scale neural activity with cellular resolution in awake, mobile mice. *Neuron* 56: 43–57.
16. Gautam SG, Perron A, Mutoh H, Knöpfel T (2009) Exploration of fluorescent protein voltage probes based on circularly permuted fluorescent proteins. *Front Neuroeng* 2: 14.
17. Lundby A, Akemann W, Knöpfel T (2010) Biophysical characterization of the fluorescent protein voltage probe VSFP2.3 based on the voltage-sensing domain of Ci-VSP. *Eur Biophys J* 39: 1625–1635.
18. Dimitrov D, He Y, Mutoh H, Baker BJ, Cohen LB, et al. (2007) Engineering and characterization of an enhanced fluorescent protein voltage sensor. *PLoS ONE* 5: e440.
19. Perron A, Mutoh H, Launey T, Knöpfel T (2009) Red-shifted voltage-sensitive fluorescent proteins. *Chem Biol* 16: 1268–1277.
20. Baker BJ, Jin L, Han Z, Cohen LB, Popovic M, et al. (2012) Genetically-encoded fluorescent voltage sensors using the voltage-sensing domain of *Nematostella* and *Danio* phosphatases exhibit fast kinetics. *J Neurosci Meth* 208(2): 190–196.
21. Villalba-Galea CA, Sandtner W, Dimitrov D, et al. (200) Charge movement of a voltage-sensitive fluorescent protein. *Biophysical J* 96:2:19–21.
22. Kralj JM, Douglass AD, Hochbaum DR, MacLaurin D, Cohen AE (2011) Optical recording of action potentials in mammalian neurons using a microbial rhodopsin. *Nat. Methods* 9: 90–95.
23. Jin L, Baker B, Mealer R, Cohen LB, Pieribone V, et al. (2011) Random insertion of split-cans of the fluorescent protein venus into Shaker channels yields voltage sensitive probes with improved membrane localization in mammalian cells. *J Neurosci Meth* 199:1:1–9.
24. Popovic MA, Foust AJ, McCormick DA, Zecevic D (2011) The spatio-temporal characteristics of action potential initiation in layer 5 pyramidal neurons: a voltage imaging study. *J Physiol (Lond)* 589: 4167–4187.

CHAPTER FIVE

TWO-PHOTON DIRECTED EVOLUTION OF GREEN FLUORESCENT PROTEINS

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

Author: Lauren M Barnett

Contributions: Mutagenesis design; performed all molecular biology, DNA sequence analysis, mutation identification and characterization, and purified protein handling; provided technical consultation on optical design regarding biological parameters; collected optical measurements; critical analysis of data; drafting and editing manuscript.

Author: Caleb Stoltzfus

Contributions: Optical design and construction; collection of optical measurements; critical analysis of data; drafting of manuscript figures; drafting and editing manuscript.

Co-Author: Mikhail Drobizhev

Contributions: Collection of optical measurements; critical analysis of data; editing manuscript.

Co-Author: Geoffrey Wicks

Contributions: Collection of optical measurements; critical analysis of data; editing manuscript.

Co-Author: Alexander Mikhaylov

Contributions: Collection of optical measurements; critical analysis of data; editing manuscript.

Co-Author: Thomas E Hughes

Contributions: Consultation on mutagenesis experimental design; consultation on optical design regarding biological parameters; critical analysis of data; editing manuscript; project supervision.

Co-Author: Aleksander Rebane

Contributions: Optical design and construction; drafting and editing manuscript; project supervision; corresponding author.

Manuscript Information Page

Caleb R. Stoltzfus¹, Lauren M. Barnett², Mikhail Drobizhev¹, Geoffrey Wicks¹,
Alexander Mikhaylov¹, Thomas E. Hughes² & Aleksander Rebane^{1,3}

¹Physics Department, Montana State University, Bozeman MT. 59717. ²Cell Biology
Neuroscience Department, Montana State University, Bozeman MT. 59717. ³National
Institute of Chemical Physics and Biophysics, Tallinn, Estonia, 12618.

Status of the manuscript:

☐ Prepared for submission to a peer-reviewed journal

☐ Officially submitted to a peer-reviewed journal

☐ Accepted by a peer-reviewed journal

☒ Published in a peer-reviewed journal

Published in Nature Scientific Reports, doi:10.1038/srep11968

SCIENTIFIC REPORTS

OPEN Two-photon directed evolution of green fluorescent proteins

Caleb R. Stoltzfus¹, Lauren M. Barnett², Mikhail Drobizhev¹, Geoffrey Wicks¹, Alexander Mikhaylov¹, Thomas E. Hughes² & Aleksander Rebane^{2,3}

Received: 08 March 2015

Accepted: 12 June 2015

Published: 06 July 2015

Directed evolution has been used extensively to improve the properties of a variety of fluorescent proteins (FPs). Evolutionary strategies, however, have not yet been used to improve the two-photon absorption (2PA) properties of a fluorescent protein, properties that are important for two-photon imaging in living tissues, including the brain. Here we demonstrate a technique for quantitatively screening the two-photon excited fluorescence (2PEF) efficiency and 2PA cross section of tens of thousands of mutant FPs expressed in *E. coli* colonies. We use this procedure to move EGFP through three rounds of two-photon directed evolution leading to new variants showing up to a 50% enhancement in peak 2PA cross section and brightness within the near-IR tissue transparency wavelength range.

EGFP is recognized as the fluorescent probe of choice in most demanding two-photon imaging experiments because of its superior photostability and high fluorescence quantum yield^{1–10}. However, the maximum 2PEF efficiency of EGFP, in particular its peak 2PA cross section of σ_{2PA} (900 nm) = 40 GM (1 GM = 10^{-50} cm⁴ s photon⁻¹)¹¹, lags behind the best values reported for some other types of FPs, notably the red FPs¹¹, leaving ample room for improvement. Here we present a method of improving σ_{2PA} of green FPs using two-photon directed evolution. Attempts to increase the 2PEF efficiency by introducing smart point mutations into the EGFP framework have been hampered by the complex and mostly unknown relationship between σ_{2PA} and the protein structure¹². Directed evolution offers an alternative route but requires fast, yet sufficiently accurate, screening of the two-photon properties of a large number of FP mutants^{13–16}. Until now, this has proven to be an exceedingly challenging task. Even though one-photon excited fluorescence (1PEF) and 2PEF share some fluorescence characteristics, such as the emission wavelength and the emission yield, the 2PEF brightness is proportional to the value of σ_{2PA} , which cannot be deduced from one-photon properties alone, making two-photon screening imperative. We address this issue by constructing a femtosecond 2PEF imaging setup that quantifies both the 1PEF and the 2PEF efficiency of tens of thousands of mutant FPs expressed in *E. coli* colonies. This allows us to pick mutants showing promising increases of σ_{2PA} , which is an important property of FPs used in two-photon imaging of living tissues^{9,15,17–20}.

The small σ_{2PA} values of FPs imply that large photon flux is needed to achieve a practical two-photon excitation rate. In a typical 2PEF microscope setup a minimum peak photon flux of $\sim 10^{28}$ photons cm⁻² s⁻¹ is obtained by using ~ 100 MHz repetition rate, ~ 100 fs duration femtosecond oscillator laser pulses focused to a nearly diffraction-limited spot, which is then raster-scanned over a sample area of ~ 1 mm² or less^{18,21–23}. However, two-photon directed evolution experiments would require measuring the 2PEF from a much larger sample area, typically a standard (9 cm diameter) Petri plate, which is hardly practical, especially considering a maximum reasonable time frame of ~ 1 h per plate (see Supplementary Information for detailed evaluation of the technical issues involved). To accomplish this large area 2PEF imaging we therefore use a 1 kHz pulse repetition rate, 150 fs pulse duration regenerative amplifier beam shaped into a 2×20 mm² stripe with an intensity of $\sim 10^{10}$ J cm⁻² s⁻¹ on the sample, as shown in Fig. 1. We can scan this stripe over an entire Petri plate area within about 20 min, while the fluorescence image

¹Physics Department, Montana State University, Bozeman MT. 59717. ²Cell Biology Neuroscience Department, Montana State University, Bozeman MT. 59717. ³National Institute of Chemical Physics and Biophysics, Tallinn, Estonia, 12618. Correspondence and requests for materials should be addressed to A.R. (email: rebane@physics.montana.edu)

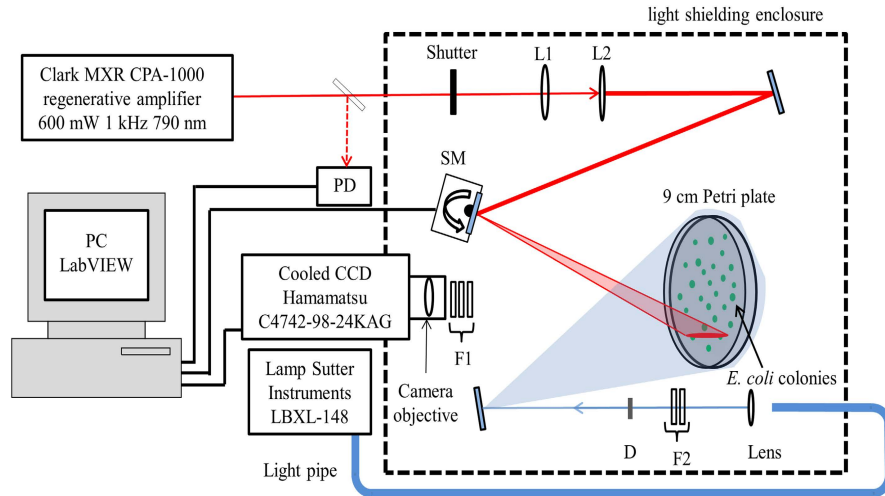


Figure 1. Schematic of the 2PEF and 1PEF imaging setup. L1, cylindrical lens; L2, spherical lens; PD, photo diode; SM, scanning mirror; F1, fluorescence detection filters; F2, one-photon excitation wavelength selection filters; D, diffuser.

of the entire plate is captured by a CCD camera (see Methods and Supplementary Information for details on laser setup and imaging methods).

In the 2PEF image, the *E. coli* colonies appear as a collection of distinct small bright areas or spots, where each spot corresponds to the fluorescence from a particular mutant FP (see Supplementary Fig. 1). The 2PEF intensity integrated over the spot area is proportional to the two-photon brightness of the corresponding FP (defined as the product of σ_{2PA} and the fluorescence yield, see Supplementary Information). The integrated fluorescence also scales linearly with the total number of the mature FP chromophores in each colony, where the last parameter varies broadly from one colony to another depending on many secondary factors such as the bacteria replication rate, FP production, folding efficiency, protein maturation rate etc.^{13,24}, and is thus notoriously difficult to determine. Fortunately, we can take advantage of the fact that the integrated 1PEF signal has the same linear dependence on the FP chromophore concentration as the 2PEF signal. By measuring both the 2PEF and 1PEF from each colony and by evaluating the ratio between the integrated 2PEF and 1PEF values, we can effectively minimize the uncertainty due to varying expression and maturation rates, which allows us to evaluate the relative two-photon brightness of FP mutants on a whole plate in a reasonable amount of time. Furthermore, by calibrating the fluorescence of the mutated FPs with respect to a reference sample containing only non-mutated EGFP colonies, we can quantitatively compare the two-photon efficiency of a whole library of mutants, usually expressed on tens of different plates and measured at different times, to the non-mutated parent FP. Bringing all the above together, the screening parameter that we use to identify the most promising mutants may be expressed as the ratio between the normalized integrated 2PEF and 1PEF signals of the n^{th} mutant given by:

$$\eta = \frac{F_{2PEF}^{norm}(n)}{F_{1PEF}^{norm}(n)} = \left(\frac{C_{2PEF}^{ref}}{C_{1PEF}^{ref}} \right) \frac{\sigma_{2PA,n}(\lambda_{2PA})}{\varepsilon_n(\lambda_{1PA})}, \quad (1)$$

where $\sigma_{2PA,n}(\lambda_{2PA})$ is the two-photon absorption cross section of the n^{th} mutant at the excitation wavelength, λ_{2PA} , $\varepsilon_n(\lambda_{1PA})$ is the molar extinction coefficient of the mutant at the one-photon excitation wavelength, λ_{1PA} , and C_{2PEF}^{ref} and C_{1PEF}^{ref} are, respectively, proportional to the average 2PEF and the average 1PEF signals of the reference sample (see Supplementary Information for details about the reference correction and this calculation). The quantity enclosed in the large brackets is constant for all samples in a particular library, which means that the screening parameter given by equation (1) turns out to be simply a constant times the ratio between the 2PA and 1PA cross section values at the respective excitation wavelengths.

The screening parameter given by equation (1) is not unique, in the sense that it depends on the choice of the excitation wavelengths λ_{2PA} and λ_{1PA} . In principle, the wavelengths can be selected to guide the evolution in different directions e.g. shifting and/or maximizing the peak two-photon wavelength. In our current experiment, however, we are restricted to $\lambda_{2PA} = 790$ nm by our laser system, which is ~ 100 nm below the 2PA peak of EGFP. We chose the one-photon wavelength to also be below the 1PA

peak, at $\lambda_{1PA} = 450$ nm, in order to maximize the effect that mutations can have on η , thus augmenting the efficacy of the screening procedure. This circumstance has consequences regarding the final evolution outcome, as will be described below.

Figure 2 summarizes the screening data from three consecutive cycles of evolution starting with EGFP, where the mutant libraries were obtained by a combination of error prone PCR and gene shuffling, and were expressed in *E. coli* colonies grown on ampicillin charcoal black agar Petri plates (see Supplementary Information). About 15,000 individual fluorescent colonies were screened in the three rounds of mutation, as summarized in Fig. 2. In the scatter graphs (left panel) each colony is plotted in the integrated and normalized coordinates of the 1PEF (horizontal axis) and 2PEF (vertical axis) signals. The red dots represent the mutagenized colonies, whereas the black dots correspond to the non-mutagenized reference colonies. The dashed diagonal line represents idealized non-mutagenized EGFP ($\eta = 1$). In the right panel the same data is arranged in the form of a histogram, which shows the frequency of a particular 2PEF/1PEF ratio, η , both for the mutants (red solid line) and the reference (black dashed line).

Because the mutation rate in the 1st round was expectedly low, approximately 1 mutation per mutant, there were only a few mutants that significantly deviated from the parent EGFP, with the majority of the red dots in Fig. 2a lining up close to $\eta = 1$. The corresponding histogram plot in Fig. 2b and Supplementary Fig. 2 shows that in about 99% of cases $\eta < 1.3$, i.e. the mutants were virtually indistinguishable from the parent EGFP. However, among the remaining population there were about 100 colonies (out of the ~7,500 colonies in the 1st library) that showed a potentially enhanced 2PEF/1PEF ratio, and thus lied above the $\eta = 1.3$ line (solid black line in Fig. 2a). 59 of these colonies were picked and subjected to further error prone PCR and gene shuffling that created the 2nd generation mutagenized library. Figure 2c shows that in the 2nd library more of the red dots are shifted above the dashed diagonal line indicating that the 2nd generation of mutants has a much larger population of colonies with useful mutations. The corresponding histogram (Fig. 2d and Supplementary Fig. 2) shows that ~42% of the colonies have $\eta > 1.3$.

The increase of η may follow either from an increase of the two-photon cross section at λ_{2PA} or from a decrease of the one-photon cross section at λ_{1PA} (or both). The fact that most of the 2nd round mutants in Fig. 2c show a substantial increase of η points in the direction of a shifting or changing 1PA spectrum, which is accompanying the change of σ_{2PA} . It is also interesting to note that on the scatter graph the mutants appear to congregate in distinct groups that have similar η . To verify this observation, we performed DNA sequencing of ~10 colonies picked from each of the ten groups (total of 96 colonies) and confirmed that each group indeed corresponds to a particular mutation.

Among the total 12 unique mutants found in the 2nd round, one labeled 2.18.01 (indicated by an arrow in Fig. 2c) featured the chromophore structure altering mutation T65S, and was thus excluded from further rounds of mutagenesis. The other 11 mutants preserved the EGFP chromophore structure, and were used as the DNA template for the 3rd and final round of error prone PCR and gene shuffling. The histogram plot in Fig. 2f and Supplementary Fig. 2 shows that about 93% of the mutagenized colonies had $\eta > 1.3$ in this final round. From those, 50 of the most promising colonies were picked and 11 new unique mutants were identified. The mutation type correlated well with the value of the screening parameter η .

Table 1 presents the peak 1PA and 2PA wavelengths and the peak 2PA cross sections of 23 unique mutants identified from the three evolution rounds. It is known that depending on the environment, GFP and related chromophores are observed in different ionization states^{2,10}. EGFP under physiological conditions is found predominantly in the anionic form with the 1PA peak at 488 nm, and only a small fraction resides in the neutral form with the 1PA peak at 395 nm. Many of the mutants showed enhanced 1PA at shorter wavelengths, which is most likely a result of our choice of λ_{2PA} and λ_{1PA} preferring the selection of the neutral form over the ionic form. Based on this, we classified the mutants into three categories: (1) analog of the anionic form of EGFP; (2) analog of the neutral form of EGFP and (3) variants that exhibit traits of both the anionic and neutral forms of EGFP. Representative mutant 2PA and 1PA spectra from each of the three categories along with the non-mutagenized EGFP are shown in Fig. 3 (see Supplementary Information and methods for measurement details). The 1PA shape and the peak extinction coefficient of mutant 3.18 (Fig. 3a) closely resemble those of EGFP (Fig. 3d), however the 2PA peak appears to be increased by about 50% to $\sigma_{2PA} = 61$ GM. The other 14 mutants from the same category showed similar features, with peak 2PA values in the range of $\sigma_{2PA} = 40$ –60 GM. In mutant 2.18.01 (Fig. 3b) the 1PA and 2PA peaks are almost entirely switched to shorter wavelengths. The 2PA spectrum closely resembles that of mAmetrine, which has a peak 2PA cross section of 56 GM at 809 nm¹¹. The DNA sequence reveals the mutation T65S which is a characteristic of FPs dominated by the neutral form^{10,25}. The spectra of mutant 3.06 (Fig. 3c) appear to be a superposition of the neutral and anionic form spectra, where the absolute cross sections of each of the forms remains hard to determine because of the unknown relative concentrations. Nevertheless, we may proceed with the comparison between different mutants if we introduce the effective 2PA cross section, $\sigma_{2PA,eff}$, and the effective 1PA extinction coefficient, ϵ_{eff} , defined respectively as:

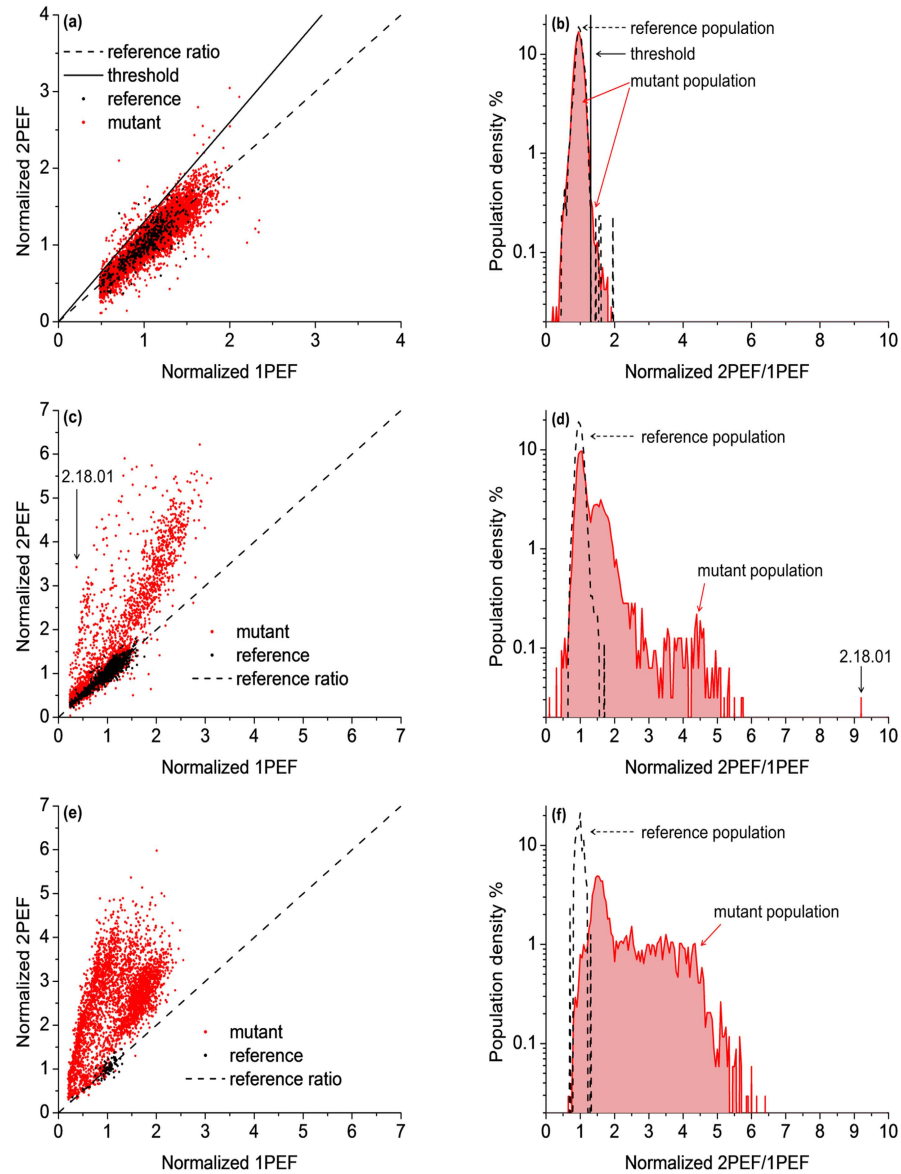


Figure 2. Fluorescence data of randomly mutagenized EGFP. Left panel: Normalized 2PEF signal plotted versus normalized 1PEF signal of the mutagenized (red symbols) and non-mutagenized (black symbols) colonies. Each point represents a single colony. The black dashed line corresponds to $\eta = 1$, the average slope of non-mutated EGFP colonies. Right panel: Histogram representation of the data shown in the left panel in terms of percentage of the colonies (vertical axis) with a particular ratio value η (horizontal axis). (a, b), The 1st generation library. The black solid line corresponds to $\eta = 1.3$. Mutants that appear above this line were used as the DNA template for the second library. (c, d), The 2nd generation library. Black arrows highlight the normalized integrated fluorescence and the normalized ratio of the colony expressing mutant 2.18.01. (e, f), The 3rd generation mutagenized library. The 1st 2nd and 3rd libraries contained 7,536, 3,192, and 3,423 colonies respectively. Colonies that could not be reliably identified, e.g. due to low fluorescence signal, spatial overlap between neighboring colonies, or close proximity to the Petri plate's outer rim, were eliminated from consideration (see Methods and Supplementary Information).

Group	FP	Mutations	Anionic form $\sigma_{2PA,an}$ (λ_{max} nm)	Neutral form $\frac{C_{ne}}{C_{an}}\sigma_{2PA,ne}$ GM (λ_{max} nm)	Anionic form 1PA λ_{max} nm	Anionic form Relative Brightness	$\Delta\mu(S_0 \rightarrow S_1)$ D
1	EGFP		42 (896)	11 (806)	488	1.0	3.5
1	3.18	V163A, Q184R	61 (936)	18 (806)	491	1.4	2.5
3	2.59.17	N121S, V163A	61 (910)	33 (788)	488	1.5	3.1
1	3.01	S72G	60 (932)	31 (806)	495	1.2	3.5
1	3.26	D117G, V163A	60 (928)	17 (798)	491	1.4	2.3
1	3.21	V68M, V163A, extra A.A. C-term	57 (932)	33 (798)	492	1.3	2.3
1	3.02	S72G, Q184R	56 (934)	27 (806)	495	1.1	3.4
1	2.59.14	V68M	54 (912)	31 (788)	490	1.3	3.0
1	2.59.38	V163A	54 (902)	22 (812)	491	1.2	3.1
1	2.18.13	D117G, V163A, S202N, V219I	53 (904)	28 (826)	492	1.2	3.0
1	3.30	S72G, D117G, V163A	52 (934)	26 (800)	498	1.0	2.5
1	2.18.08	V68M, V163A	51 (898)	31 (808)	492	1.2	2.9
1	2.18.15	V163A, S202N	46 (912)	17 (788)	491	1.0	2.7
1	2.59.12	V68M, N105S, V163A	44 (910)	31 (788)	492	0.90	2.7
1	2.18.12	E6G, S72G, V163A	42 (906)	32 (808)	498	0.80	2.9
1	2.18.19	S72G, V163A	40 (900)	27 (792)	498	0.76	2.9
3	3.06	D117G, V163A, T203I	33 (946)	47 (784)	507	0.88	3.4
3	3.12	Q80R, D117G, V163A, T203I	35 (942)	46 (806)	507	0.80	3.3
3	3.15	S72G, T203I	35 (940)	32 (782)	506	0.68	2.7
3	3.43	E6G, S72G, V163A, T203I	35 (920)	25 (798)	507	0.79	
3	3.04	V68M, V163A, T203I	27 (946)	59 (784)	507	0.68	2.8
3	2.59.01	V163A, T203I	23 (952)	48 (790)	508	0.51	2.6
3	2.59.08	T203I	19 (948)	48 (790)	507	0.44	2.4
2	2.18.01	T65S, S202N	8 (940)	61 (772)	476	0.39	

Table 1. Photophysical properties and DNA analysis data of the 23 selected mutants of EGFP. The first column on the left shows the correspondence to one of the 3 groups. In the second and third column is the name of the mutant and identity of the mutations from the DNA analysis. In the fourth and fifth columns are the peak 2PA cross sections of the anionic form and the effective peak 2PA cross section of the neutral form along with the corresponding wavelengths (in parentheses). In the sixth column is the peak 1PA wavelength of the anionic form. The last two columns on the right show the brightness of the peak 2PEF, defined as $\sigma_{2PA,an} \cdot \varphi_{rel}$ (see Supplementary Information), of the anionic form of the chromophore relative to EGFP and the permanent electric dipole moment change in the $S_0 \rightarrow S_1$ transition.

$$\sigma_{2PA,eff} = \frac{C_{ne}}{C_{an}} \sigma_{2PA,ne}(\lambda) + \sigma_{2PA,an}(\lambda) \quad (2)$$

$$\varepsilon_{eff} = \frac{C_{ne}}{C_{an}} \varepsilon_{ne}(\lambda) + \varepsilon_{an}(\lambda) \quad (3)$$

where C_{ne} and C_{an} are the relative concentration of the neutral and anionic forms, $\sigma_{2PA,ne}$ and $\sigma_{2PA,an}$ are the absolute two-photon cross sections of the two forms, and ε_{ne} and ε_{an} are the respective one-photon molar extinction coefficients. From our spectroscopic data we can calculate the effective cross sections if we assume that the one-photon extinction coefficient of the anionic form is equivalent to EGFP, $\varepsilon_{an} = 55,000 \text{ M}^{-1} \text{ cm}^{-1}$ (see Methods and Supplementary Fig. 3), and that, at 950 nm the $\sigma_{2PA,ne}$ is virtually zero, leaving only the anionic contribution to the two-photon cross section.

The mutation T203I is associated with intra-chromophore charge transfer due to breaking of a hydrogen bond with the β barrel^{6,10,25} and is likely responsible for the increase of the neutral form in all but one of the mutants from the 3rd category (3.06, 3.12, 3.15, 3.43, 3.04, 2.59.01, and 2.59.08). The V163A

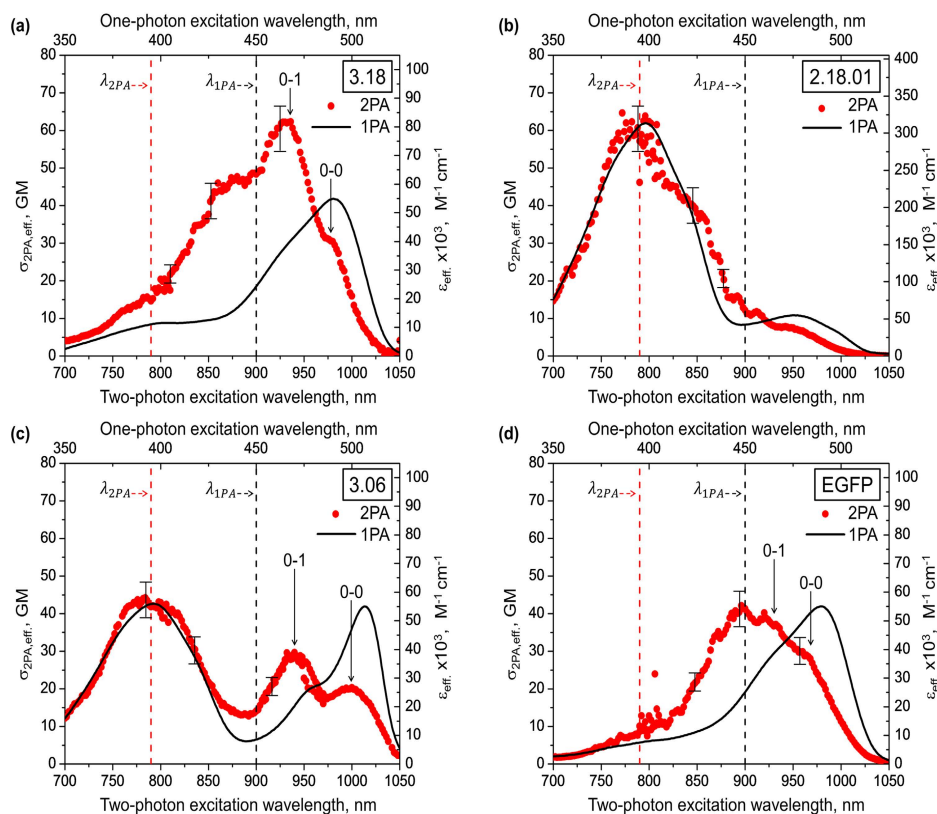


Figure 3. Absorption spectra of selected mutants of EGFP. Two-photon absorption cross section (red symbols) with 10% error (black error bars) and one-photon extinction (black solid lines) of the selected representative mutants and EGFP. Vertical dashed red and black lines represent λ_{2PA} and λ_{1PA} respectively. The vertical arrows indicate the peak wavelengths of the vibronic components. The 2PA spectra of all 23 mutants are presented in Supplementary Fig. 3. The numbers in the upper right hand corner of each plot designate different mutants.

mutation, previously reported to accelerate the protein folding²⁵, is present in 17 mutants including 2.59.38, where the V163A mutation led to a 30% increase in the peak σ_{2PA} and a 20% increase in the relative brightness of the anionic form of the chromophore.

Further comparative inspection of the σ_{2PA} spectra in Fig. 3 reveals that in the $S_0 \rightarrow S_1$ transition region 3.18 exhibits a distinct vibronic structure where the 0-1 peak prevails over the 0-0 component. This behavior has been previously observed in several FPs and chromophores and is most likely related to the Herzberg-Teller type coupling between the vibrational motion and the permanent dipole moment change ($\Delta\mu$) in the $S_0 \rightarrow S_1$ electronic transition of the chromophore²⁶. If the anionic chromophore possesses an active bond-length-alternating vibrational coordinate²⁷, then the mutations leading to 3.18 could be linked to an increased mixing between the resonance forms^{1,26}. These same mutations could also alter the local electric field inside the protein¹¹, which in turn may cause stronger vibronic coupling and therefore increase the peak 2PA value of the 0-1 transition. This tentative explanation is further supported by the observation that most mutants in the same category as mutant 3.18 had similar 2PA values for the purely electronic 0-0 transition, 30 ± 5 GM, while the value of the vibronic 0-1 transition varied from 40 GM to 61 GM (see Supplementary Fig. 3). Table 1 lists the $\Delta\mu$ values in the $S_0 \rightarrow S_1$ transition determined from the σ_{2PA} of the 0-0 component^{28,29} (see ref. 25 for calculation details).

In summary, we have developed a new high through-put *in situ* multi-photon excited fluorescence screening procedure that facilitates directed evolution of genetically encoded multi-photon probes. We put this procedure to the test by evolving new EGFP variants (V163A, Q184R and N121S, V163A) with 50% enhanced 2PA cross section and roughly 50% enhanced 2PEF brightness at a near-IR excitation

wavelength. Our method can be used to optimize the two-photon and higher-order multi-photon properties of many different FP types, especially if combined with an appropriately tunable wavelength excitation source. Developing a broad color palette of efficient multi-photon probes may become instrumental for imaging of the brain and other complex tissues that comprise many different cell types^{3,9,15,17,30–32}. By tuning both the λ_{2PA} and λ_{1PA} to the 0–0 component of the $S_0 \rightarrow S_1$ transition of the chromophore, we can potentially maximize the voltage sensitivity of FPs by increasing the change of the permanent electric dipole moment, $|\Delta\mu|$. Already at the current level of accuracy, this procedure enables a rather detailed quantitative comparison of mutant FPs not only from different Petri plates, but also across different libraries measured under different conditions. We expect that future experiments will facilitate the establishment of practical structure-property relationships between different mutation types and their corresponding multi-photon properties.

Methods

Additional methods are provided in the Supplementary Information.

Measurement of the 2PEF and 1PEF signals. For verification that the 2PEF image represents the true 2PEF of the sample the exponent of the measured fluorescent signal's power dependence was measured and found to be 2.0 ± 0.01^{33} . The Hamamatsu C474-98-24KAG cooled CCD camera and Petri plate were enclosed in a semi-light-tight box and the room lights were shut off to reduce background light. A stack of color filters with a center wavelength of 535 nm and 40 nm band pass were used in front of the CCD camera (F1 in Fig. 1) to select for the desired fluorescence emission wavelength and block any scattered excitation light. A combination of a band pass filter with a center wavelength of 450 nm and a band pass of 50 nm and a neutral density filter (F2 in Fig. 1) were used in front of the lamp source to select for the one-photon excitation wavelength and to adjust the excitation photon flux. A glass diffuser plate (D in Fig. 1) was used in front of the lamp source to provide spatially uniform intensity on the sample. A PC with Windows XP was used to control the camera, monitor the laser power, and control the Zaber T-OMG motorized x-y-axis optical mount through a custom program written in LabVIEW. In order to quantitatively compare the fluorescence images from different Petri plates in a library the illumination intensity each Petri plate receives should be relatively similar. To accomplishing this goal the laser and lamp were allowed sufficient time to reach an equilibrium temperature before each measurement was made. Before each library was scanned, the laser system was allowed to warm up for at least one hour. The camera was also given some time to reach its equilibrium CCD temperature of -30°C . While the laser was warming up, the first Petri plate was inserted into a custom built mounting plate that holds the Petri plate vertically with the *E. coli* colonies facing the camera. The LabVIEW program was initialized, and the Sutter Instruments LBXL-148 lamp source was turned on and allowed to warm up. While the lamp was reaching its optimal operating temperature, the first set of images was taken. The sample was illuminated by white light to capture a scattered light image showing the position of all of the bacterial colonies on the Petri plate including non-fluorescent colonies. Once the lamp source was at its optimal operating temperature, four images of the 1PEF were captured, averaged together in LabVIEW, and saved. For all images captured, both 1PEF and 2PEF, an 8 second exposure time was used. To measure the dark background, the lamp was shut off and four images with no illumination (both the lamp and laser were blocked) were averaged together and saved. These images were used to subtract any background signal from all of the fluorescence images. Finally, the shutter blocking the laser was opened and the 2PEF was collected. This was done by scanning the laser illumination across the Petri plate vertically (y axis in Supplementary Fig. 4b) once per image and stepping the horizontal (x axis in Supplementary Fig. 4b) position of the laser stripe after each image. In our measurement of mutagenized EGFP libraries a total of 60 horizontal steps and two laser passes (two images) per step were taken, resulting in 112 total images being used to calculate the final 2PEF image for each Petri plate. Measuring the 2PEF of one plate took approximately 20 minutes. Averaging of the images was done in LabVIEW such that the final output was a single image of the 2PEF. During the collection of the 2PEF image, the laser power as a function of position of the vertical illumination stripe on the Petri plate was recorded, using a Molectron P4-35 power detector, and saved. These steps were repeated for each Petri plate in the library being scanned. Collected data were imported into a MATLAB program that calculates the 2PEF and 1PEF signals of each colony and evaluates the 2PEF versus 1PEF ratio. Neither the camera nor the samples were disturbed while the 2PEF and 1PEF images were captured which ensured both final images overlapped exactly with each other facilitating quantitative comparison of the 1PEF and 2PEF fluorescence data. The estimated spatial resolution of the imaging setup is 7 lines per mm.

Measuring the two-photon absorption cross section. The 2PA spectra were measured using the fluorescence excitation method described in detail in³⁴. The 2PA spectra of all of the mutants selected from the three mutagenized libraries were measured with respect to the reference standard fluorescein in pH11 buffer solution. The quadratic power dependence of the 2PEF signal was checked at either 770 nm and 930 nm or 750 nm and 950 nm. The exponent of the power dependence measurement was 2.0 ± 0.05 . The one-photon absorption (1PA) spectra were measured using either a Perkin Elmer Lambda 950 or a Perkin Elmer Lambda 900 spectrometer. The quantum yields were measured by comparing the ratio of the fluorescence and optical density of the samples to that of fluorescein. The fluorescence

spectra were measured using a Perkin Elmer LS 50 B spectrofluorometer. The one-photon extinction coefficients were measured for a select number of mutants using the alkaline denaturation method³⁵. Extinction coefficients of the anionic form of the chromophore for all mutants measured were found to be $55 \pm 6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. A value of $55 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for the extinction coefficient of the anionic form of the chromophore was used for all calculations of the two-photon cross section.

References

- Bell, A. F., He, X., Wachter, R. M. & Tonge, P. J. Probing the ground state structure of the green fluorescent protein chromophore using Raman spectroscopy. *Biochemistry* **39**, 4423–4431 (2000).
- Chattoraj, M., King, B. A., Bublitz, G. U. & Boxer, S. G. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. *Proc Natl Acad Sci USA* **93**, 8362–8367 (1996).
- Chudakov, D. M., Matz, M. V., Lukyanov, S. & Lukyanov, K. A. Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol Rev* **90**, 1103–1163 (2010).
- Hanson, G. T. *et al.* Green fluorescent protein variants as ratiometric dual emission pH sensors. 1. Structural characterization and preliminary application. *Biochemistry* **41**, 15477–15488 (2002).
- Heim, R., Cubitt, A. B. & Tsien, R. Y. Improved green fluorescence. *Nature* **373**, 663–664 (1995).
- Heim, R., Prasher, D. C. & Tsien, R. Y. Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proc Natl Acad Sci USA* **91**, 12501–12504 (1994).
- Patterson, G. H., Knobel, S. M., Sharif, W. D., Kain, S. R. & Piston, D. W. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys J* **73**, 2782–2790 (1997).
- Peter, M. *et al.* Multiphoton-FLIM quantification of the EGFP-mRFP1 FRET pair for localization of membrane receptor-kinase interactions. *Biophys J* **88**, 1224–1237 (2005).
- Svoboda, K. & Yasuda, R. Principles of two-photon excitation microscopy and its applications to neuroscience. *Neuron* **50**, 823–839 (2006).
- Tsien, R. Y. The green fluorescent protein. *Annu Rev Biochem* **67**, 509–544 (1998).
- Drobizhev, M., Makarov, N. S., Tillo, S. E., Hughes, T. E. & Rebane, A. Two-photon absorption properties of fluorescent proteins. *Nat Methods* **8**, 393–399 (2011).
- Rajul, K. & Jain, R. R. Local complexity of amino acid interactions in a protein core. *Proc Natl Acad Sci USA* **101**, 111–116 (2004).
- Al, H., Baird, M. A., Shen, Y., Davidson, M. W. & Campbell, R. E. Engineering and characterizing monomeric fluorescent proteins for live-cell imaging applications. *Nat Protoc* **9**, 910–928 (2014).
- Akerboom, J. *et al.* Optimization of a GCaMP calcium indicator for neural activity imaging. *Journal of Neuroscience* **32**, 13819–13840 (2012).
- Broussard, G. J., Liang, R. & Tian, L. Monitoring activity in neural circuits with genetically encoded indicators. *Front. Mol. Neurosci.* **7**, 1–17 (2014).
- Davidson, M. W. & Campbell, R. E. Engineered fluorescent proteins: innovations and applications. *Nat Methods* **6**, 713–717 (2009).
- Bovetti, S., Moretti, C. & Fellin, T. Mapping brain circuit function *in vivo* using two-photon fluorescence microscopy. *Microsc Res Tech* **77**, 492–501 (2014).
- Hoover, E. E. & Squier, J. A. Advances in multiphoton microscopy technology. *Nat Photonics* **7**, 93–101 (2013).
- Liang, R., Broussard, G. J. & Tian, L. Imaging chemical neurotransmission with genetically encoded fluorescent sensors. *ACS Chem. Neurosci.* **6**, 84–93 (2015).
- Marblestone, A. H. *et al.* Physical principles for scalable neural recording. *Front Comput Neurosci* **7**, 1–34 (2013).
- Carriles, R. *et al.* Invited review article: Imaging techniques for harmonic and multiphoton absorption fluorescence microscopy. *J. A. Rev. Sci. Instrum.* **80**, 81101 (2009).
- Denk, W., Strickler, J. H., Webb, W. W. Two-photon laser scanning fluorescence microscopy. *Science* **248**, 73–76 (1990).
- Diaspro, A., Chirico, G. & Collini, M. Two-photon fluorescence excitation and related techniques in biological microscopy. *Q. Rev. Biophys.* **38**, 97–166 (2005).
- Elowitz, M. B., Levine, A. J., Siggia, E. D. & Swain, P. S. Stochastic gene expression in a single cell. *Science* **297**, 1183–1186 (2002).
- Cubitt, A. B., Woollenweber, L. A. & Heim, R. Understanding structure-function relationships in the Aequorea victoria green fluorescent protein. *Methods Cell Biol* **58**, 19–30 (1999).
- Drobizhev, M., Makarov, N., Tillo, S., Hughes, T. & Rebane, A. Describing two-photon absorptivity of fluorescent proteins with a new vibronic coupling mechanism. *J Phys Chem B* **116**, 1736–1744 (2012).
- Meyers, F., Marder, S., Pierce, B. & Bredas, J. Electric field modulated nonlinear optical properties of donor-acceptor polyenes: sum-over-states investigation of the relationship between molecular polarizabilities (α , β , and γ) and bond length alternation. *J. Am. Chem. Soc.* **116**, 10703–10714 (1994).
- Drobizhev, M., Tillo, S., Makarov, N., Hughes, T. & Rebane, A. Color hues in red fluorescent proteins are due to internal quadratic stark effect. *J. Phys. Chem. B* **113**, 12860–12864 (2009).
- Rebane, A. *et al.* Quantitative Prediction of Two-Photon Absorption Cross Section Based on Linear Spectroscopic Properties†. *J Phys Chem C* **112**, 7997–8004 (2008).
- Akemann, W., Mutoh, H., Perron, A., Rossier, J. & Knöpfel, T. Imaging brain electric signals with genetically targeted voltage-sensitive fluorescent proteins. *Nat Methods* **7**, 643–649 (2010).
- Akerboom, J. *et al.* Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. *Front Mol Neurosci* **6**, 1–29 (2013).
- Tillo, S. E., Hughes, T. E., Makarov, N. S., Rebane, A. & Drobizhev, M. A new approach to dual-color two-photon microscopy with fluorescent proteins. *BMC Biotechnol* **10**, 6 (2010).
- Stoltzfus, C. *et al.* A multidimensional screening method for the selection of two-photon enhanced fluorescent proteins. *Proc SPIE* **8956**, 895611–895611 (2014).
- Makarov, N. S., Drobizhev, M. & Rebane, A. Two-photon absorption standards in the 550–1600 nm excitation wavelength range. *Opt Express* **16**, 4029–4047 (2008).
- Ward, W. W. Biochemical and physical properties of green fluorescent protein. *Green fluorescent protein: properties, applications and protocols. 2nd ed.* Hoboken, USA: Wiley and Sons 39–65 (2005).

Acknowledgments

We would like to thank Nathan Shaner for the generous gift of pCP. This work was supported by NIH grant R01GM098083 and NIH/NINDS 1 R01 NS083875-01.

Author Contributions

Optical design was performed by C.R.S. and A.R. Software was written by C.R.S. and G.W. measurements were performed by C.R.S., L.B., G.W., M.D. and A.M. Biological experimental design was performed by L.B. and T.H. All molecular biology was performed by L.B. This manuscript was written by C.R.S. and A.R. with comments from all authors. A.R. and T.H. supervised the project.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Stoltzfus, C. R. *et al.* Two-photon directed evolution of green fluorescent proteins. *Sci. Rep.* 5, 11968; doi: 10.1038/srep11968 (2015).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

Two-photon Directed evolution of green fluorescent proteins

Caleb R. Stoltzfus^a, Lauren M. Barnett^b, Mikhail Drobizhev^a, Geoffrey Wicks^a, Alexander Mikhaylov^a, Thomas E. Hughes^b,
Aleksander Rebane^{a,c,*}

^aPhysics Department, Montana State University, Bozeman MT. 59717; ^bCell Biology Neuroscience Department, Montana State University, Bozeman MT. 59717, ^cNational Institute of Chemical Physics and Biophysics, Tallinn, Estonia, 12618,

*Corresponding author

Supplementary Information

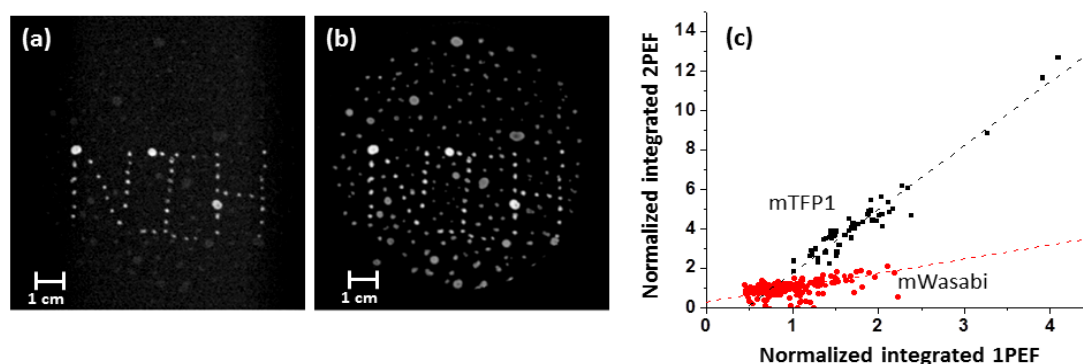
Analysis of the 2PEF and 1PEF images.

Once the data were collected for each library, the digital images of the 1PEF, 2PEF, and background were imported into a custom program written in MATLAB. The program first subtracts the background image for each Petri plate from the corresponding fluorescence images to account for scattered ambient light and noise in the camera. The 1PEF image is corrected for variations in the distribution of the lamp source illumination intensity and the 2PEF image is corrected for changes in laser power throughout the scan. The lamp source illumination variation was measured by recording the average 1PEF from a piece of uniformly fluorescent paper placed in a Petri plate and held in the Petri plate holder in the imaging setup. The paper should be placed on top of a piece of cardboard to assure that it is at the same height in the Petri plate as the *E. coli* colonies in a typical library. This average 1PEF as a function of position in a Petri plate was normalized to its peak value and any pixels outside the Petri plate in the resulting averaged 1PEF image were set to 1 such that nothing was divided by 0. The 1PEF images of the Petri plates containing the mutagenized FP expressing *E. coli* colonies can then be divided by this normalized image. Our correction factor ranged from 1 at the center of the 1PEF normalization image to 0.2 at the edge of the Petri plate. For the three libraries in the validation study, no corrections to the 2PEF images due to laser power variations were necessary. If the laser power does change over the course of the measurement of the 2PEF image this can be accounted for by multiplying each column of the image (if the laser stripe is scanned vertically as it was here) by a correction factor based on the normalized laser power recorded while that column of the image was being illuminated. When corrections must be done to the 2PEF image it is important to take into account the fact that the 2PEF depends on the square of the laser power. These corrections are necessary to assure accurate comparison of the signals from different *E. coli* colonies in different positions on a given Petri plate. Unfortunately, agar at the edge of most Petri plates climbs up the side of the plate resulting in odd artifacts in the edge of the fluorescence images. To deal

with this problem, any pixels within 2 mm of the edge of the Petri plate were set to zero. The total fluorescence of each colony was found by identifying the brightest pixel in the IPEF image, recording the position of that pixel, saving the sum of the pixel values for an area around the selected pixel in both the IPEF and 2PEF images, and then setting that area to zero in the original image such that the next brightest pixel, representing the next brightest colony's fluorescence signal, could be found. The size of the area that is summed depends on how large the colonies were allowed to grow and the camera setup used. Typically this is set once for each library to be about the size of the largest colony such that no fluorescence is missed. For the colonies we used this was typically an 11 pixel by 11 pixel area. This process was repeated until all of the fluorescing colonies were identified and their corresponding IPEF and 2PEF signals recorded. The total IPEF and 2PEF signals were all normalized relative to the corresponding average IPEF or 2PEF fluorescent signal of the colonies from the reference scan.

Demonstration of the variable absolute brightness of FP expressing *E. coli* colonies.

Supplementary Fig. 1 shows the 2PEF (a) and IPEF (b) images of a sample containing two kinds of *E. coli* colonies growing in particular patterns. The first type expressed the mTFP1 protein ("NIH" letter pattern in the center of the plate) and the second expressed the mWasabi protein (colonies distributed randomly across the plate). The individual colonies appear as bright spots on dark background. mTFP1 and mWasabi are known to have notably different 2PA properties at the excitation wavelength 790 nm: the 2PA cross section of mTFP1 is, $\sigma_{2PA}(790 \text{ nm}) = 10 \text{ GM}$, while that of mWasabi is, $\sigma_{2PA}(790 \text{ nm}) = 1 \text{ GM}$. Due to this difference, the mTFP1 colonies appear, in the 2PEF image (in Supplementary Fig. 1a) on average as brighter spots than the corresponding mWasabi colonies. At the same time, the 1PA cross section and the IPEF brightness of the two types of FPs is comparable. Accordingly, in Supplementary Fig. 1b both types show similar fluorescence signals.



Supplementary Figure 1 | Fluorescence images of a Petri plate with intermixed *E. coli* colonies. Colonies are expressing mTFP1 ("NIH" pattern) and mWasabi (randomly distributed); (a) 2PEF image; (b) IPEF image. (c) Normalized integrated 2PEF signal (vertical axis) from each of the mTFP1 (black symbols) and mWasabi (red symbols) colonies plotted versus the corresponding normalized integrated IPEF signal (horizontal axis). Each point on the graph represents an individual colony of *E. coli* bacteria expressing either one or the other FP type. Dashed lines are the linear fits to the corresponding data points.

A careful inspection of Supplementary Fig. 1a and 1b reveals a fairly large variation of the colony size as well as 2PEF brightness, some of the colonies emitting several times more overall fluorescence than other colonies expressing the same type of FP. One can imagine that if we would need to differentiate between thousands of unknown mutants, where the mutations can further affect these factors, these variations would make any quantitative comparison and selection of the best two-photon mutants highly problematic. Fortunately, the ratio between the 2PEF and 1PEF signals from the same type of FP is more or less constant and appears to be unaffected by these factors. Supplementary Fig. 1c shows the correlation plot between the 2PEF signal (vertical axis) and 1PEF signal (horizontal axis) from each individual colony from Supplementary Fig. 1a and 1b. Every point on the plot represents the normalized fluorescence intensity integrated over the whole area of the corresponding colony. The data shows that nearly all colonies fall into two distinct groups according to their type and that each group approaches a distinct linear fit line. The slope of this line can serve as a practical quantitative measure of the 2PEF efficiency that is both independent of most growth-related factors, as well as reproducible from one plate to another.

Preparation of non-mutated mixed FP plates pictured in Supplementary Fig. 1.

For the purpose of demonstrating the differences between the 1PEF and 2PEF of different known FPs, we prepared the plates shown in Supplementary Fig. 1. The encoding regions of mWasabi and TFP1.0 were first cloned into the same plasmid used for constitutive bacterial expression in our EGFP mutant screening (pCP, from Nathan Shaner) using the same ligation-independent cloning protocol used for EGFP. *E. coli* expressing mWasabi and TFP1.0 were then manually spotted on the black LB agar plates using a thin wire into a pattern constituting either the background or the “NIH” pattern, respectively. The plates used were the same 9cm Petri plates containing black LB Agar with Ampicillin as used for the mutant EGFP libraries. This method of manually spotting the *E. coli* colonies on the plate places more than one bacterium at each position leading to greater variation in final colony size after incubation than does plating *E. coli* using glass beads, as we did with our mutant libraries. The spotted *E. coli* plates were allowed to incubate overnight at 37°C and then scanned the next day using the same procedures used for scanning the mutated libraries.

Once the 2PEF and 1PEF images were captured and averaged in LabVIEW they were imported into a custom MATLAB program. This program first cropped the 1PEF and 2PEF images to include only the Petri plate. The images were then converted to matrices of pixel values. The 1PEF and 2PEF matrices were normalized to their maximum values. A threshold was used on both matrices such that small background pixels were set to a value of 1. This ensured that nothing was divided by 0. The 1PEF threshold was set to 0.17 and the 2PEF threshold was set to 0.09. The 2PEF matrix was divided by the 1PEF matrix. A threshold was imposed on the final matrix of ratios of $0.2 < \text{matrix} < 1$. All pixel values outside this range were set to 0. The 1PEF and 2PEF background pixels that were initially set to 1 in the threshold were set to 0. Finally the matrices were converted back to images and saved.

Random mutagenesis of EGFP.

All error-prone PCR was performed using Taq DNA Polymerase (New England BioLabs) in the presence of 0.04 μ M MnCl₂. All primers used to amplify EGFP and the mutant variants were complementary to the translation initiation and the 3' end of the EGFP sequence and included 15 base pair joints of sequence complementary to the cloning plasmid on the 5' and 3' ends for ligation-independent cloning (In-Fusion® HD, Clontech). The gene shuffling used in the PCR reactions of the second and third round of mutagenesis consisted of an initial series of brief elongation steps (6 rounds total) of increasing time (from 10sec to 60sec) before the start of 30 rounds of traditional PCR. All DNA sequencing was performed by GenScript (<http://www.genscript.com>).

For the first round of mutagenesis, the encoding region of EGFP was amplified using error-prone PCR. From the first round screening results, 100 colonies were selected above an arbitrary value of 2PEF/1PEF ratio.

For the second round of mutation, two reactions were used to determine the ideal DNA template for amplification. The template pool for the first reaction (named m2.59) included all 59 of the 100 colonies selected from the second screening of the first round library that reproducibly showed a larger 2PEF/1PEF ratio compared to the parent EGFP. The m2.59 reaction used only error-prone PCR. The template for the second, more exclusive reaction (m2.18) was the top 18 of the 59 reproducibly different 2PEF/1PEF ratio colonies from the second screening of the first round library. The m2.18 reaction used error-prone PCR and gene shuffling. Both libraries produced similar results, therefore 96 colonies with improved 2PEF/1PEF ratios were selected from both m2.59 and m2.18 libraries and sent off for DNA sequencing.

The 11 unique mutant GFP variants identified by DNA sequencing moved on as DNA template for the third and final round of error-prone PCR and gene shuffling.

Cloning and Library preparation.

In-Fusion cloning reactions (Clontech) were used to insert the randomly mutated FP coding region from the aforementioned PCR reactions into our cloning plasmid and then transform *E.coli* (dh5- α High Efficiency chemically competent cells, New England BioLabs). The cloning plasmid was selected for constitutive expression of his-tag proteins in bacteria (pCP, generous gift from Nathan Shaner). The transformation reactions were then diluted with SOC media and plated with glass beads on multiple (10-14) Petri plates (9cm) with black LB agar and Ampicillin. Black LB agar plates were made by adding Rublev Lamp Black pigment (a natural carbon pigment for artists, www.naturalpigments.com) to the LB agar. The Lamp Black agar plates significantly reduced laser scatter and background fluorescence from the LB agar. Plates were screened within 2 days of transforming *E. coli*. For each round, one plate of non-mutated parent EGFP was plated and screened under identical experimental conditions for reference. The parent EGFP reference corrected for day-to-day fluctuations in equilibrium laser power, changes in camera settings, and other variations in experimental conditions. By using a reference sample, the need to fully

characterize all of the detection and laser parameters of the system each day was avoided, and different libraries scanned under different conditions could be quantitatively compared.

Selection and acquisition of mutated FPs.

In all three rounds of EGFP mutation the following procedures were used to pick interesting mutants. Once the fluorescence signal was cataloged for each colony, the selection criterion to be used to screen the mutated FPs was defined. This was done using a custom MATLAB program where the user can select mutants by either setting a threshold (1.3 for the first library) for the ratio of the 2PEF and 1PEF signals or by manually selecting mutants (picked out of the apparent groups of mutants for the second and third libraries) from the correlation plot of the 2PEF versus 1PEF signals. As long as the Petri plates containing the library being analyzed are properly stored, selection of interesting mutants can be done at any time after the fluorescence signals have been recorded for as long as the *E. coli* are alive. Once the desired selection parameters are defined, the MATLAB program used generates an image showing which Petri plate each particular mutant fitting the selection criteria is from and where on that Petri plate that mutant is located. This image is used to pick the mutated colonies of interest from their respective Petri plates, label them, and keep them for use in later libraries and for further characterization. Colonies of interest were picked individually with a sterile loop and used to inoculate an overnight culture of LB broth with Ampicillin. These overnight cultures were then used to extract the plasmid DNA for each mutant using PureLink Quick Plasmid Miniprep Kit (Invitrogen).

FP purification.

E. coli colonies expressing fluorescent protein (in pCP, Nathan Shaner) were picked and grown at 34°C for 48 hours in 125mL of Circlegrow (MP Biomedicals) and Ampicillin. The expressing *E. coli* culture was pelleted and excess Circlegrow broth was removed. *E. coli* pellets were lysed using BugBuster (Novagen) and Benzonase (Novagen). Cleared lysates were then purified using Protino Ni-TED 2000 packed columns (Macherey-Nagel). Purified fluorescent proteins were eluted in 1x Protino Ni-TED stock pH8 buffer solution.

Calculation of the relative brightness.

The relative brightness was found using the following two equations:

$$\varphi_{rel.} = \frac{OD_{sample}(480nm)}{Fl_{sample}(512nm)} * \frac{Fl_{fluorescein}(512nm)}{OD_{fluorescein}(480nm)}$$

$$Brightness = \frac{\sigma_{2PA,an,sample} \varphi_{rel.,sample}}{\sigma_{2PA,an,EGFP} \varphi_{rel.,EGFP}}$$

where $\varphi_{rel.}$ is the quantum yield of the sample relative to fluorescein, OD is the optical density of the sample measured using a Perkin Elmer Lambda 950 or a Perkin Elmer Lambda 900 spectrometer, FI is the fluorescence of the sample measured using a

Perkin Elmer LS 50 B spectrofluorometer, and $\sigma_{2PA,an}$ is the two photon cross section of the anionic form of the sample as reported in Table 1.

Derivation of the 2PEF and 1PEF signals.

A diagram of the image plane of our wide field 2PEF imaging system is shown in Supplementary Fig. 4b. The y axis represents the vertical spatial direction, the x axis represents the horizontal direction, the Petri plate is represented by the gray circle, the FP expressing *E. coli* colonies are represented by the green circles, and the illuminating laser stripe is shown by the red circles. The 2PEF is integrated on the camera while scanning the laser illumination in the y direction from 0 to d_v . After one vertical pass of the laser stripe the illumination is stepped to the right and another image is captured while the laser is again scanned vertically. Images are captured at each horizontal position of the vertically scanning laser stripe until all of the colonies on the Petri plate have been illuminated. In the following derivation lower indices 1, and 2 indicate one-photon and two-photon excitation respectively. Assuming that the excitation rate is always much below the saturation limit and using the two-level approximation, the number of 2PEF photons detected by the camera, from a single Petri plate containing fluorescent colonies, integrated over the wavelength interval $\lambda_{min} - \lambda_{max}$, defined by the bandwidth of the filters in front of the camera, is given by:

$$F_{2PEF} = N_2 \int_{\lambda_{min}}^{\lambda_{max}} C_{FL}(\lambda') \phi(\lambda', x, y) d\lambda'$$

Where $C_{FL}(\lambda)$ is the differential detection efficiency (including parameters such as the efficiency of collecting fluorescence, and efficiency of the CCD camera), N_2 is the total number of molecules excited, and $\phi(\lambda, x, y)$ is the differential quantum efficiency of fluorescence as a function of position (x,y) on the Petri plate. The differential quantum efficiency of fluorescence is defined in terms of the standard quantum yield, Q, by the following equation.

$$\int_0^{\infty} \phi(\lambda) d\lambda = Q$$

The standard quantum yield is equal to the ratio of the radiative decay rate to the total decay rate of the S_1 state. Assuming that the scanning laser stripe is essentially stationary on the timescale of the femtosecond pulse, the number of molecules excited per laser pulse is given by:

$$N_2 = \frac{1}{2(h\nu_2)^2} \int \int \int \sigma_{2PA}(x, y, \lambda_{2PA}) c(x, y) I(x, y) I_{laser}^2(x, y, t) dx dy dt$$

Where h is Planck's constant, ν_2 is the laser frequency, $\sigma_{2PA}(x, y, \lambda_{2PA})$ is the two-photon cross section as a function of position on the Petri plate being illuminated with excitation wavelength, λ_{2PA} , $c(x, y)$ is the concentration of FP molecules present as a

function of position on the Petri plate, $l(x,y)$ is the thickness of any bacterial colonies present as a function of position on the plate, and I_{laser} is the intensity of the laser pulse.

With a pulse repetition rate of g , a vertical scan distance of d_v , a camera exposure time of τ_{2PEF} , and treating the pulses as delta functions in time on the time scale of the vertical scan rate, d_v/τ_{2PEF} , we find that the illuminating laser stripe moves $d_v/g\tau_{2PEF}$ between each pulse. The number of molecules excited in the n^{th} fluorescent colony on the Petri plate, during the k^{th} vertical scan of the laser is therefore given by the following summation over all pulses in that scan:

$$N_{2,n,k} = \sum_{i=0}^{g\tau_{2PEF}} \frac{\sigma_{2PA,n}(\lambda_{2PA})c_n}{2(h\nu_2)^2} \iiint_{-\infty}^{\infty} l(x,y) I_{laser}^2\left(x, y - \frac{d_v}{g\tau_{2PEF}}i, t\right) dx dy dt$$

Now that we have the number of molecules excited in the n^{th} colony per vertical scan of the laser we can write down the total number of molecules excited in that colony after a full scan of the Petri plate, $N_{2,n}$.

$$N_{2,n} = \sum_{k=0}^{S_h} \sum_{i=0}^{g\tau_{2PEF}} \frac{n_v \sigma_{2PA,n}(\lambda_{2PA})c_n}{2(h\nu_2)^2} \iiint_{-\infty}^{\infty} l(x,y) I_{laser}^2\left(x - \frac{d_h}{S_h}k, y - \frac{d_v}{g\tau_{2PEF}}i, t\right) dx dy dt \quad (1)$$

Here S_h is defined as the number of horizontal steps of the scanning laser stripe taken, n_v is the number of vertical passes of the laser stripe per horizontal step, and d_h/S_h is the size of the horizontal steps taken. These parameters are defined by the LabVIEW program controlling the scanning mirror mount. Assuming the temporal and spatial profiles of the laser stripe are well described by Gaussians we can write the spatial distribution of the laser intensity as:

$$I_{laser} = I_{laser}^{(0)} e^{-4\ln(2)\frac{t^2}{\tau^2}} e^{-4\ln(2)\frac{y^2}{\Delta y^2}} e^{-4\ln(2)\frac{x^2}{\Delta x^2}} \quad (2)$$

Where $I_{laser}^{(0)}$ is the peak laser intensity ($\text{mJ cm}^{-2} \text{s}^{-1}$), τ is the temporal pulse width (FWHM), Δy is the vertical spatial beam width (FWHM), and Δx is the horizontal spatial beam width (FWHM). Assuming that each colony of bacteria is spherically symmetric, with a diameter of Δr_n (FWHM), a maximum height of $l_{0,n}$, position on the Petri plate of (x_0, y_0) , and can be described by Gaussian functions, we can define the thickness of the n^{th} colony as:

$$l(x,y) = l_0 e^{-4\ln(2)\frac{(x-x_0)^2 + (y-y_0)^2}{\Delta r^2}} \quad (3)$$

Using this definition of the thickness of the n^{th} colony, we can find the volume of that colony, V_n , using:

$$V_n = \iint_{-\infty}^{\infty} l(x, y) dx dy = \iint_{-\infty}^{\infty} l_0 e^{-4 \ln(2) \frac{(x-x_0)^2 + (y-y_0)^2}{\Delta r^2}} dx dy = \frac{l_{0,n} \Delta r_n^2 \pi}{\ln(16)} \quad (4)$$

Using equations (2), (3), and (4) we can solve the integral in equation (1) and write the number of molecules excited as:

$$N_{2,n} = \sigma_{2PA,n} (\lambda_{2PA}) c_n \tau_{2PEF} V_n (I_{laser}^{(0)})^2 \sum_{k=0}^{S_h} \sum_{i=0}^{g \tau_{2PEF}} \frac{\tau n_v}{\tau_{2PEF} 2 (h\nu_2)^2} \sqrt{\frac{\pi}{2 \ln(16)}} e^{-8 \ln(2) \left(\frac{\left(\frac{k d_h - x_0}{S_h} \right)^2}{2 \Delta r_n^2 + \Delta x^2} + \frac{\left(\frac{i d_v - y_0}{g \tau_{2PEF}} \right)^2}{2 \Delta r_n^2 + \Delta y^2} \right)} \frac{1}{\sqrt{\left(1 + \frac{2 \Delta r_n^2}{\Delta x^2}\right) \left(1 + \frac{2 \Delta r_n^2}{\Delta y^2}\right)}}$$

If we define the function $\kappa_{scan}(\Delta r_n, \Delta x, \Delta y, S_h, \tau, g, \tau_{2PEF}, n_v, d_h, d_v, x_0, y_0)$ as the summation that takes into account all of the laser parameters and the stripe scanning parameters defined by the user in the LabVIEW program controlling the scanning mirror mount, we can write the equation for the 2PEF of the n^{th} colony of bacteria on the Petri plate as:

$$F_{2PEF}(n) = \sigma_{2PA,n} (\lambda_{2PA}) c_n V_n (I_{laser}^{(0)})^2 \kappa_{scan} \int_{\lambda_{min}}^{\lambda_{max}} C_{FL}(\lambda') \varphi_n(\lambda') d\lambda'$$

For the sake of simplicity we will assume that the differential detection efficiency and differential quantum yield is approximately constant over the bandwidth of the detection wavelengths. If we define λ_{cw} to be the center wavelength of the detection filters in front of the camera and $\Delta\lambda$ to be the bandwidth of the detection filters, we can write:

$$\int_{\lambda_{min}}^{\lambda_{max}} C_{FL}(\lambda') \varphi_n(\lambda') d\lambda' \approx C_{FL}(\lambda_{cw}) \varphi_n(\lambda_{cw}) \Delta\lambda \quad (5)$$

$$F_{2PEF}(n) = C_{FL}(\lambda_{cw}) V_n c_n \sigma_{2PA,n} (\lambda_{2PA}) \varphi_n(\lambda_{cw}) \Delta\lambda \kappa_{scan} (I_{laser}^{(0)})^2$$

We note that the parameter C_{FL} that takes into account all of the detection efficiency parameters and the parameter κ_{scan} that takes into account the laser parameters, are both constant from colony to colony on a given Petri plate. We can define $\varphi_n = \varphi_n(\lambda_{cw}) \Delta\lambda$ which allows us to express the 2PEF as:

$$F_{2PEF}(n) = C_{FL} V_n c_n \sigma_{2,n} (\lambda_{2PA}) \varphi_n \kappa_{scan} (I_{laser}^{(0)})^2$$

In a similar fashion we can find the 1PEF. We will start from the equation for the 1PEF of the entire Petri plate, given by:

$$F_{1PEF} = N_I \tau_{1PEF} \int_{\lambda_{min}}^{\lambda_{max}} C_{FL}(\lambda') \varphi(\lambda', x, y) d\lambda'$$

Where N_I is the number of molecules excited per unit time and τ_{1PEF} is the exposure time of the camera. Note that $\eta(\lambda)$, and $\varphi(\lambda, x, y)$, will be the same functions as in the 2PEF case. The number of molecules excited per unit time by one-photon absorption is given by:

$$N_I = \iint_{-\infty}^{\infty} 3.82 \times 10^{-21} \varepsilon(x, y, \lambda_{1PA}) c(x, y) l(x, y) \frac{I_{lamp}}{h\nu_1}(x, y) dx dy$$

Where $\varepsilon(x, y, \lambda_{1PA})$ is the one-photon extinction coefficient as a function of position on the Petri plate and one-photon excitation wavelength λ_{1PA} . The one-photon extinction coefficient is related to the one-photon cross section by: $\varepsilon = 3.82 \times 10^{-21} \sigma_{1PA}$. $c(x, y)$ and $l(x, y)$ represent the same concentration of FPs and thickness of the colony of bacteria as before, I_{lamp} represents the intensity of the lamp source, and ν_1 is the lamp frequency. Under lamp illumination the illumination light is fixed with respect to the Petri plate and can be considered constant over the area of a single colony. Using the function for the thickness of the n^{th} colony we defined in equation (3), we can write down the number of molecules excited per unit time, N_I .

$$N_I = 3.82 \times 10^{-21} \varepsilon_n(\lambda_{1PA}) c_n \frac{I_{lamp}}{h\nu_1} \iint_0^\infty l_0 e^{-4 \ln(2) \frac{(x-x_0)^2 + (y-y_0)^2}{\Delta x^2}} dx dy \quad (6)$$

By substituting equations (4) and (5) into Equation (6) we find that the 1PEF for the n^{th} colony is given by:

$$F_{1PEF}(n) = 3.82 \times 10^{-21} \tau_{1PEF} C_{FL}(\lambda_{cw}) V_n c_n \varepsilon_n(\lambda_{1PA}) \Delta \lambda \varphi_n(\lambda_{cw}) \frac{I_{lamp}}{h\nu_1}$$

We can absorb some parameters into the constant C_{FL} that now takes into account all of the camera settings, multiplicative factors and detection efficiency parameters that are constant from colony to colony on a given Petri plate to write:

$$F_{1PEF}(n) = C_{FL} V_n c_n \varepsilon_n(\lambda_{1PA}) \varphi_n I_{lamp} \tau_{1PEF}$$

In our experiment we take special care that the excitation and the fluorescence detection conditions remain constant during both 1PEF and 2PEF signal collection. This allows us to make good use of a reference sample, consisting of a Petri plate

with *E. coli* colonies expressing a non-mutated FP such as EGFP. A fresh reference sample is prepared along with each new library. We normalize both the 1PEF and 2PEF signals from each mutagenized library relative to the corresponding average 1PEF and 2PEF signals obtained from the reference sample under identical conditions. Normalization of the 1PEF and 2PEF signals makes comparison of mutant FPs to the non-mutated FP being evolved much simpler. The normalized integrated 1PEF signal may be expressed as:

$$F_{1PEF}^{norm}(n) = \frac{F_{1PEF}(n)}{C_{FL} \varepsilon_{ref}(\lambda_{1PA}) \varphi_{ref} \langle V_r c_r \rangle_{ref} I_{lamp} \tau_{1PEF}} = C_{1PEF}^{ref} V_n c_n \varepsilon_n(\lambda_{1PA}) \varphi_n, \quad (7)$$

where $\langle \dots \rangle_{ref}$ stands for the average over all colonies in the reference sample. The normalized integrated 2PEF signal is:

$$F_{2PEF}^{norm}(n) = \frac{F_{2PEF}(n)}{C_{FL} \sigma_{2PA}^{ref}(\lambda_{2PA}) \varphi_{ref} \langle V_r c_r \rangle_{ref} \kappa_{scan} I_{laser}^2 \tau_{2PEF}} = C_{2PEF}^{ref} V_n c_n \sigma_{2PA}(\lambda_{2PA}) \varphi_n, \quad (8)$$

By taking the ratio of equations (7) and (8) we end up with equation (1) from the main text:

$$\frac{F_{2PEF}^{norm}(n)}{F_{1PEF}^{norm}(n)} = \left(\frac{C_{2PEF}^{ref}}{C_{1PEF}^{ref}} \right) \frac{\sigma_{2PA,n}(\lambda_{2PA})}{\varepsilon_n(\lambda_{1PA})},$$

Estimation of laser parameters

Using the scanning parameters of our imaging setup we can estimate the required laser parameters needed to image a Petri plate in a reasonable amount of time. We will assume the following criteria are needed for realistic two-photon protein evolution: 1) the fluorescence signal measured from each colony needs to be detected with a minimum signal-to-noise ratio, S/N = 10, and 2) the screening process must have a minimum throughput of at least ten Petri plate samples per day (~1 sample per hour). We use these basic metrics, together with known general properties of FPs to evaluate what type of femtosecond laser is best suited to accomplish the named tasks.

If we write out the number of detected 2PEF photons for the n^{th} colony of bacteria on a Petri plate, with its explicit dependence on all of the laser, optics, and scanning parameters we find the following equation:

$$2PEF_n = \frac{\eta_c T_f \Omega \Delta \lambda \sigma_{2PA,n}(\lambda_{2PA}) V_n c_n \varphi_n n_L^2}{2\sqrt{2} (\Delta x \Delta y)^2 \tau} \left(\frac{\ln(16)}{\pi} \right)^{5/2} \sum_{k=0}^{S_h} \sum_{i=0}^{g\tau_{2PEF}} n_v e^{\frac{-8 \ln(2) \left(\frac{\left(\frac{k d_h}{S_h} - x_0 \right)^2}{2 \Delta r_n^2 + \Delta x^2} + \frac{\left(\frac{i d_v}{g\tau_{2PEF}} - y_0 \right)^2}{2 \Delta r_n^2 + \Delta y^2} \right)}{\sqrt{\left(1 + \frac{2 \Delta r_n^2}{\Delta x^2} \right) \left(1 + \frac{2 \Delta r_n^2}{\Delta y^2} \right)}}$$

This expression is in terms of the number of photon in each pulse, n_L . If the number of laser pulses, $g\tau_{2PEF}$, per horizontal step of the scanning laser stripe is large we can approximate the sum over the laser pulses as an integral. This approximation holds only for high repetition rate lasers.

$$2PEF_n \approx \frac{\eta_c T_f \Omega \Delta \lambda \sigma_{2PA,n}(\lambda_{2PA}) V_n c_n \varphi_n n_L^2 \left(\frac{\ln(16)}{\pi} \right)^{5/2} \sum_{k=0}^{S_h} \int_0^{g\tau_{2PEF}} n_v \frac{e^{-8 \ln(2) \left(\frac{\left(\frac{k d_h - x_0}{S_h} \right)^2 + \frac{\left(\frac{i d_v - y_0}{g\tau_{2PEF}} \right)^2}{2\Delta r_n^2 + \Delta x^2} \right)}}{\sqrt{\left(1 + \frac{2\Delta r_n^2}{\Delta x^2} \right) \left(1 + \frac{2\Delta r_n^2}{\Delta y^2} \right)}} di$$

We will use the following typical laser and scanning parameters in our analysis of our imaging technique:

- The quantum efficiency of the camera: $\eta_c = 0.8\%$
- The transmission of the collection optics (including color filters): $T_f = 0.8\%$
- The solid angle of collection (in terms of percentage of fluorescence collected):
 - $\Omega = 0.001\%$ (for our wide field two-photon imaging setup)
 - $\Omega = 0.14\%$ (for a two-photon microscope with an objective with a numerical aperture of 0.7)
- Bandwidth of the detection filters: $\Delta\lambda = 50$ nm
- Two-photon excitation wavelength: $\lambda_{2PA} = 790$ nm
- Two-photon cross section at λ_{2PA} : $\sigma_{2PA} = 40$ GM
- Concentration of FPs: $c_n = 5 \times 10^{15}$, (assuming 10% of the total volume is occupied by GFP molecules)
- Differential quantum yield of fluorescence: $\varphi = 0.1\%$
- Pulse duration of the laser: $\tau = 150$ fs (We will assume this does not change with the rep rate of the laser)
- Average laser power of the 1 kHz system: $P = 0.6$ W (We will vary this along with the rep rate of the laser from 0.1 W to 10 W in our analysis of this imaging technique.)
- Number of illumination photons per pulse: $n_L = \frac{P}{g} \left(\frac{hc}{\lambda_{2PA}} \right)^{-1}$
- Typical bacterial colony diameter: $\Delta r = 1$ mm
- Bacterial colony volume: $V_n = 1.1$ mm³
- Vertical scan distance: $d_v = 100$ mm
- Horizontal scan distance: $d_h = 100$ mm
- Number of vertical laser passes at each horizontal position: $n_v = 2$
- Position of the colony (doesn't change the measured 2PEF so put it at the center of the Petri plate): $x_0 = y_0 = 50$ mm

Now that we have defined typical values for all of the constant terms we can make some assumptions about our scan parameters to lower our number of unknowns. If we assume that the illuminating laser spot is spherically symmetric we can make the following definition:

- Laser stripe width: $\Delta x = \Delta y$

We also know that if we turn up the intensity of the laser much more than it is in the current setup the colonies will burn. We can thus define the optimal laser beam height using the following equation:

- Laser stripe height: $\Delta y = \sqrt{\frac{n_L}{\sqrt{C_P \tau}}}$

Here C_P is a constant that is related to the maximum laser intensity desired. For our setup this value is approximately 2.4×10^{40} . This should be kept below the limit of cell damage. We will assume that the Rayleigh range of the focused laser cannot be less than the thickness of the colonies of bacteria, typically ~ 1 mm, this means the lower limit on Δy is 0.016 mm. We will also assume that we want to have a minimum signal to noise ratio of 10. If the signal to noise is limited by the camera noise we can invert equation (8) which determines the vertical scan speed, as:

- Camera noise limited camera exposure time:

$$\tau_{2PEF} = \frac{14320 d_v \sqrt{2} (\Delta x \Delta y)^2 \tau (\ln(16))^{-5/2} \sqrt{\ln(4) \left(1 + \frac{2\Delta r_n^2}{\Delta x^2}\right)}}{g \eta_c T_f \Omega \Delta \lambda \sigma_{2PA,n} (\lambda_{2PA}) V_n c_n \varphi_n n_L^2 \Delta y \pi^5 \left(\text{Erf} \left(\frac{2y_0 \sqrt{\ln(4)}}{\sqrt{\Delta y^2 + 2\Delta r_n^2}} \right) - \text{Erf} \left(\frac{2(y_0 - d_v) \sqrt{\ln(4)}}{\sqrt{\Delta y^2 + 2\Delta r_n^2}} \right) \right)}$$

If the signal to noise ratio is limited by the laser amplification noise then the camera exposure time is determined by the number of pulses that must be averaged. This was measured for our system and occurs when the size of the vertical steps is at least $1/200^{\text{th}}$ the size of Δy . Therefore, the camera exposure time is given by:

- Laser amplification noise limited camera exposure time: $\tau_{2PEF} = 200 \frac{d_v}{g \Delta y}$

We will assume that the number of horizontal steps is such that the horizontal step size is $1/10^{\text{th}}$ the size of Δx :

- Number of horizontal steps: $S_h = \frac{10 d_h}{\Delta x}$

We will assume that for a 1 kHz repetition rate laser the signal to noise ratio is limited by the laser amplification process and is a measurable quantity that is independent of average laser power. To measure this quantity, for the laser used in our system, a Petri plate containing *E. coli* colonies expressing EGFP was used. The 1PEF and 2PEF signals were collected, and the ratio of the 2PEF/1PEF was calculated for each colony on the Petri plate. The noise in the ratio of the two signals was assumed to be dominated by the noise from the laser amplification process. The signal to noise ratio was calculated by taking the average 2PEF/1PEF signal and dividing it by the standard deviation of the 2PEF/1PEF signal. The laser amplification limited signal to noise ratio for our 1 kHz laser system was found to be 9.

Finally, we will assume that for a 100 MHz repetition rate laser the signal to noise ratio is limited by the noise intrinsic to the detection system. The noise of the camera used in our system was measured by taking multiple images with no illumination on the camera and an 8 second exposure time. The standard deviation of the resulting pixel values was calculated and found to be 17.9 counts. Using this number the signal to noise ratio for the 2PEF image taken with a high repetition rate laser can be found using the following equation:

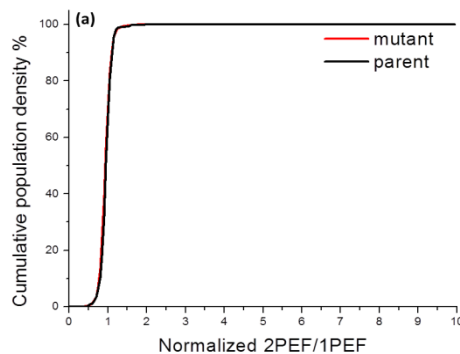
$$\text{camera limited signal to noise ratio} = \frac{2PEF_k}{17.9 * 10}$$

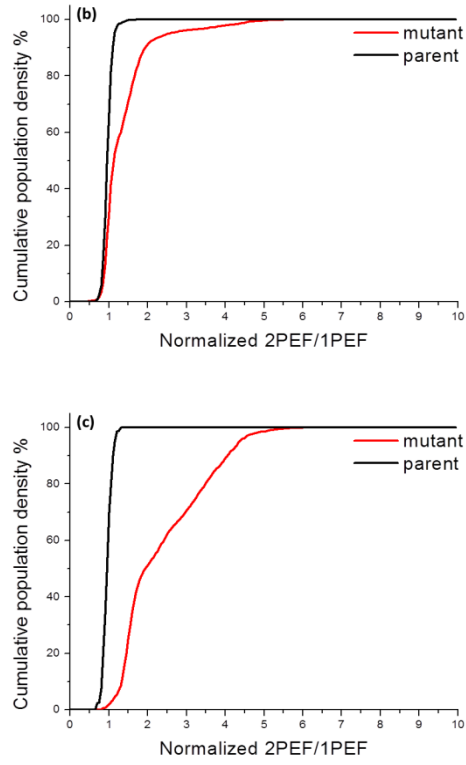
In this equation $2PEF_k$ is the fluorescence detected from one colony of bacteria in one vertical pass of the illuminating laser stripe. The factor of 10 multiplying the noise per camera pixel per image comes from the fact that for this camera each colony of bacteria is about 10 pixels across.

Supplementary Fig. 4a shows the estimated maximum number of samples that can be screened per day as a function of the average laser power based on the above assumptions and calculations. The red dashed lines correspond to an ultrafast oscillator laser with a 100 MHz pulse repetition rate and the black dashed lines correspond to a 1 kHz pulse repetition rate typical of a femtosecond regenerative amplifier. In both cases we assume that the pulse duration is 100 fs. The open red circle in Supplementary Fig. 4a shows the maximum screening rate using a generic 2PEF microscope setup utilizing a diffraction-limited focus spot and 10 mW average power, if we assume that the typical video-rate spatial beam scanning could be extrapolated from 100x100 μm^2 area to 9x9 cm^2 sample area. Note that increasing the average laser power (thin red line) from 10 mW to 100 mW would, in principle, be sufficient to meet the minimum average sample imaging rate (solid thick blue horizontal line). However, when arriving at this apparently favorable estimate we had to assume that all *E. coli* colonies grow such that they have identical spatial properties. In practice, the growth is always heterogeneous, and individual colonies end up having very different thicknesses, sizes and shapes. In addition, the concentration of FP expressed in each colony is different from one colony to another. To achieve the necessary quantitative reproducibility, the fluorescence signal from the FPs needs to be excited and collected uniformly throughout the whole thickness of each colony. As a result, the Rayleigh range of the excitation beam focus cannot be very short, and must be at least as large as the maximum thickness of the colonies, about 1 mm. Furthermore, for the

purpose of quantitative evaluation of the 2PEF efficiency, it is necessary to calibrate the 2PEF signal with respect to the 1PEF signal collected from the same colony, which also implies that the two-photon excitation photon flux must be uniform throughout the thickness of the sample. Our model calculation shows that under such realistic conditions the optimum spot diameter for the 100 MHz excitation is about 16 – 200 μm , depending on the laser power. The thick red dashed line in Supplementary Fig. 4a shows the minimum average power that accounts for the heterogeneity of colony growth. The result indicates that with the 100 MHz femtosecond oscillator about 1W of average power would be needed to meet, simultaneously, the minimum screening rate as well as the minimum S/N value.

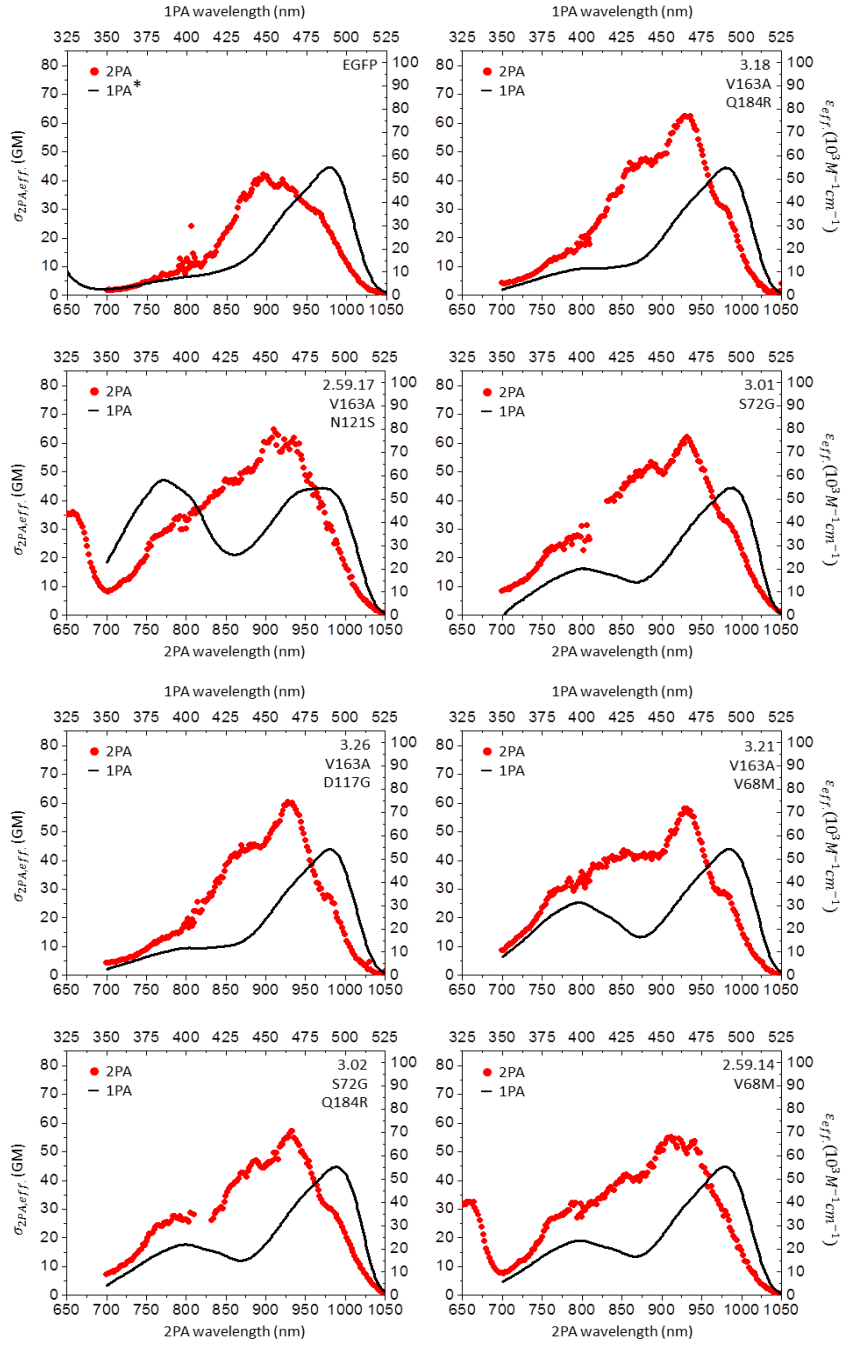
A spot diameter of 16 μm means that about 10^7 beam positions will be needed to cover the area of a standard Petri plate. This type of high precision, large area, focused beam scanning may become technically involved. An alternative would be a generic 1 kHz pulse rate regeneratively amplified femtosecond laser. This type of femtosecond amplifier typically operates at about 10^5 times lower pulse repetition rate (1 kHz) than the femtosecond oscillators, while providing a factor of about 10^5 higher peak photon flux. The higher peak intensity allows the laser spot diameter to be expanded by several orders of magnitude, up to several millimeters, while maintaining the required minimum S/N ratio. Supplementary Fig. 4b shows an example of how just about 20x20 partially overlapping beam positions can cover the entire sample. A smaller number of excitation beam positions is the key for achieving high accuracy and fast throughput. The fundamental drawback of this approach is that amplified lasers exhibit quite high, up to 10%, root mean square energy fluctuations, whereas in the 100 MHz oscillator pulses such fluctuations are minimal. To maintain the required minimum S/N ratio, the 2PEF signal obtained using an amplified femtosecond laser source requires additional averaging and therefore longer collection time. The upper limit of the shaded area in Supplementary Fig. 4a shows the minimum average laser power in the case of an ideal noise-free amplifier, and the lower limit shows the corresponding limit in the case of 10% root mean square energy fluctuations. Comparison of the shaded area to the red dashed line shows that even with a relatively high noise level the 1 kHz laser system offers a distinct advantage over the 100 MHz pulses.

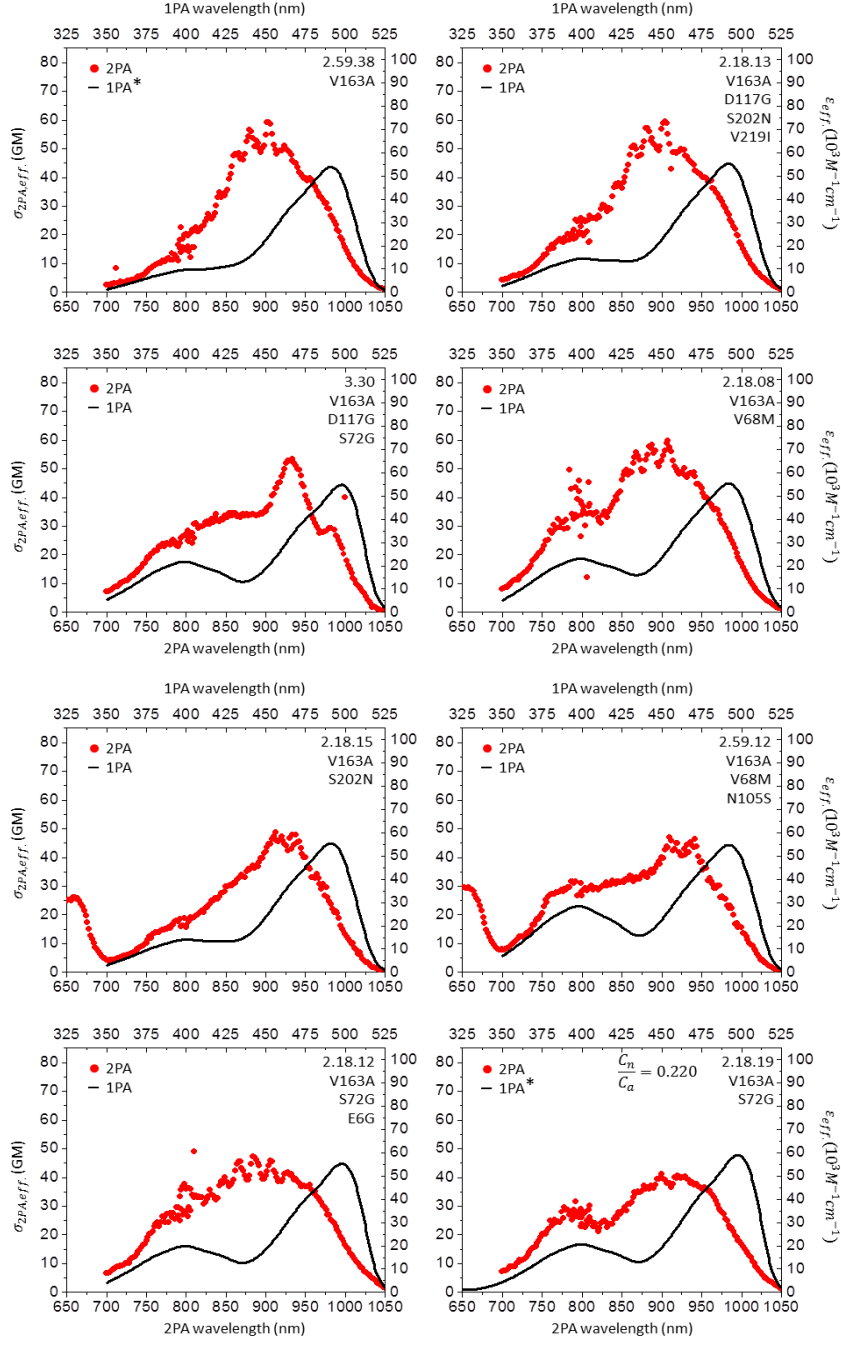


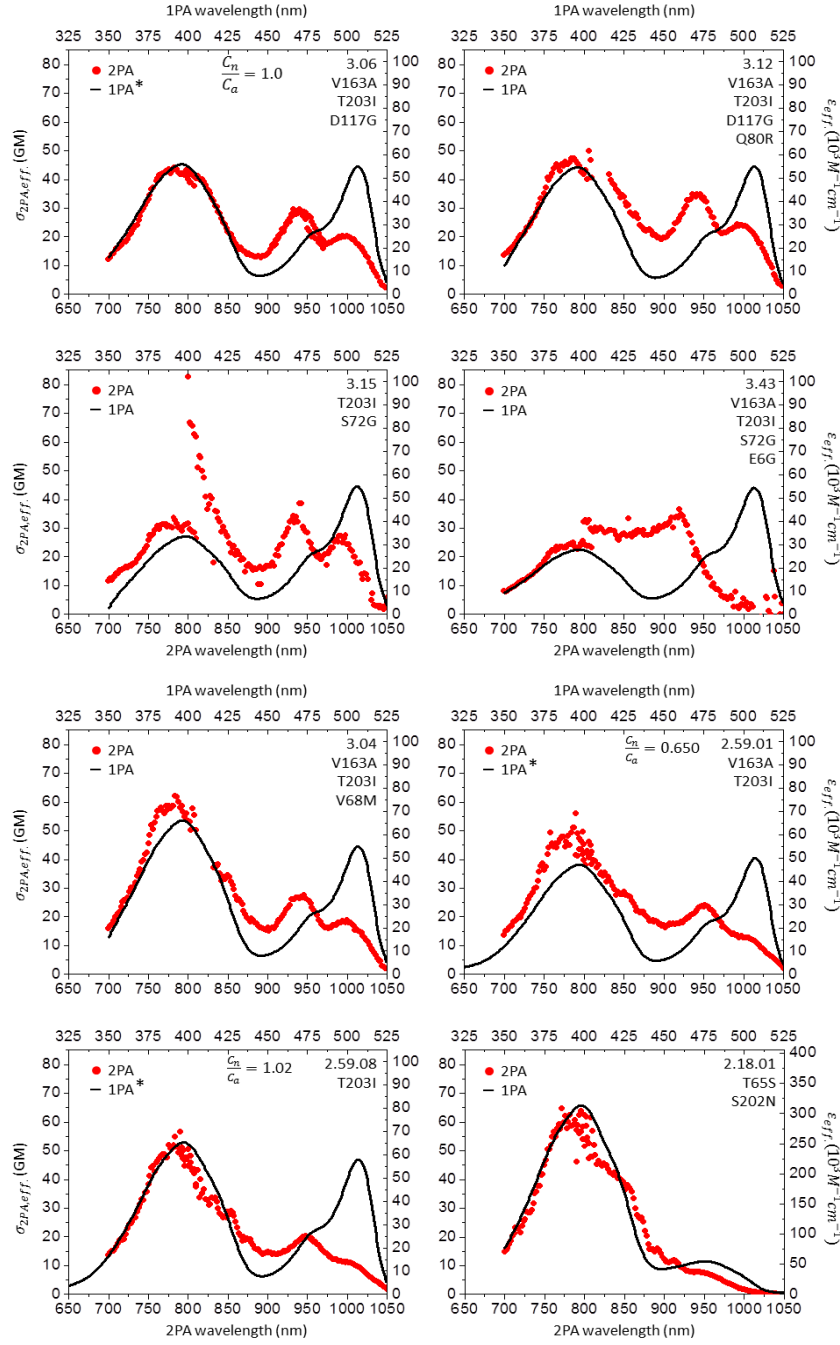


Supplementary Figure 2I Cumulative distribution functions of fluorescence data of randomly mutagenized EGFP. Cumulative percentage of the colonies (vertical axis) with a particular ratio value η (horizontal axis). (a), The 1st generation library. (b), The 2nd generation library. (c), The 3rd generation mutagenized library.

Plots of the effective two-photon cross section for selected mutants.

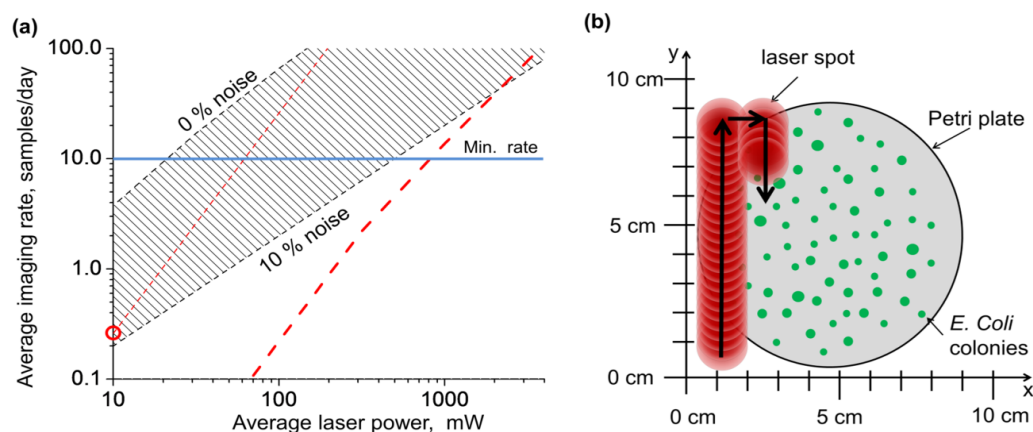






Supplementary Figure 3| Absorption spectra. Here all of the effective two-photon cross section spectra are shown. The effective extinction coefficients spectral shapes are overlaid on the plots for reference. The plots are arranged in the same order as in Table 1 in the main text. *For 1PA spectra with a star the absolute values of the extinction coefficients were explicitly measured using the method outlined in Supplementary

Information. For all other mutants the peak IPA of the anionic form of the chromophore was assumed to be $55,000 \text{ M}^{-1} \text{ cm}^{-1}$. The ratio of the concentration of the two forms is shown for those mutants for whom it was measured. The numbers in the upper right hand corner of each plot designate different mutants.



Supplementary Figure 4| Imaging rate estimation versus laser parameters. (a) Estimated average imaging rate (vertical axis) as a function of the average laser power (horizontal axis) assuming $S/N=10$. The thick blue solid line shows the required minimum imaging rate of 10 samples per day. The red circle represents a 100 MHz repetition rate laser using a 2PEF microscope setup with diffraction-limited focusing and video rate scanning. The thin red line represents an extrapolation to higher average powers. The red dashed line represents a 100 MHz laser, taking into account heterogeneous growth of *E. coli* colonies and the associated decrease of imaging rate due to an increase of the minimum laser spot size. The black shaded area represents a 1 kHz pulse rate amplified laser with root mean square energy fluctuations between 10% (lower dashed line) and 0% (upper dashed line) (b) Schematic representation of the spatial beam scanning with a 1 kHz laser beam (red circles). The shaded gray area is the Petri plate containing colonies of *E. coli* bacteria (green dots). The whole plate is covered by less than 400 beam positions.

CHAPTER SIX

FUTURE RESEARCH DIRECTIONS

Voltage Sensor Optimization

The optimization of genetically-encoded fluorescent Ca^{2+} sensors has far surpassed what has been done in genetically-encoded fluorescent voltage sensors for a couple of reasons. First, the GCaMP sensors are cytosolic proteins. They are relatively easy to purify and study spectroscopically. Second, the crystal structures have been resolved, in both Ca^{2+} free and Ca^{2+} -saturated states (Akerboom et al. 2008; Ding et al. 2014). Voltage sensors are based on complex proteins with both a structured membrane domain and highly mobile cytosolic domain. They have not yet been fully crystallized or studied spectroscopically because of their complex structure and conformational voltage dependence. Third, because voltage sensors can only be tested in an electrically active cell membrane, the throughput to screen for better voltage sensors is very low (Storace et al. 2016). The number of voltage sensor variants that have been screened over the last 20 years is comparatively small.

To date, several voltage sensor designs have been able to achieve the speed and/or membrane trafficking efficiency that is necessary to image action potentials in mammalian cells, but a voltage sensor with a large change in fluorescence on level of the GCaMP5 or GCaMP6 sensors still seems to be a long way off. That said, there are several protein engineering groups that are aggressively working toward reaching this benchmark. Achieving or getting closer to this goal will most likely require: 1) an

understanding of the molecular mechanism causing the fluorescence change in these sensors; and/or 2) a high-throughput functional screen that can facilitate the exploration of a large number of voltage-sensor designs. Significant steps forward have been made in both of these areas (Kang & Baker 2016; Park et al. 2013; Wardill et al. 2013; Platisa et al. 2017), but there is still a lot of work to be done. Looking for clues in known, similarly designed biosensor mechanisms, open data sharing for design successes and failures, multi-group collaborations, and development and sharing of large scale screening technologies will all be essential in developing a voltage sensor on the scale of the current GCaMP sensors.

Multi-Color Imaging

To image and distinguish the activity of specific sets of cell types in relation to one another, neuroscientists will need suites of multicolored biosensors. This will require multiple colors of the same biosensor, reliable and reproducible genetic labeling techniques to deliver a unique biosensor to specific cell types, and an imaging modality to excite and observe the fluorescence changes of all unique biosensors at once. To date, the single fluorescent protein based Ca^{2+} sensors have the most diverse color options available: green, yellow, red and blue GCaMP-like designs have been published (Akerboom et al. 2013; Zhao et al. 2011; Wu et al. 2014; Dana et al. 2016; Inoue et al. 2014). However, this palette of sensors has some limitation in application. Most importantly, the magnitude of the Ca^{2+} -dependent fluorescence change and the Ca^{2+} -binding kinetics are widely varied between the different sensors (Akerboom et al. 2013;

Zhao et al. 2011; Wu et al. 2014; Dana et al. 2016; Inoue et al. 2014). Based on the size of the fluorescence signal, only the green and red versions of these Ca^{2+} sensors are useful for *in vivo* imaging (Dana et al. 2016; Chen et al. 2013; Akerboom et al. 2012). Furthermore, the red fluorescent proteins undergo very fast photobleaching in multi-photon excitation (Drobizhev et al. 2014). It is possible that different multi-photon imaging regimes, or new red fluorescent proteins, will circumvent this problem, but at the moment only the green Ca^{2+} sensors are useful for multi-photon imaging of intact brains and circuits. The obvious need for different colored biosensors combined with the limitations of the current fluorescent proteins available, begs for the exploration of alternative approaches. GFPs are relatively photostable in both one- and two-photon excitation, and the Ca^{2+} sensors that are based on them are the most robust tools we have.

It may be possible to exploit the neutral and anionic equilibrium in GFP-based biosensors to color-code neutral activity via ratiometric excitation rather than using multiple color fluorescent proteins. As illustrated by my GCaMP6m mutants in Chapter 3, amino acid mutations made in the beta-barrel surrounding the chromophore can alter the ratio of neutral and anionic chromophore concentrations via a change in the acid dissociation constant of the chromophore (pK_a) (Warshel 1981). The fluorescence response of these GCaMP6m mutants are unique when observing the ratio of the neutral and anionic forms of the chromophore, which requires ratiometric excitation at 400 nm and 470 nm excitation while collecting the 515 nm fluorescence emission. These mutants could be used as an alternative approach to multicolor imaging neural activity, particularly for two-photon imaging applications. It should be noted that pH fluctuations

from cell to cell and rapid photobleaching of the mutant GCaMP sensors, due to increased excitation, could be two potential pitfalls of this approach.

Two-Photon Biosensor Imaging

Screening and optimizing for brighter two-photon fluorescent proteins is important, but separately screening and optimizing fluorescent biosensors for two-photon imaging is necessary as well. For example, there have been many efforts to improve GCaMP sensors. Significant improvements in the speed of Ca^{2+} -binding/dissociation make it possible to image individual action potentials (Chen et al. 2013; Sun et al. 2013; Badura et al. 2014; Helassa et al. 2015; Helassa et al. 2016), and large changes in fluorescence (Akerboom et al. 2012; Chen et al. 2013) make it possible to image in ever more demanding applications (Sofroniew et al. 2016; Ji et al. 2016). However, relatively little work has focused on improving the performance of these sensors for two-photon absorption.

The GCaMP6 series of sensors are remarkably bright fluorescent tools in one-photon excited imaging applications and sufficient in two-photon imaging, there is still significant room for improvement in two-photon Ca^{2+} sensor development. Just as there are different optimal fluorescent proteins for two-photon versus one-photon imaging applications, the biosensors designs will be different as well. Especially for the future voltage sensors, this will be particularly important to consider given then increased excitation light and high frame rates that will be required to image action potentials.

REFERENCES CITED

- Abdelfattah, A.S. et al., 2016. A Bright and Fast Red Fluorescent Protein Voltage Indicator That Reports Neuronal Activity in Organotypic Brain Slices. *Journal of Neuroscience*, 36(8), pp.2458–2472.
- Ai, H.-W. et al., 2014. Engineering and characterizing monomeric fluorescent proteins for live-cell imaging applications. *Nature protocols*, 9(4), pp.910–928.
- Ai, H.-W. et al., 2008. Hue-shifted monomeric variants of *Clavularia* cyan fluorescent protein: identification of the molecular determinants of color and applications in fluorescence imaging. *BMC biology*, 6(1), p.13.
- Akemann, W. et al., 2010. Imaging brain electric signals with genetically targeted voltage-sensitive fluorescent proteins. *Nature methods*, 7(8), pp.643–649.
- Akerboom, J. et al., 2008. Crystal Structures of the GCaMP Calcium Sensor Reveal the Mechanism of Fluorescence Signal Change and Aid Rational Design. *The Journal of biological chemistry*, 284(10), pp.6455–6464.
- Akerboom, J. et al., 2013. Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. *Frontiers in molecular neuroscience*, 6, pp.1–29.
- Akerboom, J. et al., 2012. Optimization of a GCaMP Calcium Indicator for Neural Activity Imaging. *Journal of Neuroscience*, 32(40), pp.13819–13840.
- Ast, C. et al., 2015. Single-fluorophore membrane transport activity sensors with dual-emission read-out. *eLife*, 4, p.e07113.
- Ataka, K. & Pieribone, V.A., 2002. A Genetically Targetable Fluorescent Probe of Channel Gating with Rapid Kinetics. *Biophysical journal*, 82(1), pp.509–516.
- Badura, A. et al., 2014. Fast calcium sensor proteins for monitoring neural activity. *Neurophotonics*, 1(2), p.025008.
- Baird, G.S., Zacharias, D.A. & Tsien, R.Y., 1999. Circular permutation and receptor insertion within green fluorescent proteins. *Proceedings of the National Academy of Sciences*, 96(20), pp.11241–11246.
- Baker, B.J. et al., 2008. Genetically encoded fluorescent sensors of membrane potential. *Brain cell biology*, 36(1-4), pp.53–67.
- Baker, B.J. et al., 2012. Genetically encoded fluorescent voltage sensors using the voltage-sensing domain of *Nematostella* and *Danio* phosphatases exhibit fast kinetics. *Journal of neuroscience methods*, 208(2), pp.190–196.

- Barnett, L. et al., 2012. A Fluorescent, Genetically-Encoded Voltage Probe Capable of Resolving Action Potentials. *PloS one*, 7(9), p.e43454.
- Barnett, L.M., Hughes, T.E. & Drobizhev, M., 2017. Deciphering the molecular mechanism responsible for GCaMP6m's Ca^{2+} -dependent change in fluorescence. *PloS one*, 12(2), p.e0170934.
- Bell, A.F. et al., 2000. Probing the ground state structure of the green fluorescent protein chromophore using Raman spectroscopy. *Biochemistry*, 39(15), pp.4423–4431.
- Belousov, V.V. et al., 2006. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nature methods*, 3(4), pp.281–286.
- Berg, J., Hung, Y.P. & Yellen, G., 2009. A genetically encoded fluorescent reporter of ATP:ADP ratio. *Nature methods*, 6(2), pp.161–166.
- Bernstein, J.G. & Boyden, E.S., 2011. Optogenetic tools for analyzing the neural circuits of behavior. *Trends in cognitive sciences*, 15(12), pp.592–600.
- Bezánilla, F., 2008. How membrane proteins sense voltage. *Nature reviews. Molecular cell biology*, 9(4), pp.323–332.
- Bezánilla, F., 1986. Voltage-Dependent Gating. In R. Latorre, ed. *Ionic Channels in Cells and Model Systems*. Series of the Centro de Estudios Científicos de Santiago. Springer US, pp. 37–52.
- Bezánilla, F., 2007. Voltage-Gated Ion Channels. In S.-H. Chung, O. S. Andersen, & V. Krishnamurthy, eds. *Biological Membrane Ion Channels*. Biological and Medical Physics Biomedical Engineering. Springer New York, pp. 81–118.
- Bhargava, Y. et al., 2013. Improved genetically-encoded, FlnG-type fluorescent biosensors for neural cGMP imaging. *Frontiers in molecular neuroscience*, 6, p.26.
- Bovetti, S., Moretti, C. & Fellin, T., 2014. Mapping brain circuit function in vivo using two-photon fluorescence microscopy. *Microscopy research and technique*, 77(7), pp.492–501.
- Boyd, A.M. et al., 2015a. Broadcasting of Cortical Activity to the Olfactory Bulb. *CellReports*, 10(7), pp.1032–1039.
- Brejč, K., Sixma, T.K., Kitts, P.A., Kain, S.R., Tsien, R.Y., Ormö, M. & Remington, S.J., 1997. Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America*, 94(6), pp.2306–2311.
- Broussard, G.J., Liang, R. & Tian, L., 2014. Monitoring activity in neural circuits with genetically encoded indicators. *Frontiers in molecular neuroscience*, 7, p.97.

- Carriles, R. et al., 2009. Invited review article: Imaging techniques for harmonic and multiphoton absorption fluorescence microscopy. *The Review of scientific instruments*, 80(8), p.081101.
- Chalfie, M. et al., 1994. Green fluorescent protein as a marker for gene expression. *Science*. Available at: <http://www.sciencemag.org/content/263/5148/802.short>.
- Chattoraj, M. et al., 1996a. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. *Proceedings of the National Academy of Sciences of the United States of America*, 93(16), pp.8362–8367.
- Chen, Q. et al., 2012. Imaging neural activity using Thy1-GCaMP transgenic mice. *Neuron*, 76(2), pp.297–308.
- Chen, T.-W. et al., 2013. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature*, 499(7458), pp.295–300.
- Chen, Y. et al., 2013. Structural insight into enhanced calcium indicator GCaMP3 and GCaMPJ to promote further improvement. *Protein & cell*, 4(4), pp.299–309.
- Chin, D. & Means, A.R., 2000. Calmodulin: a prototypical calcium sensor. *Trends in cell biology*, 10(8), pp.322–328.
- Chow, B.Y., Han, X. & Boyden, E.S., 2012. Genetically encoded molecular tools for light-driven silencing of targeted neurons. *Progress in brain research*, 196, pp.49–61.
- Chudakov, D.M. et al., 2010. Fluorescent proteins and their applications in imaging living cells and tissues. *Physiological reviews*, 90(3), pp.1103–1163.
- Cubitt, A.B., Woollenweber, L.A. & Heim, R., 1998. Chapter 2: Understanding Structure—Function Relationships in the *Aequorea victoria* Green Fluorescent Protein. *Methods in cell biology*, 58, pp.19–30.
- Cui, G. et al., 2013. Concurrent activation of striatal direct and indirect pathways during action initiation. *Nature*, 494(7436), pp.238–242.
- Dana, H. et al., 2016. Sensitive red protein calcium indicators for imaging neural activity. *eLife*, 5, pp.1–24.
- Dana, H. et al., 2014. Thy1-GCaMP6 Transgenic Mice for Neuronal Population Imaging In Vivo. *PloS one*, 9(9), pp.e108697–9.
- Davidson, M.W. & Campbell, R.E., 2009. Engineered fluorescent proteins: innovations and applications. *Nature methods*, 6(10), pp.713–717.

- Davila, H.V., Cohen, L.B. & Waggoner, A.S., 1973. A large change in axon fluorescence that provides a promising method for measuring membrane potential. *Nature New Biology*, 241, pp. 159-60.
- Day, R.N. & Davidson, M.W., 2009. The fluorescent protein palette: tools for cellular imaging. *Chemical Society reviews*, 38(10), pp.2887–2921.
- De Michele, R. et al., 2013. Fluorescent sensors reporting the activity of ammonium transceptors in live cells. *eLife*, 2, p.e00800.
- Denk, W., Strickler, J.H. & Webb, W.W., 1990. Two-photon laser scanning fluorescence microscopy. *Science*, 248(4951), pp.73–76.
- Denk, W. & Svoboda, K., 1997. Photon upmanship: why multiphoton imaging is more than a gimmick. *Neuron*, 18(3), pp.351–357.
- Diaspro, A., Chirico, G. & Collini, M., 2005. Two-photon fluorescence excitation and related techniques in biological microscopy. *Quarterly reviews of biophysics*, 38(2), pp.97–166.
- Dimitrov, D. et al., 2007. Engineering and characterization of an enhanced fluorescent protein voltage sensor. *PloS one*, 2(5), p.e440.
- Ding, J. et al., 2014a. Structural basis of the ultrasensitive calcium indicator GCaMP6. *Science China. Life sciences*, 57(3), pp.269–274.
- Dombeck, D.A. et al., 2007. Imaging large-scale neural activity with cellular resolution in awake, mobile mice. *Neuron*, 56(1), pp.43–57.
- Drobizhev, M. et al., 2009. Color hues in red fluorescent proteins are due to internal quadratic Stark effect. *The journal of physical chemistry. B*, 113(39), pp.12860–12864.
- Drobizhev, M. et al., 2012. Describing two-photon absorptivity of fluorescent proteins with a new vibronic coupling mechanism. *The journal of physical chemistry. B*, 116(5), pp.1736–1744.
- Drobizhev, M. et al., 2015. Long- and Short-Range Electrostatic Fields in GFP Mutants: Implications for Spectral Tuning. *Scientific reports*, 5, p.13223.
- Drobizhev, M. et al., 2014. Multiphoton photochemistry of red fluorescent proteins in solution and live cells. *The journal of physical chemistry. B*, 118(31), pp.9167–9179.
- Drobizhev, M. et al., 2011. Two-photon absorption properties of fluorescent proteins. *Nature methods*, 8(5), pp.393–399.

- Elowitz, M.B. et al., 2002. Stochastic gene expression in a single cell. *Science*, 297(5584), pp.1183–1186.
- Fenno, L., Yizhar, O. & Deisseroth, K., 2011. The development and application of optogenetics. *Annual review of neuroscience*, 34, pp.389–412.
- Frommer, W.B., Davidson, M.W. & Campbell, R.E., 2009. Genetically encoded biosensors based on engineered fluorescent proteins. *Chemical Society reviews*, 38(10), p.2833.
- Gautam, S.G. et al., 2009. Exploration of fluorescent protein voltage probes based on circularly permuted fluorescent proteins. *Frontiers in neuroengineering*, 2, p.14.
- Giepmans, B.N.G. et al., 2006. The fluorescent toolbox for assessing protein location and function. *Science*, 312(5771), pp.217–224.
- Göppert-Mayer, M., 1931. Über Elementarakte mit zwei Quantensprüngen. *Annalen der Physik*, 401(3), pp.273–294.
- Grienberger, C. & Konnerth, A., 2012. Imaging Calcium in Neurons. *Neuron*, 73(5), pp.862–885.
- Grover, D., Katsuki, T. & Greenspan, R.J., 2016. Flyception: imaging brain activity in freely walking fruit flies. *Nature methods*, 13(7), pp.569–572.
- Halaszovich, C.R., Schreiber, D.N. & Oliver, D., 2009. Ci-VSP is a depolarization-activated phosphatidylinositol-4, 5-bisphosphate and phosphatidylinositol-3, 4, 5-trisphosphate 5'-phosphatase. *The Journal of biological chemistry*, 284(4), pp.2106–2113.
- Hanson, G.T. et al., 2002. Green fluorescent protein variants as ratiometric dual emission pH sensors. 1. Structural characterization and preliminary application. *Biochemistry*, 41(52), pp.15477–15488.
- Han, Z. et al., 2014. Mechanistic studies of the genetically encoded fluorescent protein voltage probe ArcLight. *PloS one*, 9(11), p.e113873.
- Heckscher, E.S. et al., 2015. Even-Skipped+ Interneurons Are Core Components of a Sensorimotor Circuit that Maintains Left-Right Symmetric Muscle Contraction Amplitude. *Neuron*, 88(2), pp.314–329.
- Heim, R., Cubitt, A.B. & Tsien, R.Y., 1995. Improved green fluorescence. *Nature*, 373(6516), pp.663–664.
- Heim, R., Prasher, D.C. & Tsien, R.Y., 1994. Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America*, 91(26), pp.12501–12504.

- Helassa, N. et al., 2016. Design and mechanistic insight into ultrafast calcium indicators for monitoring intracellular calcium dynamics. *Scientific reports*, 6, p.38276.
- Helassa, N. et al., 2015. Fast-Response Calmodulin-Based Fluorescent Indicators Reveal Rapid Intracellular Calcium Dynamics. *Scientific reports*, 5, p.15978.
- Hinckley, C.A., Alaynick, W.A., Gallarda, B.W., Hayashi, M., Hilde, K.L., Driscoll, S.P., Dekker, J., et al., 2015. Spinal Locomotor Circuits Develop Using Hierarchical Rules Based on Motorneuron Position and Identity. *Neuron*, 87(5), pp.1008–1021.
- Hoover, E.E. & Squier, J.A., 2013. Advances in multiphoton microscopy technology. *Nature photonics*, 7(2), pp.93–101.
- Hung, Y.P. et al., 2011. Imaging Cytosolic NADH-NAD⁺ Redox State with a Genetically Encoded Fluorescent Biosensor. *Cell metabolism*, 14(4), pp.545–554.
- Inoue, M. et al., 2014. Rational design of a high-affinity, fast, red calcium indicator R-CaMP2. *Nature methods*, 12(1), pp.64–70.
- Jain, R.K. & Ranganathan, R., 2004. Local complexity of amino acid interactions in a protein core. *Proceedings of the National Academy of Sciences of the United States of America*, 101(1), pp.111–116.
- Ji, N., Freeman, J. & Smith, S.L., 2016. Technologies for imaging neural activity in large volumes. *Nature neuroscience*, 19(9), pp.1154–1164.
- Jin, L. et al., 2010. Genetically Encoded Protein Sensors of Membrane Potential. In M. Canepari & D. Zecevic, eds. *Membrane Potential Imaging in the Nervous System*. New York, NY: Springer New York, pp. 157–163.
- Jin, L. et al., 2011. Random insertion of split-cans of the fluorescent protein venus into Shaker channels yields voltage sensitive probes with improved membrane localization in mammalian cells. *Journal of neuroscience methods*, 199(1), pp.1–9.
- Jin, L. et al., 2012. Single action potentials and subthreshold electrical events imaged in neurons with a fluorescent protein voltage probe. *Neuron*, 75(5), pp.779–785.
- Jung, G. & Zumbusch, A., 2005. The photophysics of green fluorescent protein: influence of the key amino acids at positions 65, 203, and 222. *Biophysj*, 88(3), pp.1932–1947.
- Kandel, E.R. et al., 2013. Neuroscience thinks big (and collaboratively). *Nature reviews. Neuroscience*, 14(9), pp.659–664.
- Kang, B.E. & Baker, B.J., 2016. Pado, a fluorescent protein with proton channel activity can optically monitor membrane potential, intracellular pH, and map gap junctions. *Scientific reports*, 6, p.23865.

- Kohout, S.C. et al., 2008. Subunit organization and functional transitions in Ci-VSP. *Nature structural & molecular biology*, 15(1), pp.106–108.
- Kralj, J.M. et al., 2011. Optical recording of action potentials in mammalian neurons using a microbial rhodopsin. *Nature methods*, 9(1), pp.90–95.
- Kuboniwa H, Tjandra N, Grzesiek S, Ren H, Claude B, Bax A, 1995. Solution structure of calcium-free calmodulin. *Nature structural biology*, 2(9). Available at: <http://spin.niddk.nih.gov/bax/lit/spdf/225.pdf>.
- Lakowicz, J.R., 2006. *Principles of Fluorescence Spectroscopy* 3rd ed., New York: Springer.
- Liang, R., Broussard, G.J. & Tian, L., 2015. Imaging chemical neurotransmission with genetically encoded fluorescent sensors. *ACS chemical neuroscience*, 6(1), pp.84–93.
- Lin, J.Y., 2011. A user's guide to channelrhodopsin variants: features, limitations and future developments. *Experimental physiology*, 96(1), pp.19–25.
- Lin, M.Z., 2011. Beyond the rainbow: new fluorescent proteins brighten the infrared scene. *Nature methods*, 8(9), pp.726–728.
- Lin, M.Z. & Schnitzer, M.J., 2016. Genetically encoded indicators of neuronal activity. *Nature neuroscience*, 19(9), pp.1142–1153.
- Li, Y. & Tsien, R.W., 2012. pHTomato, a red, genetically encoded indicator that enables multiplex interrogation of synaptic activity. *Nature neuroscience*, 15(7), pp.1047–1053.
- Looger, L.L. & Griesbeck, O., 2012. Genetically encoded neural activity indicators. *Current opinion in neurobiology*, 22(1), pp.18–23.
- Lossau, H. et al., 1996. Time-resolved spectroscopy of wild-type and mutant Green Fluorescent Proteins reveals excited state deprotonation consistent with fluorophore-protein interactions. *Chemical physics*, 213(1), pp.1–16.
- Lundby, A. et al., 2008. Engineering of a Genetically Encodable Fluorescent Voltage Sensor Exploiting Fast Ci-VSP Voltage-Sensing Movements. *PloS one*, 3(6), p.e2514.
- Lundby, A., Akemann, W. & Knöpfel, T., 2010. Biophysical characterization of the fluorescent protein voltage probe VSFP2.3 based on the voltage-sensing domain of Ci-VSP. *European biophysics journal: EBJ*, 39(12), pp.1625–1635.
- Makarov, N.S., Drobizhev, M. & Rebane, A., 2008. Two-photon absorption standards in the 550–1600 nm excitation wavelength range. *Optics express*, 16(6), pp.4029–4047.

- Marblestone, A.H. et al., 2013. Physical principles for scalable neural recording. *Frontiers in computational neuroscience*, 7, p.137.
- Martin, M.E., Negri, F. & Olivucci, M., 2004. Origin, Nature, and Fate of the Fluorescent State of the Green Fluorescent Protein Chromophore at the CASPT2//CASSCF Resolution. *Journal of the American Chemical Society*, 126(17), pp.5452–5464.
- Marvin, J.S. et al., 2011. A genetically encoded, high-signal-to-noise maltose sensor. *Proteins: Structure, Function, and Bioinformatics*, 79(11), pp.3025–3036.
- Marvin, J.S. et al., 2013. An optimized fluorescent probe for visualizing glutamate neurotransmission. *Nature methods*, 10(2), pp.162–170.
- Matz, M.V., Lukyanov, K.A. & Lukyanov, S.A., 2002. Family of the green fluorescent protein: journey to the end of the rainbow. *BioEssays: news and reviews in molecular, cellular and developmental biology*, 24(10), pp.953–959.
- Meyers, F. et al., 1994. Electric-Field Modulated Nonlinear-Optical Properties of Donor-Acceptor Polyenes-Sum-Over-States Investigation of the Relationship Between Molecular Polarizabilities (Alpha, Beta, and Gamma) and Bond-Length Alternation. *Journal of the American Chemical Society*, 116(23), pp.10703–10714.
- Miesenböck, G., De Angelis, D.A. & Rothman, J.E., 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*, 394(6689), pp.192–195.
- Murata, Y. et al., 2005. Phosphoinositide phosphatase activity coupled to an intrinsic voltage sensor. *Nature cell biology*, 435(7046), pp.1239–1243.
- Muto, A. et al., 2011. Genetic visualization with an improved GCaMP calcium indicator reveals spatiotemporal activation of the spinal motor neurons in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, 108(13), pp.5425–5430.
- Mutoh, H. et al., 2011. Optogenetic monitoring of membrane potentials. *Experimental physiology*, 96(1), pp.13–18.
- Nagai, T. et al., 2001. Circularly permuted green fluorescent proteins engineered to sense Ca²⁺. *Proceedings of the National Academy of Sciences*, 98(6), pp.3197–3202.
- Nagai, T., 2004. Expanded dynamic range of fluorescent indicators for Ca²⁺ by circularly permuted yellow fluorescent proteins. *Proceedings of the National Academy of Sciences*, 101(29), pp.10554–10559.
- Nakai, J., Ohkura, M. & Imoto, K., 2001. A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. *Nature biotechnology*, 19(2), pp.137–141.

- Nikolaev, V.O., Gambaryan, S. & Lohse, M.J., 2006a. Fluorescent sensors for rapid monitoring of intracellular cGMP. *Nature methods*, 3(1), pp.23–25.
- Odaka, H. et al., 2014. Genetically-Encoded Yellow Fluorescent cAMP Indicator with an Expanded Dynamic Range for Dual-Color Imaging. *PloS one*, 9(6), pp.e100252–7.
- Ohkura, M. et al., 2005. Genetically Encoded Bright Ca²⁺-Probe Applicable for Dynamic Ca²⁺-Imaging of Dendritic Spines. *Analytical chemistry*, 77(18), pp.5861–5869.
- Okamura, Y. & Dixon, J.E., 2011. Voltage-sensing phosphatase: its molecular relationship with PTEN. *Physiology*, 26(1), pp.6–13.
- Okumoto, S., Jones, A. & Frommer, W.B., 2012. Quantitative imaging with fluorescent biosensors. *Annual review of plant biology*, 63(1), pp.663–706.
- Ormö, M. et al., 1996. Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science*, 273(5280), pp.1392–1395.
- Park, J. et al., 2013. Screening fluorescent voltage indicators with spontaneously spiking HEK cells. *PloS one*, 8(12), p.e85221.
- Patterson, G.H. et al., 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophysical journal*, 73(5), pp.2782–2790.
- Perron, A. et al., 2009. Red-shifted voltage-sensitive fluorescent proteins. *Chemistry & biology*, 16(12), pp.1268–1277.
- Peterka, D.S., Takahashi, H. & Yuste, R., 2011. Imaging voltage in neurons. *Neuron*, 69(1), pp.9–21.
- Peter, M. et al., 2005. Multiphoton-FLIM quantification of the EGFP-mRFP1 FRET pair for localization of membrane receptor-kinase interactions. *Biophysical journal*, 88(2), pp.1224–1237.
- Pieribone, V. & Gruber, D.F., 2005. *Aglow in the Dark: The Revolutionary Science of Biofluorescence*, Harvard University Press.
- Platisa, J. et al., 2017. Directed evolution of key residues in fluorescent protein inverses the polarity of voltage sensitivity in the genetically-encoded indicator ArcLight. *ACS chemical neuroscience*. Available at: <http://pubs.acs.org/doi/abs/10.1021/acschemneuro.6b00234>.
- Popovic, M.A. et al., 2011. The spatio-temporal characteristics of action potential initiation in layer 5 pyramidal neurons: a voltage imaging study. *The Journal of physiology*, 589(17), pp.4167–4187.

- Rebane, A. et al., 2008. Quantitative Prediction of Two-Photon Absorption Cross Section Based on Linear Spectroscopic Properties. *Journal of Physical Chemistry C*, 112(21), pp.7997–8004.
- Reiner, A. & Isacoff, E.Y., 2013. The Brain Prize 2013: the optogenetics revolution. *Trends in neurosciences*, 36(10), pp.557–560.
- Sakai, R. et al., 2001. Design and characterization of a DNA-encoded, voltage-sensitive fluorescent protein. *The European journal of neuroscience*, 13(12), pp.2314–2318.
- Sakata, S. et al., 2016. Voltage-dependent motion of the catalytic region of voltage-sensing phosphatase monitored by a fluorescent amino acid. *Proceedings of the National Academy of Sciences of the United States of America*, 113(27), pp.7521–7526.
- Sankaranarayanan, S. et al., 2000. The use of pHluorins for optical measurements of presynaptic activity. *Biophysical journal*, 79(4), pp.2199–2208.
- Sarkisyan, K.S. et al., 2015. Green fluorescent protein with anionic tryptophan-based chromophore and long fluorescence lifetime. *Biophysical journal*, 109(2), pp.380–389.
- Scott, J.N. & Callis, P.R., 2013. Insensitivity of tryptophan fluorescence to local charge mutations. *The journal of physical chemistry. B*, 117(33), pp.9598–9605.
- Shaner, N.C., Steinbach, P.A. & Tsien, R.Y., 2005. A guide to choosing fluorescent proteins. *Nature methods*, 2(12), pp.905–909.
- Shen, Y. et al., 2014. pHuji, a pH-sensitive red fluorescent protein for imaging of exo-and endocytosis. *The Journal of cell biology*, 207(3), pp.419–432.
- Sherrington, C., 1941. MAN ON HIS NATURE. *The Journal of nervous and mental disease*, 94(6), p.762.
- Shimomura, O., Johnson, F.H. & Saiga, Y., 1962. Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous Hydromedusan, Aequorea. *Journal of cellular and comparative physiology*, 59(3), pp.223–239.
- Shu, X. et al., 2006. Novel chromophores and buried charges control color in mFruits. *Biochemistry*, 45(32), pp.9639–9647.
- Siegel, M.S. & Isacoff, E.Y., 1997. A genetically encoded optical probe of membrane voltage. *Neuron*, 19(4), pp.735–741.
- Sofroniew, N.J. et al., 2016. A large field of view two-photon mesoscope with subcellular resolution for in vivo imaging. *eLife*, 5, p.e14472.

- Stoltzfus, C. et al., 2014. A multidimensional screening method for the selection of two-photon enhanced fluorescent proteins. In SPIE BiOS. International Society for Optics and Photonics, pp. 895611–895611–7.
- Stoltzfus, C.R. et al., 2015. Two-photon directed evolution of green fluorescent proteins. *Scientific reports*, 5, p.11968.
- Storace, D. et al., 2016. Toward Better Genetically Encoded Sensors of Membrane Potential. *Trends in neurosciences*, 39(5), pp.277–289.
- St-Pierre, F., Marshall, J.D., Yang, Y., et al., 2014. High-fidelity optical reporting of neuronal electrical activity with an ultrafast fluorescent voltage sensor. *Nature neuroscience*, 17(6), pp.884–889.
- Stryer, L. & Haugland, R.P., 1967. Energy transfer: a spectroscopic ruler. *Proceedings of the National Academy of Sciences of the United States of America*, 58(2), pp.719–726.
- Sun, X.R. et al., 2013. Fast GCaMPs for improved tracking of neuronal activity. *Nature communications*, 4, p.2170.
- Svoboda, K. & Yasuda, R., 2006. Principles of two-photon excitation microscopy and its applications to neuroscience. *Neuron*, 50(6), pp.823–839.
- Tallini, Y.N., 2006. Imaging cellular signals in the heart in vivo: Cardiac expression of the high-signal Ca²⁺ indicator GCaMP2. *Proceedings of the National Academy of Sciences*, 103(12), pp.4753–4758.
- Tewson, P. et al., 2012. Simultaneous detection of Ca²⁺ and diacylglycerol signaling in living cells. *PloS one*, 7(8), p.e42791.
- Tewson, P.H., Quinn, A.M. & Hughes, T.E., 2013. A multiplexed fluorescent assay for independent second-messenger systems: decoding GPCR activation in living cells. *Journal of biomolecular screening*, 18(7), pp.797–806.
- Theis, L., Berens, P., Froudarakis, E., Reimer, J., Rosón, M.R., et al., 2016. Benchmarking Spike Rate Inference in Population Calcium Imaging. *Neuron*, 90(3), pp.471–482.
- Tian, L. et al., 2009. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nature methods*, 6(12), pp.875–881.
- Tillo, S.E. et al., 2010. A new approach to dual-color two-photon microscopy with fluorescent proteins. *BMC biotechnology*, 10, p.6.
- Topell, S., Hennecke, J. & Glockshuber, R., 1999. Circularly permuted variants of the green fluorescent protein. *FEBS letters*, 457(2), pp.283–289.

- Treger, J.S., Priest, M.F. & Bezanilla, F., 2015. Single-molecule fluorimetry and gating currents inspire an improved optical voltage indicator. *eLife*. Available at: <http://elifesciences.org/content/4/e10482.abstract>.
- Tsai, P.S. et al., 2002. Principles, Design, and Construction of a Two-Photon Laser-Scanning Microscope for In Vitro and In Vivo Brain Imaging. In R. D. Frostig, ed. *In vivo optical imaging of brain function*. Boca Raton: CRC Press, pp. 113–171.
- Tsien, R.Y., 1998. The green fluorescent protein. *Annual review of biochemistry*, 67, pp.509–544.
- Tsutsui, H. et al., 2008. Improving membrane voltage measurements using FRET with new fluorescent proteins. *Nature methods*, 5(8), pp.683–685.
- Villalba-Galea, C.A., Sandtner, W., et al., 2009. Charge Movement of a Voltage-Sensitive Fluorescent Protein. *Biophysical journal*, 96(2), pp.L19–L21.
- Villalba-Galea, C.A., Miceli, F., et al., 2009. Coupling between the voltage-sensing and phosphatase domains of Ci-VSP. *The Journal of general physiology*, 134(1), pp.5–14.
- Wang, Q. et al., 2008. Structural basis for calcium sensing by GCaMP2. *Structure*, 16(12), pp.1817–1827.
- Wardill, T.J. et al., 2013. A neuron-based screening platform for optimizing genetically-encoded calcium indicators. *PloS one*, 8(10), p.e77728.
- Ward, W.W., 2005. Biochemical and Physical Properties of Green Fluorescent Protein. In M. Chalfie & S. R. Kain, eds. *Green Fluorescent Protein*. Hoboken, NJ, USA: John Wiley & Sons, pp. 39–65.
- Warp, E. et al., 2012. Emergence of patterned activity in the developing zebrafish spinal cord. *Current biology: CB*, 22(2), pp.93–102.
- Warshel, A., 1981. Calculations of enzymatic reactions: calculations of pKa, proton transfer reactions, and general acid catalysis reactions in enzymes. *Biochemistry*, 20(11), pp.3167–3177.
- Whitaker, M., 2010. Genetically encoded probes for measurement of intracellular calcium. *Methods in cell biology*, 99, pp.153–182.
- Wu, J., Abdelfattah, A.S., Miraucourt, L.S., Kutsarova, E., Zhou, H., et al., 2014. A long Stokes shift red fluorescent Ca²⁺ indicator protein for two-photon and ratiometric imaging. *Nature communications*, 5, p.5262.

- Xie, D. & Zeng, J., 2005. Electronic excitations of green fluorescent proteins: protonation states of chromophore model compound in solutions. *Journal of computational chemistry*, 26(14), pp.1487–1496.
- Xu, C., 1996. Multiphoton fluorescence excitation: New spectral windows for biological nonlinear microscopy. *Proceedings of the National Academy of Sciences*, 93(20), pp.10763–10768.
- Xu, C. & Webb, W.W., 1996. Measurement of two-photon excitation cross sections of molecular fluorophores with data from 690 to 1050 nm. *JOSA B*, 13(3), pp.481–491.
- Zhang, M.-Y. et al., 2013. Theoretical study of the proton transfer wires influence on the one- and two-photon absorption properties of green fluorescent protein chromophore. *Organic & biomolecular chemistry*, 11(8), pp.1414–1422.
- Zhao, Y. et al., 2011. An Expanded Palette of Genetically Encoded Ca²⁺ Indicators. *Science*, 333(6051), pp.1888–1891.
- Zipfel, W.R., Williams, R.M. & Webb, W.W., 2003. Nonlinear magic: multiphoton microscopy in the biosciences. *Nature biotechnology*, 21(11), pp.1369–1377.