CHARACTERIZING FLUORESCENT FUSION PROTEINS: mCHERRY – GCAMP2

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by
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ABSTRACT

The work presented here is focused on the characterization of the fusion protein created by genetically linking the two fluorescent proteins, mCherry and GCaMP2. This protein was cloned into the pRSET bacterial expression vector for expression in *Escherichia coli* cells in which the protein was amplified and subsequently extracted for experimentation. Four different mutations of GCaMP2, as well as an undisturbed GCaMP2 sequence, were paired separately with a previously optimized mCherry sequence for testing. The fluorescent activity of each of the five proteins was tested in the presence of calcium ions (Ca$^{2+}$) to determine a calibration curve of calcium concentration versus fluorescence intensity. This measurement and analysis was also conducted in the presence of interfering magnesium ions (Mg$^{2+}$) and at varying pHs to determine the effects of these different environments on the fluorescent activity of the protein. Mutations exhibited a sigmoid – like response with the increase of [Ca$^{2+}$], and magnesium ions caused distortion of this sigmoid – like signal. Substitution of the M13 calmodulin (CaM) binding moiety with CaMKII CaM binding moiety resulted in loss of the sigmoid – like signal but showed an increased dynamic range. The proteins showed a functional pH range between ~6.5 and ~11.5.
BIOGRAPHICAL SKETCH

Evan L. Graham completed his undergraduate studies at Cornell University in Biological Engineering, and completed also a minor in Biomedical Engineering. Evan’s undergraduate research was primarily focused on molecular biology and its applications in the development of recombinant bacterial strains and biosensor development. The laboratory skills that he developed as an undergraduate were put to good use in the Beauty R&D department of the Proctor and Gamble Company where he worked as an intern in summer of 2008. Here, he worked on the creation and optimization of new shave gel formulae and helped create formulae that were used in subsequent customer trials.

From his undergraduate studies, Evan entered the graduate program in Biological Engineering at the Masters level, again at Cornell University, and has focused his coursework around biomedical sciences and molecular biology applications. He has taken a large variety of courses ranging from embryology in his natural engineering course to biosensor design to soft tissue biomechanics to stochastic and deterministic mathematical modeling. The work here presented was completed in the Biomedical Sciences department of the Cornell University College of Veterinary Medicine and has given him an opportunity to apply what he has learned in the classroom in a laboratory setting. Evan’s graduate work has, however, taught him more than just science. It has given him the opportunity to learn from masters of their fields how to be a better scientist, and for this opportunity he is truly thankful.
ইয়েশিমের জন্য।
আমার প্রিয় বন্ধু, আমার সোনা, আমার ভালোবাসা।
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LIST OF ABBREVIATIONS

Amp – Ampicillin
AV – Atrioventricular
BFP – Blue Fluorescent Protein
CaM – Calmodulin
CaMKII – Calmodulin – dependent protein kinase II
CaEGTA – Calcium enriched ethylene glycol tetraacetic acid
CFP – Cyan Fluorescent Protein
cDNA – Complementary DNA
cpEGFP – Circularly Permutated Enhanced Green Fluorescent Protein
ddH₂O – Distilled and deionized water
E. coli – Escherichia coli
EDTA - ethylenediaminetetraacetic acid
eGFP – Enhanced Green Fluorescent Protein
FRET – Fluorescence Resonance Energy Transfer
GFP – Green Fluorescent Protein
GPCR – G-protein coupled receptor
HCl – Hydrogen chloride
HEK-293 – Human Embryonic Kidney cell line
HTS – High Throughput Screein
IPTG - isopropyl β-D-1-thiogalactopyranoside
KCl - Potassium chloride
K₆ – Michaelis-Menten constant
K₂EGTA – Potassium enriched ethylene glycol tetraacetic acid
KOH – Potassium Hydroxide
LB – Luria – Bertani culture media
MgCl₂ – Magnesium chloride
MOPS - 4-Morpholinepropanesulfonic acid
MWCO – Molecular weight cutoff
M13 – Calmodulin binding fragment of myosin light chain kinase
PCR – Polymerase Chain Reaction
polyHis – Poly-Histidine
SA – Sinoatrial
SNR – Signal to Noise Ratio
TAE - Tris-acetate-EDTA
UV – Ultraviolet
YFP – Yellow Fluorescent Protein
1. INTRODUCTION

1.1 The Importance of Intracellular Calcium Concentration

The concentration of intracellular free calcium ions [Ca$^{2+}$] has been implicated as a universal second messenger in muscle and neuronal cells (1). In cardiac muscle cells, the Ca$^{2+}$ current is responsible for the depolarizing phase of the action potential created in the sinoatrial (SA) and the atroventricular (AV) nodes and is also the basis of the contraction for all cardiomyocytes (3). Studying the changes in concentrations of intracellular calcium can lead to a greater understanding of the ionic activity of individual neurons and/or muscle cells as well as further elucidating the ionic communication between neuronal and muscular cells in the heart and other parts of the body.

1.1.1 Action Potential Generation and Muscle Contraction in Cardiomyocytes

The Ca$^{2+}$ current is generated by the migration of calcium ions across the cell membrane via slow calcium-sodium ion channels which stay open for several tenths of a second (3). During this relatively long duration in which the calcium-sodium channels are open they allow both calcium and sodium (Na$^+$) ions to pass through creating a prolonged depolarized state in the cell which causes a plateau in the action potential. Additionally, unlike in skeletal muscle cells, the Ca$^{2+}$ ions that enter the cell during this time directly initiate muscle contraction.

The contractile process of cardiac (and skeletal) muscle is hinged on this excitation-contraction coupling process by which the action potential causes the muscles to contract. This process begins in much the same way as skeletal muscle with the action potential propagated through the entire muscle fiber syncytium by means of the muscle cell’s transverse tubules. The signal from the transverse tubules is then transmitted to the longitudinal membranes of the
sarcoplasmic tubules which causes the release of calcium ions into the muscle’s sarcoplasm from the sarcoplasmic reticulum. These Ca$^{2+}$ ions then diffuse into the myofibrils and catalyze the chemical interactions between the actin and myosin filaments which create the “sliding” mechanism which causes muscle contraction. The primary difference in this process between skeletal and cardiac muscle is a secondary effect of Ca$^{2+}$ in cardiac cells that is absent in skeletal muscle cells. While many Ca$^{2+}$ ions are released from the cisternae of the sarcoplasmic reticulum in both skeletal muscles cells and cardiomyocytes, in cardiac muscle the flux of Ca$^{2+}$ ions from outside of the cell is a significant source of total Ca$^{2+}$ required to catalyze muscle contraction. This extra source of calcium is necessary in cardiomyocytes due to the less developed structure of the cardiac sarcoplasmic reticulae.

1.1.2 Neuronal Cell Calcium Dependence

At the point when an action potential is created in nerve cells, calcium gated channels at the end of the presynaptic terminal open allowing large amounts of Ca$^{2+}$ to pass through. These calcium ions then go on to initiate the release of various neurotransmitter molecules from vesicles maintained within the cell. The amount of neurotransmitter molecules that are released is directly related to the concentration of intracellular Ca$^{2+}$ ions. It is believed that when the Ca$^{2+}$ ions enter the cell, they are associated with protein molecules on the inside surface of the presynaptic membrane. This binding between the calcium ions and these release sites causes the sites to open and allow transmitter vesicles to release their transmitter into the synaptic cleft and subsequently carry the action potential signal on to the next neuron. In the case of acetylcholine, between 2000 and 10,000 molecules can be found inside one vesicle, and there are enough
vesicles held within the presynaptic terminal to transmit from a few hundred to upwards of 10,000 action potentials.

1.2 Detecting Changes in Calcium Ion Concentration

Because of the physiological importance of calcium ions, various methods for calcium detection have been devised over the past few decades. Today, the most widely used detection methods focus around fluorescent bioprobes, but before these methods were discovered, electrophysiological methods and bioluminescent probes were employed for calcium ion detection. The electrophysiological methods focused primarily on patch clamp techniques, but these have only proven marginally successful in high throughput screening. The luminescent probes showed greater promise, but were limited by a less specific scope of detection as well as a low sensitivity. They also exhibited a propensity for signal interference.

1.2.1 Electrophysiological Methods for Calcium Ion Detection

Measuring the concentration of intracellular calcium by electrophysiology means to measure the current that is carried across the cell membrane by calcium gated channels. This method makes use of the patch (or voltage) clamp technique. In this technique, an electronic feedback device is used that adjusts an applied current to match and counter the membrane current so that the membrane voltage is effectively held constant (5, 9, 11). In this way, the number of ions that enter the cell can be calculated by integrating the current over the surface of the cell membrane, however, using this method it is still impossible to calculate the intracellular ion concentration achieved because this depends highly on intracellular buffering effects. Still, these methods can provide precise information about the sources of the rise in [Ca^{2+}]. However,
in order to use this technique for any sort high throughput screening (HTS) however, it is necessary to consider information from more than one cell. Recent researchers have developed new automated patch clamp methods that make HTS more feasible (4-5).

Many companies have created an automated, two-electrode system. Most of these methods have been optimized for use with *Xenopus* oocytes because the ion channels of these cells have been widely expressed and studied. This approach was first introduced commercially by Axon Instruments in the form of the Multiclamp 700B which was capable of recording from two cells simultaneously by means of sophisticated software with automated features such as series resistance and capacitance compensation. This method yielded a small improvement in throughput, but was not capable of being used as a HTS tool (18).

With this new impetus to increase the throughput of their electrophysiological methods, companies began to develop methods to automate a whole-cell voltage clamp. For this, two main approaches have been used. The first attempts to automate the near-conventional approach using patch electrodes, software, and robotics. Flyion, a company founded as a spin-off from the Institute of Physiology at the University of Tübingen, specializes in developing patch clamp and ion channel technologies and they have developed a relatively novel approach in which the cell itself is placed into an electrode and lodged in the opening for recording purposes. This is different from the conventional patch clamp techniques in which the electrode is aligned and place on the cell. This instrument called a Flyscreen® enables whole cell recordings without the need for micromanipulators and also produces giga-ohm seals with solution exchange in low volumes. The second major design for an automated electrophysiological system makes use of a planar arrangement of micron-sized holes in a plastic or glass substrate which serves to replace the conventional electrode or pipette, with miniaturized recording interfaces. In most
approaches, the cell interface is constructed of two chambers that are separated by a substrate with a single micron-sized hole. The wells are then arranged in a 2D format providing a large number of measurement chambers. The cells are loaded into one chamber and negative pressure is used to pull the cells into the holes to form the high resistance (giga-ohm) seals. The membrane within the whole is then mechanically, electrically, or chemically perforated by circulating an intracellular solution containing an ionophore or detergent to obtain a whole-cell recording configuration. This system, unlike the traditional patch clamp, does not need a micromanipulation system, microscope, or vibration isolation mechanism. These factors combine to simplify the design and therefore enable parallel processing to increase the level of throughput. Other technologies have been developed that can record ion channel function by patch clamping up to 16 cells simultaneously on a planar array (5).

While these electrophysiological methods are advancing in terms of their ability to analyze ion channel functionality, and thus their ability to measure calcium ion concentration, at this stage in technological development the bioluminescent and fluorescent probes are still more widely used.

1.2.2. Fluorescent Probes

Because of the difficulties associated with the application of electrophysiological methods for HTS of calcium concentration, classically, the principle technology used to measure cystolic levels of Ca$^{2+}$ utilizes probes whose fluorescent response changes in the presence of calcium ions. The probes that have been used to carry out these methods are low molecular weight dyes, including Fura-2, Fluo-4, Fluo-8, and Calcium 3 (10, 12, 13). These dyes chelate
Ca\(^{2+}\) and, upon chelation, change their fluorescent properties to the extent that is directly proportional to the concentration of free Ca\(^{2+}\) in the sample.

The Ca\(^{2+}\)-chelating dyes have been used in the past two decades as a mode of measuring the changes in calcium levels in response to G-protein coupled receptors (GPCRs). The acetoxymethyl ester forms of the dyes are cell permeable and therefore perfuse into the cell freely after a long incubation period. Once the dyes are inside the cells, they are de-esterified to form a less permeable form and are therefore maintained on the interior of the cell for the duration of the experiment. When the dyes come in contact with calcium ions, their emission wavelengths change which provides a means of detecting the change in Ca\(^{2+}\) levels. A light source is needed to excite the dye in the cells. It is this difference in the luminescent emission when the dye is bound versus when the dye is unbound that gives the measure of the change in [Ca\(^{2+}\)].

Advances in this technology have come in the form of more effective dyes, automated detection systems such as the fluorometric imaging plate reader (32), and the implementation of procedures which obviate extensive cellular washing to remove extracellular dye. The primary limitation of these dyes is the inability for these dyes to be implemented \textit{in vivo}. In recent years, one of the new aims of calcium detection has been to create genetically linked detection mechanisms that can detect changes in calcium concentration in a truly physiological environment. Because these dyes cannot be loaded into tissue samples, it is necessary to create some sort of technology that can precisely and accurately detect small changes in calcium concentration \textit{in vivo}. Consequently, the most recent advances in calcium detection technology have made use of specialized photoproteins to generate signals in the presence of Ca\(^{2+}\).
1.3 Fluorescent Proteins

The use of proteins as a method for calcium concentration detection has become more viable with the discovery of bioluminescent proteins such as aequorin which naturally emit light with changes in [Ca$^{2+}$]. The primary difference between the use of these proteins and the use of the fluorescent dyes comes from the fundamental difference between fluorescence and bio- or chemiluminescence. Because the light emitted from a bioluminescent element comes from an inherent property of the molecule, no exothermic chemical reaction is necessary to create the signal. This also means though, that there is also a much higher background signal. This typically results in a low signal to noise ratio (SNR) which also makes detection of smaller signals more difficult. In the case chemiluminescence however, the light signal emitted is a consequence of a supplementary chemical reaction and consequently yields a much higher SNR because of the absence of a substantial background signal (15, 16).

A major advantage of fluorescent proteins though, is that they maintain a high quantile efficiency such that it takes only a few photon emissions to be detected by sensitive devices (10). This means that in order to be detected only a few intracellular bio-emitters need to be expressed. Proteins expressed at levels as low as in the attomolar range have been used to detect intracellular [Ca$^{2+}$] (9). This advantage also makes miniaturization much more feasible because of the ability to carry out experimentation in small fluid volumes. Also, because these are intracellular proteins, by using recombinant DNA techniques, there is a much greater ability to localize experimentation in animal models. Another major advantage of this technology is that the photoproteins have a much lower buffering effect than the chelating dyes on the Ca$^{2+}$ ions. This limits the level of intracellular Ca$^{2+}$ that is sequestered, thus helping to maintain the cell’s functional integrity.
1.3.1 The Green Fluorescent Protein

Originally discovered in a species of the jellyfish *Aequorea* aequorea, Green Fluorescent Protein (GFP) and its derivatives have in the past 40 years become extremely powerful, and some of the most widely used fluorescent sensing proteins (29-31). GFP was discovered in 1961 by Osamu Shimomura during his studies with the Friday Harbor Laboratories of the University of Washington, “on a small island near Victoria, British Columbia, Canada” (6). He and his colleagues were, at the time, primarily focused on extracting and characterizing a substance, now known as the bioluminescent protein aequorin, which emitted a blue light in the presence of Ca\(^{2+}\). However, during the purification of aequorin, they also noticed a green fluorescent substance in the light organs of the jellyfish which they then recognized as a protein and aptly named it Green Fluorescent Protein or GFP (30, 34).

The biological function of GFP is to “shift the color of bioluminescence from blue to green in luminous coelenterates (jellyfish, hydroids, sea pansies, and sea pens)” (6). This protein has been implicated as a gene marker to be able to tell whether or not a gene is being expressed. It has also been widely used as a genetic fusion partner with host proteins to monitor that proteins location and biological fate. The ideal fusion protein will maintain the biological function of the endogenous protein but also be fluorescent. At this point, GFP has been successfully targeted to every major organelle. However, GFP has also been used as an active indicator by itself.
GFPs that act as indicators of their environment have been created via combinations of directed and random mutagenesis (33). The first such indicator was created by fusion of a GFP molecule with the Shaker potassium channel (9). In this way depolarization of the cell membrane would yield an optical signal. The most effective use of this technology however, has come in conjunction with the exploitation of the fluorescent resonance energy transfer (FRET) of GFPs with different colors. FRET is a phenomenon that occurs when two fluorophores are in molecular proximity and the emission spectrum of one, the donor, overlaps with the excitation
spectrum of the other, the acceptor. When this occurs, the energy from the donor causes the acceptor to emit and effectively quench the donor, which would normally emit light in the absence of the acceptor. Any biochemical molecule that changes the distance between these two fluorophores or changes the orientation of their transition dipoles will change the efficiency of the energy transfer.

The first useful biochemical indicators that were based on GFP were the Ca\textsuperscript{2+} sensors independently, though almost simultaneously, developed by Romoser et. al. and Miyawaki et. al. (17, 23). Romoser et. al. (23) linked class 6 BFP and class 2 GFP mutants with a 26-residue spacer to the calmodulin (CaM)-binding domain from the avian smooth muscle myosin light chain kinase. The addition of the spacer allowed FRET to occur between the BFP and the GFP. Addition of the CaM moiety interrupted the FRET. These proteins were then microinjected into HEK-293 cells in which elevations of cystolic [Ca\textsuperscript{2+}] resulted in a direct change in the emission spectra. Miyawaki et. al. (17) fused class 5 CFP (BFP) with class 2 GFP or class 4 yellow fluorescent protein (YFP) to the C-terminus of M13 which is the CaM binding peptide from the skeletal muscle myosin light chain kinase. The binding of Ca\textsuperscript{2+} to the CaM caused it to associate with the M13 which increased the FRET. Then, by creating mutations specialized for mammalian expression, these indicators were made sufficiently bright to be introduced into cells via DNA transfection rather than the protein microinjection protocols that had been used previously. These two studies effectively solidified GFP and its derivatives as viable methods for detection of Ca\textsuperscript{2+}, however there are advantages and disadvantages for using GFP-based sensors and for using FRET techniques.

GFP-based Ca\textsuperscript{2+} indicators are advantageous because they are applicable to most organisms and can even be targeted toward different tissues. However in order to target those
tissues, gen transfection is necessary. GFPs are not likely to diffuse so well in the cell as to blur spatial gradients, but the maximum change in emission ratio is, at present, less than the change observed for small-molecule dyes. With GFPs, modular construction is improved by mutagenesis and there are also good optical properties such as visible excitation, the ability to generate an emission ratio, and high photostability. Also, cDNAs or improved sequences are cheap to replicated and distribute. However, the binding kinetics of GFPs are somewhat slower and the CaM and M13 moieties may have additional biological activity (12, 13).

Using FRET as a signal transduction technique is advantageous because it is suitable for in vitro studies and, except in yeast, it can be applied in vivo. It can respond dynamically to post-translational modifications, has high temporal and spatial resolution, and interacting proteins need not be confined to the nucleus of the cell. However, in order for any FRET to occur, fusion proteins must be created in which both proteins are fluorescently active. There is also a limit as to how far away from one another the two fluorophores can be. Even when there is no association, spectral overlap will contribute some signal at the FRET wavelengths which will make uncommon interactions difficult to detect. There is a drastic need for positive and negative controls as references, and homodimerization is much more difficult to study than heterodimerization. A great advantage though, is that the efficiency of FRET at 100% complexation can also give some structural information (36).

Because of the advantages associated with GFPs, specifically their ability to be expressed in vivo, numerous studies have focused on creating sensing proteins for various applications. Two such proteins, GCaMP2 and mCherry, will be the focus of the remainder of this paper.
1.4 GCaMP2 – Structure and Development

The GCaMP sensor is composed of a circularly permutated enhanced GFP moiety (cpEGFP) bound to a calmodulin (CaM) moiety and the CaM-binding peptide M13pep from the myosin light chain kinase (Fig. 2). It was developed by Junichi Nakai et. al. (19) using cpEGFP because of its apparent advantages in fluorescence as compared to FRET-based Ca\(^{2+}\) probes. In this protein, the N-terminus of the cpEGFP is connected to the M13 fragment of the myosin light chain kinase and the C-terminus is associated with the CaM moiety. The binding of Ca\(^{2+}\) to calmodulin increases its affinity for the M13 region causing a conformational change in the protein which then creates a change in the fluorescence intensity. During the development of GCaMP, Nakai et. al. also tested a probe with the CaM linked to the N-terminus of the cpEGFP, but this conformation only resulted in small responses to Ca\(^{2+}\) (19). The probes were screened in HEK cells, and confocal imaging showed that the proteins are expressed in the cytosol. After being purified from bacteria, the excitation and emission spectra of GCaMP were found to be 489 nm and 509 nm respectively which are similar to those of EGFP. When Ca\(^{2+}\) was added, the fluorescence intensity was increased by ~4.5 fold and Ca\(^{2+}\) titration of GCaMP yielded an apparent K\(_d\) for Ca\(^{2+}\) of 235 nM and a Hill coefficient of 3.3 (at 0.3 μM [protein]).
This first sensor was unfortunately extremely dim and folded poorly at physiological temperatures however. Through stabilizing mutations and random mutagenesis, GCaMP2 was developed with significant improvements on the folding and fluorescence of its predecessor. The first three mutations of GCaMP (or GCaMP1 as it was later called) were V163A, S175G, and A206K. V163A and S175G had been described previously as mutations that would improve the temperature stability of GFP and A206K was introduced to inhibit GFP dimerization. In GCaMP1, V163A and S175G resulted in improvement in the brightness of the molecule, however, neither these mutations nor A206K were able to correct the instability of the molecule above 30°C. A 35-residue polyHis plasmid leader sequence (pRSET) was then linked to the M13 moiety at the N-terminus for purification and this newly mutated GCaMP1 (termed GCaMP1.6) underwent PCR-based random mutagenesis and bacterial colonies displaying the brightest fluorescence at 37°C were selected and sequenced.

Figure 3 Crystal structure of GCaMP2 at different angles. Showing the relationship between the cpEGFP moiety and the CaM moiety. From Wang, et. al. 2008 (35).
The addition of this sequence was found to be crucial in the improvement of the thermal stability of this molecule, as removal of this leader sequence resulted in loss of fluorescence at 37°C. This random mutagenesis also yielded two new mutations, D180Y and V93I in separate β-sheets which improved the brightness of the cpEGFP molecule. This new pRSET-M13-cpEGFP-CaM molecule, GCaMP2 is ~200 times brighter than GCaMP1 and ~6 times brighter than GCaMP1.6. It displays similar 4- to 5-fold increase in signal between 0 and saturating Ca²⁺, and is thermally stable at 37°C. Titrations of purified GCaMP2 proteins with fixed Ca²⁺ solutions indicate highly cooperative Ca²⁺ with K_d = 146 nM and a Hill coefficient of 3.8. There was also no shown Mg²⁺ dependence (35).

The crystal structure of GCaMP2 was solved and analyzed to provide a structural basis for the improvements made on GCaMP1-1.6 (Fig. 3). Akerboom et. al. (1) and Wang et. al. (35) determined crystal structures of both the calcium-free and calcium-bound states of the molecule, and used these structures to understand the molecular mechanism of Ca²⁺ - dependent fluorescence. They first found that GCaMP2 exists in a monomeric and dimeric form and that these two forms have different absorption and fluorescence properties. This suggests an altered chromophore environment between the states. It was also found that the dimeric species have significantly lower chromophore absorption and fluorescence. This group generated various GCaMP2 mutants (on the context of GCaMP2-T116V), each used to investigate one of three different hypotheses:

a) mutating the CaM or the cpEGFP-CaM (GGTGGS) linker to block solvent access to the EGFP chromophore would improve brightness

b) disrupting the cpEGFP-CaM interfaces seen in the crystal structure would decrease sensor performance, and
c) mutating EGFP β-barrel positions observed to be solvent-exposed in the GCaMP2 structure (though not in EGFP itself) due to circular permutation would alter sensor function

GCaMP2-T116V was used for all the experiments because it had been previously described to increase the wild type EGFP excited state proton transfer. Along with this mutation, eighteen other mutations, through three mutant classes, were created by site-directed mutagenesis and were subsequently analyzed by measuring fluorescence emissions of purified protein. From these mutations, it was found that large scale conformational changes which alter relative domain orientations serve to change the stereochemical functionality presented to the chromophore at the cpEGFP barrel opening, and also serve to control the access of solvent molecules. Also, though it was found that GCaMP2 can exist in both a monomeric and dimeric form, the biophysical characterization in solution showed that, at physiological concentrations, GCaMP2 exists in a predominately monomeric form. The crystal structures also demonstrated the close proximity of the M13-cpEGFP linker to the EGFP chromophore relative to the cpEGFP-CaM linker. In the M13-cpEGFP linker, it was noted in the original design of GCaMP1 that positively charged side chains at position 61 of the chain abolished calcium response, whereas serine or threonine residues in that position resulted in photoisomerization.

The mutations on GCaMP2 have helped researchers to further understand the molecular mechanisms of the fluorescence in this molecule. While further study is needed to completely optimize and understand GCaMP2, at this point, this protein has been used for *in vivo* sensing (30), and in this investigation has been genetically linked to another fluorescent protein, mCherry, to make a dimerized, duel-wavelength emission sensor.
1.5 mCherry – Structure and Development

The bioluminescent protein, mCherry, a red monomeric protein originally derived from Discosoma sp. and more directly derived from a second generation protein, mRFP1 (25-27). MRFP1 was derived from DsRed and successfully overcame DsRed’s tetramerization and sluggish maturation and also exceeded DsRed’s excitation and emission wavelength. However, during the evolution of mRFP1, the extinction coefficient, fluorescence quantum yield, and photostability was decreased. As with the development of GCaMP2 from GCaMP1, mRFP1 underwent numerous rounds of directed evolution.

The red chromophore of DsRed, and subsequently mCherry, is a result of post-translational modification of the Gln$^{66}$, Tyr$^{67}$, and Gly$^{68}$ residues into an imidazolinione heterocycle with $\mu$-hydroxybenzylidene and acylimine substituents (14, 20). The first step to improving the brightness of mRFP1 was the construction of a directed library containing residues surrounding the chromophore, including at position 66, which were randomized. From this process, the mutation Q66M was determined which promoted more complete maturation and provided an additional 5 nm red-shift of both the excitation and the emission spectra relative to mRFP1. Then next step was to reduce the sensitivity of this new mRFP1.1 to N-terminal fusions. Because GFP is relatively indifferent to N- or C-terminal fusions, by replacing the first seven amino acids of mRFP1.1 with the corresponding residues from eGFP (MVSKGEE) followed by a spacer sequence (NNMA), and appending the last seven amino acids of GFP to the C-terminus, a new mRFP1.3 was developed. This new mutant not only maintained the high level of fluorescence as mRFP1.1, but did so regardless of fusions to its N-terminus. More rounds of screening random libraries based on mRFP1.3 and wavelength-shifted mRFP variants yielded the beneficial folding mutations V7I and M128K which were used to create mRFP1.4. Further
randomization, this time at position 163 in mRFP1.4 led to the substitution M163Q which resulted in a nearly complete disappearance of the absorbance peak at ~510 nm. This fifth generation mutant underwent two more rounds of directed evolution to produce the final substitutions N6aD, R17K, K194N, T195V, and D196N which were then implemented in the final, optimized, mCherry.

It was necessary to test whether or not the introduction of the GFP-type termini into the mRFP variants would benefit the fusion proteins expressed in mammalian cells so mRFP1 and mCherry were fused to the N-terminus of α-tubulin. The proteins were expressed in HeLa cells and mCherry was found to have successfully been incorporated into the cell’s microtubules, while mRFP was present in the cytoplasm (27). Similar experiments were carried out in Madin-Darby canine kidney and primary human fibroblasts and resulted in equivalent results. The emission and excitation maxima for mCherry are 610 nm and 587 nm respectively. The fluorescence quantum yield is 0.22, and the extinction coefficient is 72,000 M⁻¹cm⁻¹. Because of its excitation and emission maxima, its long wavelengths, high photostability, fast maturation times, high extinction coefficient, tolerance for N-terminal fusions, and excellent pH resistance, even though mCherry has a relatively low quantum yield, it has still rendered its predecessor obsolete.

1.6 GCaMP2 – mCherry Fusion Protein

In order to harness the fluorescence power of both of these proteins, in this study, the fusion of the two via recombinant DNA techniques has been explored. In past cases of calcium sensing, it has been possible to detect certain levels of calcium ions in vivo by simply using a monomeric fluorescent protein (31, 33). However, because of the various levels of expression in
each cell, false positive results can occur due to high levels of protein expression. That is to say that though a high signal is observed, it may sometimes be a result of higher levels of protein expression in a given cell rather than an actual increase in the concentration of intracellular calcium ions.

In order to overcome this obstacle, the two proteins, GCaMP2 and mCherry are fused together, and expressed in bacterial cells so as to generate a fluorescence ratio between the two molecules. Since mCherry does not inherently react to calcium, there exists a base level of red fluorescence. When mCherry is fused to GCaMP2, in the presence of calcium, the calcium induced emission from GCaMP2 will interact with that from mCherry and the ratio of fluorescence between mCherry and GCaMP2 will quantitatively display the presence of Ca$^{2+}$ regardless of the protein concentration. This is an important step in the direction of \textit{in vivo} sensing because taking the ratio effectively eliminates a dependent variable in the calculation of calcium concentration. Also, because the intracellular protein concentration does not need to be determined, use of this fusion protein will take time off of experimental procedures by simplifying experimental design.
2. METHODS

2.1 Plasmid Acquisition – Amplification, Digestion, DNA purification, Ligation

Four separate mutations were studied. Each mutation was expected to increase the level of calcium interaction. The mutations were as follows:

1. T203V in EGFP and D78Y in CaM
2. T203V in EGFP

In mutants 3 and 4 the M13 moiety was replaced the CaMKII peptide (KII):

KII DNA sequence
TTCAATGCCCCGCGCAAACTGAAAAGGCGCCATTCTGACCACATATGCTGCAACCCGC

3. N105T in EGFP
4. T203V in EGFP and D78Y in CaM

The first step in creating the bacterial plasmids to be expressed in the competent E. coli cells was to extract the mCherry-GCaMP2 region from the mammalian expression vector that it was originally in and insert it into the pRSET bacterial expression vector. The protocol is as follows:

Digestion

1. Aliquot 10 μL of the mammalian expression vector containing the desired insert region into a 1.5 mL eppendorf tube
2. To this add 5 μL of each of the restriction enzymes EcoRI and HindIII ensuring that the total enzyme volume is < 1/10 total volume
3. Add 10 μL 10X Digestion Buffer from Fermentas
4. Add 70 μL H2O to bring the volume to 100 μL.
5. Incubate this mixture for 2 hr. at 37°C

Separation
1. Create agarose gel by adding 1% UltraPure Agarose Gel powder to 100 mL TAE
2. Mix by swirling
3. Microwave for 50 s, shake, microwave for 50 s
4. Add 4 μL of ethidium bromide and mix
5. Let cool ~10 minutes
6. Pour solution into gel mold and let sit for 30 min to allow cross-linking to occur
7. Mix sample with 6x dye solution
8. Load solution into gel electrophoresis wells
9. Run gel for 20 – 30 minutes
10. Take a photo (Fig. 4) of the gel in UV light for insertion into lab notebook (Fig 1)

Figure 4 Electrophoretic gel separation of the insert region from the mammalian cell expression vector. Two distinct bands show the successful separation of the two regions. The smaller band is the insert to be purified.

Since the insert region is only a small portion of the entire portion we know that the band to be cut and purified from the gel is the lower band. Previous gel purifications with the use of a DNA ladder have told us this.

Purification – Procedure modified from QIAquick user manual
1. Add 3 volumes of Buffer QG (Binding Buffer) to 1 volume of sample and mix

2. Check to see if the color of the solution is yellow, if not, add 10 μL of 3 M sodium acetate, pH 5.0 and mix

3. Place QIAquick spin column in 2 mL collection tube

4. To bind DNA, apply sample to QIAquick column and centrifuge for 30-60s

5. Discard flow through and place QIAquick column back into same tube

6. To wash, Add 0.75 mL Wash Buffer to the QIAquick column and centrifuge for 30-60 s. Repeat 2 to 3 times.

7. Discard flow through and place the QIAquick column back in same tube. Centrifuge column for 1 min. Make sure that flow through is discarded before additional centrifugation

8. Place QIAquick column in a clean 1.5 mL microcentrifuge tube

9. To elute DNA, add 50 mL Buffer EB (Elution Buffer) to the center of the membrane and incubate for 2 min.

10. Centrifuge for 1 min and collect flow through

Once the insert region was purified it was stored at −20°C. Next, the vector was prepared using the same procedure as above, except that, in this instance, a pRSET expression plasmid was used instead of the mammalian expression plasmid. For gel purification, the larger band was purified. Once the DNA was extracted and purified, DNA ligation was employed to form a viable plasmid for transformation into *E. coli* cells.

**Ligation**

1. Add 6 μL of vector material and 5 μL insert to a small tube.
2. Add 2 μL 10X Buffer as well as 6 μL ddH₂O.
3. Add 1 μL DNA Ligase and allow to incubate at room temperature (~25°C) for no less than 2 hr.

2.2 Plasmid Transformation and Purification

From the ligation product a viable plasmid was amplified via bacterial transformation into DH5α cells. The final cell transformation would be in BL21*DE3 cells but because of their cost, and the high level of uncertainty associated with the ligation process, the less expensive DH5α cells were used to harvest the plasmid.

Plasmid Amplification – Following Ligation

1. Add ligation product to 20 μL cell solution.
2. Flick then jerk to push cells to the bottom of the tube
3. Incubate cells on ice for 30 min
4. Heat shock the cells at 42°C for 1 min.
5. Put directly on ice for 2 min.
6. Add 1 mL Luria-Bertani (LB) media to the tube
7. Incubate and shake for 1 hr at 37°C
8. Plate on ampicillin (Amp) resistant Agar-LB plates and incubate overnight (< 16 hr) at 37°C.
9. If colonies are detected the next day, pick one up and use to inoculate 5 mL LB media with 1:1000 dilution of ampicillin
10. Incubate overnight while shaking at 37°C
After incubation, the Fermentas Mini-Prep kit was used to purify the plasmids for transformation into BL21*DE3 cells.

**MiniPrep – Procedure Adapted from Fermentas MiniPrep Kit**

1. Resuspend cells in 250 μL of resuspension solution
2. Transfer cells to a microcentrifuge tube ensuring by vortexing or pipetting up and down that the bacterial solution itself is completely resuspended
3. Add 250 μL of Lysis solution and invert the tube 4 to 6 times
4. Add 350 μL Neutralization Solution and invert 4 to 6 times
5. Centrifuge for 5 min to pellet cellular debris and chromosomal DNA
6. Avoid disturbing the pellet and transfer supernatant to GeneJET spin column and centrifuge for 1 min
7. Discard flow through and add 500 μL Wash solution and centrifuge for 30 – 60 s. Repeat two times
8. Discard flow through and centrifuge once more and then place spin column into a fresh 1.5 mL microcentrifuge tube.
9. Add 50 μL Elution Buffer.
10. Incubate for 2 minutes at room temperature (~25°C)
11. Centrifuge for 2 minutes to elute. Collect flow through.

**Plasmid Transformation**

1. Add 2 μL of plasmid to 20 μL cell solution.
2. Flick then jerk to push cells to the bottom of the tube
3. Incubate cells on ice for 30 min
4. Heat shock the cells at 42°C for 1 min.
5. Put directly on ice for 2 min.

6. Add 1 mL Luria-Bertani (LB) media to the tube

7. Incubate and shake for 1 hr at 37°C

8. Plate on ampicillin (Amp) resistant Agar-LB plates and incubate overnight (< 16 hr) at 37°C.

In order to ensure that the bacterial expression plasmid contains the desired insert sequence, the entire plasmid was sent out to be sequenced.

**Sequencing**

1. Add 5 – 10 μL of the purified plasmid to a small eppendorf tube

2. To this add 8 μL primer

Below is shown a figure depicting an annotated primary structure of the fusion protein.

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**Figure 5** Annotated primary structure of GCaMP2-mCherry fusion protein. Shown are the distinct moieties inherent in the protein whose characteristics combine to generate the fluorescent signal. The mutations are primarily created in the green
Once colonies of BL21 plasmid-containing cells had been achieved, they were cultured in LB media to increase the number of cells expressing the target protein. In this step the desire is to induce the cells to produce as much protein as possible so that there is a high enough concentration to carry out subsequent experimentation. In order to induce the cells, a protein expression inducing agent, isopropyl β-D-1-thiogalactopyranoside (IPTG), was added. IPTG was chosen because of its ability to induce gene expression in E. coli cells (2).

Protein Amplification

1. Inoculate 25 mL of LB media with 0.001% ampicillin by volume with 80 – 120 clone colonies.

2. Incubate for 4 – 6 hours at 37°C while shaking

3. Transfer solution to a 500 mL flask of LB media plus 0.001% ampicillin

4. Incubate at 37°C for 4 – 6 hours while shaking

5. Add IPTG at a 1:1000 dilution

6. Shake the solution for 16 – 20 hrs at room temperature

7. Harvest cells using 50 mL collection tubes via centrifugation

The harvested cells were then frozen down using liquid nitrogen (N₂). After freezing, the cells were lysed via sonication. The sonication was continued until no visible ice was seen in the tube. The protein was then purified via nickel affinity resin. The resin used makes use of the 6X His tag inherent in the protein from the plasmid from which it was transcribed. Because the His tag is positively charged, the protein will be attracted to the negatively charged, nickel-based stationary phase thus pulling the protein out of solution. In order to elute, a highly concentrated
imidazole solution is used whose similar functional group competes with the His tag of the protein thus displacing the protein back into solution, allowing it to be collected.

**Protein Purification**

1. Freeze cells with liquid N₂
2. Sonicate cells until no visible ice is left in the solution
3. Centrifuge the tubes at high speed for 15 min to separate out any cellular debris or chromosomal DNA.
4. Add 3 mL of Ni affinity resin ensuring that the resin was washed using filter sterilized ddH₂O prior to use
5. Allow the affinity beads to bind the protein by rotating the tube for 5 to 10 minutes
6. Centrifuge at low speed (< 2000G) at 4°C
7. Discard the supernatant and resuspend the resin in 5 to 8 mL of binding buffer solution
8. Transferred solution to an empty chromatography column in a 15 mL tube
9. Centrifuged the column and its contents for one minute at 4°C at low speed to wash.
   Repeat 4 to 6 times.
10. Placed the column inside a new 15 mL tube for elution
11. Add 1.5 mL of the elution buffer the column and allow it to incubate for 2 minutes
12. Centrifuged for 1 to 2 minutes at low speed at 4°C to elute. Collect flow through in a 15 mL tube.

This eluted protein was then concentrated using a Centriplus Centrifugal or VIVASPIN Filter Unit (5,000 MWCO). This unit is designed with a pore size such that water and salts can pass
through but that the protein is contained inside the unit’s loading chamber. This step is meant to concentrate the protein, but also to cleanse the protein of imidizole and extraneous salts.

**Protein Concentration**

1. Load sample into VIVASPIN Filter unit housed in a 50 mL collection tube
2. Fill the rest of the unit with PBS and centrifuge at high speed for ~1.5 hours or until the volume of the solution reaches 0.75 mL.
3. Refill with PBS and centrifuge at high speed. Repeat 2 – 3 times.
4. Transfer concentrated protein to a 1.5 mL eppendorf tube. If not for immediate use, flash freeze with liquid N₂ and store at –80°C

Before any sort of fluorescence measurements could be taken, it was necessary to determine the concentration of each protein in the solution. To do this the Biorad plate reader was used in conjunction with Pierce 660 solution. This solution changes the fluorescence emission wavelength of its protein solution to 660 nm, the intensity of which is determined by the concentration of the protein. In this way, fluorescence is used to generate concentration data.

**Concentration Determination**

1. Add 10 μL of Pierce 660 reagent to 100 μL of sample in a 96 well plate
2. Incubate at room temperature for 5 min
3. Read the plate at 660 nm to generate fluorescence data for concentration acquisition

**2.3 Calcium, Magnesium, and pH Titration**

The calcium titration was conducted using the calcium calibration buffer kit from Biotium Inc. This kit was used to create a dilution series of free calcium ions that could be used to determine the fusion protein’s calcium dependence. The kit uses two components. The first
(Component A) is a solution of 10 mM potassium enriched ethylene glycol tetraacetic acid (K$_2$EGTA), 100 mM potassium chloride (KCl) and 10 mM 4-Morpholinepropanesulfonic acid (MOPS) at pH 7.20 at room temperature. The second (Component B) is a solution of 10 mM CaEGTA, 100 mM KCl, and 10 mM MOPS at pH 7.20. By mixing different ratios of these two components, any desired free calcium concentration between zero and 40 μM can be theoretically produced as indicated by the formula:

$$[Ca^{++}] = K_d^{EGTA} \times \left( \frac{[CaEGTA]}{K_2^{EGTA}} \right)$$

where $K_d^{EGTA}$ is the dissociation constant of CaEGTA whose value is also a function of temperature, ionic strength and pH. A table of these values was provided with the protocol at 20°C and 37°C and at different pHs. In this experiment, this kit was used to generate 11 stock solutions of calcium indicator buffers with known free calcium concentrations ranging from zero to 39.8 μM.

The 11 calibration solutions contained 0 to 10 mM CaEGTA and were created by simply diluting the 10 mM Component B solution with the 0 mM Component A solution. The calibration curves were used to build up a picture of the calcium response to different calcium concentrations. However, in future studies it will be good to have these data as a point of reference.

Magnesium titration solutions were created in the same way as the calcium calibration solutions except that a 1 M solution of MgCl$_2$ was added to each solution to a final concentration of 1 mM MgCl$_2$. These solutions were then used to create the Mg$^{2+}$ dependence curves.

The pH calibration solutions were created by using Component B of the calcium calibration buffer kit and simply varying the pH of these 10 mM solutions with HCl and KOH.
2.4 Fluorescence Measurement

The solutions created above were used to generate the fluorescence data for the calcium dependence. In order to do this the protein solutions were diluted to a constant concentration and the mixed with the necessary solution for the experiment being performed. The fluorescence measurements were carried out at 485 nm and 575 nm because these two wavelengths are the ones at which GCaMP2 and mCherry are excited respectively.

Fluorescence Measurement

1. Dilute all samples to > 5 μM
2. Add < 1/10 of the final volume to the calcium/magnesium/pH solution in black, flat bottom 96 well plates. Ensure that final concentration of protein is > 500 nM
3. Read fluorescence using plate reader testing 485 nm and 575 nm wavelengths
4. Create calibration curve by graphing fluorescence intensity by calcium concentration or by pH depending on the experiment

The data collected here were then graphed using Microsoft Excel. Each experiment was completed once so the variability of the results cannot be determined, however, the experiments presented here are meant to serve as a point of departure for further studies and, from the trends to be presented below, we have a much better idea of this protein’s calcium-related characteristics.
3. RESULTS AND DISCUSSION

3.1 Plasmid Design

The coding regions corresponding to GCaMP2 and mCherry were amplified by means of traditional PCR methods and subsequently cloned into the pRSET expression plasmid discussed in Nakai et. al.

The pRSET plasmid (Fig. 6) is a pUC-derived expression vector which contains the necessary origin of replication and expresses under a T7 promoter. It contains a selection marker for ampicillin, and is typically used in this type of study because of its ability to yield high expression in E. coli cells (2). Using this plasmid it is possible to insert DNA downstream as well as in frame with a sequence that will encode the N-terminal fusion peptide. The sequence contains an ATG codon for translation initiation as well as a poly-His tag which functions as a metal binding domain in the translated protein. In addition to these sequences, there is a transcript stabilizing sequence from gene 10 of the T7 phage, the Xpress epitope, and also an enterokinase cleavage recognition sequence.
Figure 6 Map of the pRSET bacterial expression vector. This shows the components of the pRSET vector used to express the inserted gene encoding for the mCherry-GCaMP2 fusion protein. This plasmid is designed to be successfully expressed in E. Coli cells. The 6X His tag is necessary for the purification process because of the positive charge that it gives the protein which is then attracted to the negatively charged Ni stationary phase. It is also necessary for the elution of the protein via imidizole competition (21).

The mutations were characterized based on their fluorescence emission spectra in the presence of calcium, without inhibitor, with competitive Mg$^{2+}$ inhibition, and lastly with high calcium and varying pH levels.

The mutations were chosen based on information from Akerboom et. al. (2009). The primary mutation, eGFP T203V, had been shown previously to increase wild-type eGFP excited-state proton transfer (1). Another mutation, cam D78Y, had been expected to block solvent access to the chromophore by introducing a bulkier amino acid side chain near the cpeGFP opening. Akerboom et. al. reported that this mutation showed brighter apo state and a brighter calcium-bound state. This mutant showed the highest dynamic range of all of the mutants tested. Absorbance and fluorescence scans also showed that this mutation has a lower proportion of protonated chromophore in the calcium-bound state which helps to improve the signal to noise ratio. Position 381 of GCaMP2 (cam 78) is located on the flexible region of the calmodulin.
moiety between the two calcium binding lobes and thus packs directly against the cpEGFP opening in the Ca\(^{2+}\)-monomer structures. The side chain of a tyrosine at this position was thought to extend into the opening of the cpEGFP beta barrel and come within a few angstroms of the chromophore and therefore influence fluorescence.

In the last mutations (3 and 4) discussed above, the M13 moiety is changed to a calmodulin-dependent protein kinase II (CaMKII) moiety. This change was made based on information from Yamniuk and Vogel (2004) which says that because of the increase unwinding in the central region of this calmodulin peptide sequence, there is an increase binding of calmodulin (28, 37). The thought is that because of the increased binding affinity, the response to calcium will be more sensitive and increase the dynamic range of the protein.

3.2 Calcium Calibration Curve

The curves shown below (Fig. 7 – 16) are overlaid graphs depicting the fluorescence behavior for the four mutations and the control when excited with light at wavelengths of 485 (green) and 575 (red) nm. These wavelengths were chosen because of the fused nature of the protein being tested. Because the mCherry subunit absorbs at 575 nm and the GCaMP2 subunit absorbs at 485 nm, these two were measured so that a ratio of the emissions can be taken to determine an absolute concentration of the \([\text{Ca}^{2+}]\) without any dependence on intracellular protein concentration. For the first three samples, A, B, and C (control), the protein exhibited a sigmoid-like response with increased calcium with respect to excitation at 485 nm. For all of the samples, excitation at 575 nm resulted in a relatively constant emission. This indicates that the mCherry subunit is not affected by changes in calcium when fused to the GCaMP2 protein.
**Figure 7** Fluorescence response for mutation A (T203V in EGFP and D78Y in CaM). Sigmoid-like response with the excitation at 485nm (Green) and a relatively constant response at excitation at 575nm (Red). Dynamic range between 0.1 μM and 1 μM. Data reported from 1 replicate.

**Figure 8** Fluorescence response for mutation B (T203V in EGFP). Like A, sigmoid-like response with excitation at 485nm (Green) and constant response with excitation at 575nm (Red). Data reported from 1 replicate.
Figure 9 Fluorescence response for mutation C (control). Like A and B, sigmoid-like response with excitation at 485nm (Green) and constant response with excitation at 575nm (Red). Data reported from 1 replicate.

In the next two samples, the M13 moiety of the protein was substituted with the CaMKII moiety. Both samples exhibited relatively linear response. Mutation D showed a relatively constant response when excited at both wavelengths whereas mutation E, in accordance with all of the other samples, showed a relatively constant response when excited at 575 nm. However, there was a relatively linear response exhibited when excited at 485 nm. The relatively constant response of mutation D to both wavelengths makes this mutation a poor one for use in determining ratios for the purpose of calcium concentration calculation. Since this mutation seems to be unresponsive to changes in calcium, it will not be a sufficient calcium indicator. Mutation E proves much more responsive to calcium, and because of its relatively linear response, is a much better candidate for use as an indicator due to its increased dynamic range.
Figure 10 Fluorescence response for mutation D (N105T in EGFP). Relatively constant response at 485nm (Green) and constant response with excitation at 575nm (Red). Using this mutation would be difficult to determine a ratio between intensities of green and red spectra because of the small gap between green and red intensities. Data reported from 1 replicate.

Figure 11 Fluorescence response for mutation E (T203V in EGFP and D78Y in CaM). There was again a relatively, pseudo-constant response to excitation at 575 nm. The linear response to excitation at 485 nm is favorable, because this effectively increase the dynamic range of the protein. The large gap between intensities also makes this a good candidate for ratio-based calcium concentration determination. Data reported from 1 replicate.
In analyzing the five different emission spectra for excitation at 575 nm, we see that all exhibit relatively constant intensity responses, but by graphing them next to each other we can get a measure of the average constant intensity for each. We can see that mutations A, B, and C have the greatest background intensities and that D and E have relatively low emission intensities at 575 nm. This variation in the intensity of the emission may have to do with the proximity of one fluorescent moiety to the other. Since the concentrations of protein in each sample was the same, quenching effects may be at play causing this anomalous variation.

![Fluorescent data comparing the five different samples’ intensities when excited at 575 nm. Here samples A, B, and C maintain the highest average intensities, while D and E are only slightly lower than C (control).](image)

**Figure 10** Fluorescent data comparing the five different samples’ intensities when excited at 575 nm. Here samples A, B, and C maintain the highest average intensities, while D and E are only slightly lower than C (control).

The idea behind creating a fusion protein is to determine the ratio of the intensities of the emission spectra collected at each wavelength. The graph below shows these ratios graphed against \([\text{Ca}^{2+}]_{\text{free}}\) to create such standard curves for each of the mutations. We can calculate the dynamic range by inspection of the linear portions of each curve. For mutations A through C the ranges are between 0.1 and 1 \(\mu\text{M}\). Mutation D can be considered horizontal and therefore devoid
of a dynamic range, and the dynamic range of mutation E can be taken as 0.01 µM to ~40 µM. These data show that mutation E has the highest dynamic range, however mutation A has an increase sensitivity than the control. While the primary goal was to increase the dynamic range of the indicator, this increase in sensitivity exhibited in mutation A also begs further study. This supports the hypothesis that D78Y increases the binding affinity of this moiety thus increasing the protein’s ability to bind calcium. In sum, this means that, in future studies, it may be beneficial to substitute the M13 moiety for the CaMKII moiety to possibly increase the dynamic range, or to mutate the existing calmodulin molecule to increase sensitivity. However, both should be further tested due to the apparent lack of sensitivity of mutation D, in which this substitution has also been made. These tests should also be completed again in the future to analyze the variability of the results overall.
Figure 11 Fluorescence data from each mutation. The ratios of intensities at 485 nm to those at 575 nm for each of the five mutations. The sigmoidal behavior is not lost for mutations A through C, and are directly related to calcium concentration. Mutation D shows a constant ratio of ~1 showing the relative insensitivity to Ca^{2+} while mutation E again has the most linear nature.

3.3 Mg^{2+} Interference

In the next experiment, the same calcium titration was used, but in each sample was added 1 mM MgCl$_2$ to generate Mg$^{2+}$ ions which would compete with the Ca$^{2+}$ ions from the calcium buffer solutions. In these trials, there was still a relatively constant response for excitation at 575 nm, but the response to excitation at 485 nm was different compared to the no Mg$^{2+}$ condition. In all cases there was still an upward trend, but as would be expected, at lower [Ca$^{2+}$] the response was less uniform. This would predicate the possibility that the Mg$^{2+}$ does indeed have an effect on the emission spectra of the proteins, but, because the curves did not lose their basic shapes, the effect does not seem to be debilitating.
Figure 12  Fluorescence data for mutation A. (A) Here the fluorescence data for the Mg^{2+} and no Mg^{2+} conditions are graphed. The trends have not been completely distorted by the Mg^{2+} competition. (B) The dynamic range has become limited by the Mg^{2+}. Data reported from 1 replicate.
**Figure 13** Fluorescence data for mutation B. (A) Here the fluorescence data for the Mg$^{2+}$ and no Mg$^{2+}$ conditions are graphed. The trends have not been completely distorted by the Mg$^{2+}$ competition. Basal level of emission at 575 nm has decreased and the max at 485 nm has also decreased (B) The dynamic range has become limited by the Mg$^{2+}$ and the ratio shows how the peak response is limited. Data reported from 1 replicate.
Figure 14 fluorescence data for mutation C (control). (A) Here the fluorescence data for the Mg2+ and no Mg2+ conditions are graphed. The trends have not been completely distorted by the Mg2+ competition. Like mutations A and B, the 485 nm max has been lowered, but unlike those, there was a limited effect on the emission at 575 nm (B) The dynamic range has become limited by the Mg2+ as with the two previous mutations. Data reported from 1 replicate.
Figure 15 Fluorescence data for mutation D. (A) Here the fluorescence data for the Mg\(^{2+}\) and no Mg\(^{2+}\) conditions are graphed. The trends have not been completely distorted by the Mg\(^{2+}\) competition. Here there is little effect by Mg\(^{2+}\) which is to be expected because there is also little effect by Ca\(^{2+}\). (B) The fluctuation of this graph is anomalous but this curve is in accordance with the trend that Mg\(^{2+}\) distorts the fluorescence signal. Data reported from 1 replicate.
Figure 16 Fluorescence data for mutation E. (A) Here the fluorescence data for the Mg2+ and no Mg2+ conditions are graphed. The trends have not been completely distorted by the Mg2+ competition. Here there is little effect by Mg2+ but the shape of the curve in both cases is the same. As with the previous mutations, the signal is shifted. (B) The ratio of the intensities as the two wavelengths is relatively constant. Data reported from 1 replicate.
3.4 pH Response

The next phase of experimentation was to test the proteins’ responses to calcium at varying levels of pH (Fig 17 – 22). From these graphs, a pattern of activity was observed focusing around the physiological pH of 7.4. This is in accordance with the hypothesis that the protein will be effective as an in vivo measure. It is important to know that the protein will be able to function at physiological pH in order to implement it in future transgenic experimentation. For all of the mutations, the activity increases the pH increases towards physiological pH and then decreases dramatically as the pH is increases to 12. For low pHs, the emissions at 575 nm is relatively constant while the emissions at 485 nm are increasing. This shows that at lower pHs, compared to the response of the GCaMP2 moiety, the function of the mCherry moiety is relatively unaffected. This shows also that it is indeed the calcium function that is being affected by the change in pH in these experiments. For this experiment, it is very important to carry out subsequent experimentation to analyze the variability of these results. Because of the relatively sporadic distribution of the signal with the change in pH, it is important to understand whether or not there is some sort of trend that is not readily exemplified here because of limited replication of the experiment. From these data though, the hypothesis can be made that the protein is ineffective below a pH of 5 and that from this pH, the response should increase as the pH range increases to the physiological pH. At this pH it will start to lose its effectiveness and then, as the pH increase to 12 and beyond, the protein will again be rendered completely ineffective.
Figure 17 Calcium response for varying pH for mutation A. As the pH increase towards physiological pH the intensity increases. The drop in pH at 7.6 is not in accordance with the hypothesis, but most of the mutations show this trend. As the pH increases further, the intensity decreases and finally falls completely as the pH reaches 12 and beyond. Again, calcium response of mCherry moiety seems to be unaffected until the entire protein loses its response at the denaturing, high pH condition. Data reported from 1 replicate.

Figure 18 Calcium response for varying pH for mutation B. As the pH increase towards physiological pH the intensity increases. The drop in pH at 7.6 is not in observed in this mutation, but instead the intensity seems to level until the pH increases to 12. Again, calcium response of mCherry moiety seems to be unaffected until the entire protein loses its response at the denaturing, high pH condition. Data reported from 1 replicate.
Figure 19 Calcium response for varying pH for mutation C (control). As the pH increase towards physiological pH the intensity increases. The drop in pH at 7.6 is not observed in this mutation, but instead the intensity seems to drop steadily as the pH increases, this is most in accordance with the hypothesis that the protein is most calcium sensitive (i.e.) most active at physiological pH. The calcium response of mCherry moiety seems to be unaffected at low pHs but shows some sporadic jumps as the pH increases. This may be solved by simply having more replicates of the experiment, but it is noteworthy to mention. Also, as with the other mutations, once the entire protein loses its response at the denaturing, high pH condition the emission at 575 nm also decreases to zero. Data reported from 1 replicate.

Figure 20 Calcium response for varying pH for mutation D. As the pH increase towards physiological pH the intensity increases. The drop in pH at 7.6 is not observed in this mutation, but instead the intensity shows the same pattern as the control. This is also in accordance with the hypothesis that the protein is most calcium sensitive (i.e. most active) at physiological pH. The calcium response of mCherry moiety seems to again be unaffected at low pHs but shows some strange behavior as the pH increases and again, once the entire protein loses its response at the denaturing, high pH condition the emission at 575 nm also decreases to zero. Data reported from 1 replicate.
Figure 21 Calcium response for varying pH for mutation E. The same trends are shown in this mutation, but in this mutation, as in the first mutation (A), there was a dip in the intensity around physiological pH. The intensity increase again until the pH reached 12, but at low pH the same behavior exists as with the other 4 samples. Data reported from 1 replicate.

Creating a ratio between the two different emission wavelengths lead to the following graph of all 5 samples. This graph shows the strange dip in intensity in the vicinity of physiological pH. This may simply be due to lack of variation of the experimentation. However, because the intensities for each of samples which have shown some response to calcium (all except mutation D) show the same pattern, this trend begs further exploration.

Figure 22 Ratios of emissions at 575 nm to those at 485 nm for constant calcium concentration (10 mM) and varying pH. We see there is a basic realm of activity focused around physiological pH of 7.4, but that there is a strange dip in intensity exactly 7.6. This is against hypotheses that the protein will be most active at physiological pH, but this anomaly may be due to limited variability in the results.
4. CONCLUSIONS

From these data, we can first see that using the pRSET vector is a good method for expressing this protein in the bacterial cells. Because of the histidine tag, it is possible to effectively purify the protein which is arguably the most important step of the entire process.

Regarding the responses to calcium, magnesium and pH, in each experiment the responses showed that the mCherry moiety did indeed show no response to calcium while the GCaMP2 response varied with the variation of calcium. Mutation A, showed the most specificity while mutation E had the widest dynamic range with respect to calcium. However, with the addition of magnesium ions, the signal is distorted and shifted. In the cases of the first three mutations, the dynamic range is also limited. These data also show that the replacement of the M13 moiety with the CaMKII moiety limits the sigmoidal response to calcium thus increasing the dynamic range of the protein. It is also apparent that the mutations which result in calcium-active proteins have similar responses to pH. Also, regarding pH, these data show that below a pH of 5 and above a pH of 12 the protein is ineffective at sensing calcium. It was hypothesized that the protein would have a peak intensity at physiological pH, but from the ration between the intensities observed at 575 nm to those at 485 nm there was a distinct drop in intensity around physiological pH. However, just below and just above this pH, there was a high intensity, giving the intensity curve an “M”–like shape.

In all of these experiments more replicates would need to be conducted to create more viable data, however, the data presented here serve as a good basis for further study and more informed hypothesis generation.
5. REFERENCES


chromophore formation of engineered Ca2+ binding green fluorescent proteins. *Archives of Biochemistry and Biophysics*, 486, 27-34.


