Rational Design and Application of Genetically Encoded Fluorescent Reporters in Cellular Physiology

Shen Tang
Georgia State University

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ABSTRACT

Fluorescent protein based genetically encoded fluorescent reporters play an important role in understanding the cellular physiology by directly monitoring real-time cellular signaling pathways with fluorescent microscope.

Quantitative analysis of Ca^{2+} fluctuations in the endoplasmic/sarcoplasmic reticulum (ER/SR) is essential to defining the mechanisms of Ca^{2+}-dependent signaling under physiological and pathological conditions. Here, we developed a novel class of genetically encoded indicators by designing a Ca^{2+} binding site in the enhanced green fluorescent protein (EGFP). One of them, CatchER (Calcium sensor for detecting high concentration in the ER), exhibits unprecedented Ca^{2+} release kinetics with an off-rate estimated at around 700 s^{-1} and appropriate Ca^{2+} binding affinity, likely due to local, Ca^{2+}
induced conformational changes around the designed Ca\textsuperscript{2+} binding site and reduced chemical exchange between two chromophore states. CatchER reported considerable differences in ER Ca\textsuperscript{2+} dynamics and concentration among epithelial HeLa, kidney HEK 293, and muscle C2C12 cells, enabling us to monitor SR luminal Ca\textsuperscript{2+} in flexor digitorum brevis (FDB) muscle fibers to determine the mechanism of diminished SR Ca\textsuperscript{2+} release in aging mice. Moreover, the structure of CatchER has been investigated by nuclear magnetic resonance spectroscopy (NMR) and high-resolution X-ray crystal structures to understand the novel mechanism of Ca\textsuperscript{2+} induced fluorescent enhancement of GFP.

It is crucial to investigate the metal selectivity of Ca\textsuperscript{2+}/Mg\textsuperscript{2+} of these metalloproteins to understand cellular physiology. The major Mg\textsuperscript{2+} binding sites of proteins have been reviewed and classified based on structural differences, and identified several key factors to determine Mg\textsuperscript{2+}/Ca\textsuperscript{2+} selectivity with binding constants difference up to 10\textsuperscript{4} in several types of metalloproteins.

Thrombin is involved in numerous cellular signaling pathways and plays a crucial role in blood coagulation. I designed a novel class of single EGFP-based thrombin sensors by inserting a thirty-amino acid short peptide with a thrombin cleavage site into the fluorescent sensitive location of EGFP. These designed protease sensors exhibited optimized \(k_{\text{cat}}/K_m\) up to 10\textsuperscript{4} magnitudes higher than that of small peptide based absorption indicator EGR-pNA. The measured \(K_m\) value is in below 10 µM, in the same magnitude as that of natural thrombin substrate Fibrinogen A.

INDEX WORDS: Biosensor, Calcium signaling, Green fluorescent protein
RATIONAL DESIGN AND APPLICATION OF GENETICALLY ENCODED Fluorescent REPORTERS IN CELLULAR PHYSIOLOGY

by

SHEN TANG

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by

SHEN TANG

Committee Chair: Jenny Yang

Committee: Osvaldo Delbono
Giovanni Gadda
Donald Hamelberg
Mary Wagner

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
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LIST OF ABBREVIATIONS

GFP: green fluorescent protein
EGFP: enhanced green fluorescent protein
BFP: blue fluorescent protein
CFP: cyan fluorescent protein
YFP: yellow fluorescent protein
RFP: red fluorescent protein
DsRed: tetramer form of red fluorescent protein from coral
DsRed2-ER: commercial available ER-targeted DsRed fluorescent protein
mCherry: mCherry fluorescent protein
mCherry-ER: ER targeted mCherry protein
FRET: fluorescent resonance energy transfer
ESPT: excited state proton transfer
Ca$^{2+}$: Ca$^{2+}$ ion, Calcium
Gd$^{3+}$: Gadolinium
Tb$^{3+}$: Terbium
Ln$^{3+}$: Lanthanide
CaM: Calmodulin
TnC: Troponin C
ER: Endoplasmic reticulum
SR: Sarcoplasmic reticulum
EC-coupling: excitation contraction coupling
CatchER: Calcium sensor for detecting high concentration in the ER
Asp: aspartate, D, aspartic acid
Glu: glutamate, E, glutamic acid
$K_d$: dissociation constant
$k_{off}$: dissociation rate
$k_{on}$: association rate
ms: milli-second
EGTA: ethylene glycol tetraacetic acid
BAPTA: 1,2-bis(o-aminophenoxy)ethane-n,n,n',n'-tetraacetic acid
4-CmC: 4-Chloro-m-Cresol
InsP3: IP3, Inositol 1,4,5-trisphosphate
RyR: ryanodine receptor
RyR1: ryanodine receptor isoform-1
SERCA: sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase
FDB: flexor digitorum brevis
TEA: tetraethylammonium
di-8-ANEPPS: di-8-amino napthyl ethenyl pyridinium
CPA: cyclopiazonic acid
LB medium: lysogeny broth medium, luria broth, lennox broth, luria-bertani medium.
SV medium: minimal medium of bacterial expressing isotopic labeling protein
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
NMR: nuclear magnetic resonance (spectroscopy)
HSQC: heteronuclear single-quantum correlation (spectroscopy)
HNCA: The magnetization of the amide proton of an amino acid residue is transferred to the amide nitrogen, and then to the alpha carbons of both the starting residue and the previous residue in the protein’s amino acid sequence.

CBCACONH: A special NMR 3D experiment designed to correlate the $^1$H and $^{15}$N amide resonances of one residue with both $^{13}$CA and $^{13}$CB resonances of its preceding residue via the intervening $^{13}$CO spin by means of the $^1$J(NH), $^1$J(N, CO), $^1$J(CA, CO) and optional $^1$J(CA, CB) coupling constants.

vol/vol: The ratio in terms of volume of two solution.

PDB: protein data bank

HEPES: N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid

IPTG: Isopropyl-β-D-thio-galactoside

Tau C: $\tau_c$, correlation time

SCT-CCR: shared constant-time cross-correlated relaxation

$T_1$: longitudinal relaxation

$T_2$: transverse relaxation

NOE: nuclear overhauser effect

CSP: chemical shift perturbation

PFG: pulse-field-gradient

O.D.: optical density

SV medium: minimal medium for bacteria expression of isotopic labeling protein.

FDB: Flexor digitorum brevis muscle fibres

BTS: N-benzyl-p-toluene sulphonamide

PVP: poly (N-vinyl-2-pyrrolidone)
CHAPTER 1: GENERAL INTRODUCTION: FLUORESCENT PROTEINS AND THEIR APPLICATION IN MODERN CELLULAR BIOLOGY

1.1 The discovery of fluorescent proteins (FPs) and their structural properties.

The green fluorescent protein (GFP) is a photosensitive protein composed of 238 amino acids first isolated from Aequorea Victoria jellyfish(3). GFP has a cylindrical beta-can structure folded by eleven β-strands connected with mainchain hydrogen bonds. The chromophore is formed by autocatalytic cyclization of residues S65, T66 and G67 and is deeply buried inside the hydrophobic core of GFP. The complex of hydrogen bonds between chromophore and its neighboring residues is assumed to generate the distinguished fluorescence of GFP, which exhibits an emission wavelength at 510 nm and an excitation of 398 or 475 nm. GFP and its variants have been widely expressed in established cell lines, primary cells and transgenic animals for the targeted imaging or detection of specific molecules(8).

The structure of GFP is a sheet of eleven strands, which is folded to form a barrel. The strands are antiparallel according to the structure showed below (Figure 1.1). In the center part of the sheet the strands are linked together to form a Greek key motif, also called a jellyroll(3).
The most important part of GFP is the chromophore, which emits the green fluorescence and is also an important indicator of the activity or the correct folding of protein. It is reported that the chromophore of GFP consists of 3 residues: Ser65, Tyr66 and Gly67. The mechanism for the intramolecular biosynthesis is shown in (Figure 1.2) After these three residues correctly fold with the help of an internal tripeptide motif, the protein is able to emit fluorescence without other cofactors. The formation of chromophore is a slow and calling for high requirement of the environment.

**Figure 1.1** The secondary structure of green fluorescent protein (GFP). (A) spatial beta-can structure of enhanced green fluorescent protein (EGFP) (pdb code: 1EMA). (B) The secondary structure of sequential 238 amino acids of GFP, including beta sheets (green arrow), helices (red rod), loops (black line). Adopted from Wikipedia.
Figure 1.2 Mechanism for the intramolecular biosynthesis of the GFP chromophore, with rate constants estimated for the Ser65 to Thr mutant. Adapted from (3)
1.2 Application of fluorescent proteins in cell biology.

The discovery of the GFP is a milestone in cell biology. Currently the majority of applications of GFP fluorescent properties are focused on the labeling or indication in clinical or medicinal areas. GFP can also be used as a tag in protein expression to reflect the host protein expression after it is fused with the host protein. A whole pattelet of fluorescent proteins covering UV to infrared emission spectra has been created by site-directed mutagenesis based on green fluorescent protein discovered in Aequorea Victoria jellyfish, and DsRed isolated from coral (5)(Figure 1.3), which has significantly revoluted the whole cellular imaging field, dramatically facilitating the spontaneous multicolor imaging, and providing sufficient scaffold proteins for designing genetically encoded fluorescent reporters. The special optical properties of fluorescent protein, like excited-state proton transfer (ESPT)(9), or fluorescence resonance energy transfer (FRET)(10) have been harnessed to design single or dual fluorescent protein based reporters. The native proteins specially binding to the ligands of interest with considerable conformational change were inserted into the fluorescent sensitive location of FP to generate single FP based reporter, or flanked with FPs in its N- and C- terminals to form FRET pair based reporters. EGFP variants are the mutation of wild type of EGFP. EGFP is the enhanced mutation of GFP with S65T mutation exhibiting stronger green fluorescent emission intensity(11).
1.3 Biological roles of Ca\textsuperscript{2+} and its relevance to diseases and aging.

Calcium is a second messenger in regulating many biological processes by the interaction with calcium binding protein in cellular signal transduction. The biological functions of calcium include cell division, differentiation and apoptosis. The regulation of calcium is influenced by the intracellular calcium concentration, which ranges from submicromolar to millimolar levels. Ca\textsuperscript{2+} binding affinity to specific proteins has been shown to be important in determining the concentration of calcium. The dynamic effects of Ca\textsuperscript{2+} binding have been investigated using EF-hand proteins, which are engineered into the enhanced green fluorescence protein (EGFP) to see the fluorescence signal change.

Ca\textsuperscript{2+} is the most ubiquitous signaling molecule in the human body, regulating numerous biological functions including heart beat, muscle contraction, neural function, cell development, and proliferation, by fluxing between the subcellular compartments with different amplitudes and durations\(^{(12)}\). This signaling is encoded by changes in Ca\textsuperscript{2+} concentration and fluxes between the intracellular and extracellular space and intracellular organelles\(^{(13, 14)}\). The time scale for cytosolic calcium transients varied from us for neuron transmitter release or 0.1 ms
for muscle contraction to days for fertilization/development(15). Such temporal and spatial changes of calcium signaling are controlled by communication among different cellular compartments such as extracellular space, intracellular stores, endosomes, and golgi via channels, gap junctions and pumps. The membrane-based organelle endoplasmic reticulum (ER), functioning as the primary intracellular Ca\(^{2+}\) store, can produce intrinsic Ca\(^{2+}\) release and propagation of Ca\(^{2+}\) oscillations(16-18). Ca\(^{2+}\)-mobilization agonists such as ATP, ionomycin, histamine, and glutamine will activate the Ca\(^{2+}\) receptors and pumps, such as inositol 1,4,5-trisphosphate receptor (IP\(_3\)R), to release Ca\(^{2+}\) from ER into cytosol(19-21), which results in a rapid decrease of ER (from mM at the resting state to µM in excited state). The removal of these agonists will help Ca\(^{2+}\) refill the ER through membrane channels such as sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). The alternation of Ca\(^{2+}\) concentration activates various intracellular Ca\(^{2+}\) sensing (trigger) proteins, such as calmodulin (CaM), troponin C (TnC) and other ion channels, by their conformational changes upon binding to Ca\(^{2+}\)(22). These activated Ca\(^{2+}\)-sensor receptors will further regulate numerous cellular processes and events. Recent studies indicate that Ca\(^{2+}\) signaling is important for homeostatic handling of cardiovascular functions(23-25). In cardiomyocytes, cardiac relaxation and contraction is regulated by the periodic change of intracellular Ca\(^{2+}\) concentration and the proteins associated with the sarcoplasmic reticulum (SR), a homologue of ER(26, 27). The cardiac ryanodine receptor (RyR2), inositol (1,4,5)-trisphosphate receptor (IP\(_3\)R) and the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a (SERCA2a) are three pivotal portals for the Ca\(^{2+}\) mobilization during this agonist-induced process. Heart failure caused by dysfunction of these two proteins, featured with abnormal Ca\(^{2+}\) handling, is proved with increasing evidence collected both from animals and humans(28-31)
Cellular aging is a fundamental biological process to cause the loss of skeletal muscle strength during contractility in vivo and in vitro. The mechanisms underlying the impairment of the aging skeletal muscle, termed sarcopenia, are partially understood. The original hypothesis

Figure 1.4 The Ca\(^{2+}\) pathways in the cell. Cellular distribution of representative calmodulin (CAM) binding proteins at different cellular compartments including nuclei, ER and mitochondria. Calmodulin has been reported to interact with membrane proteins (e.g., ion channels, pumps, and gap junctions), cytoskeletal proteins, and a variety of enzymes in the cytoplasm. (Courtesy of Dr. Yubin Zhou)
on the relationship between excitation contraction uncoupling and sarcopenia was proposed by Delbono and co-workers(32), suggesting that the number of RYR1 (ryanodine receptor 1) uncoupled to DHPR (dihydropyridine receptor) increases with age, and the SR Ca\(^{2+}\) depletion during the EC coupling declined in aging skeletal muscle. This hypothesis was initially demonstrated by high affinity ligand binding studies in soleus, extensor digitorum longus (EDL) and in a pool of several skeletal muscles consisting of a mixture of fast- and slow- twitch muscle fibers in middle-aged (14-month) and old (28-months) Fisher 344 Brown Norway F1 hybrids rats. The number of DHPR, RYR1, the coupling between both receptors expressed as the DHPR/RYR1 maximum binding capacity, and their dissociation constant for high affinity ligands were measured. The DHPR/RYR1 ratio was significantly reduced in the three groups of muscles(32). Furthermore, using cytosolic Ca\(^{2+}\) indicator Rhod-2, the significant reduction (around 50%) in the peak myoplasmic Ca\(^{2+}\) concentration efflux from SR was directly measured in skeletal muscle fibers at 40 mV from the flexor digitorum brevis (FDB) of 21-24-month-old FVB mice in compared to 5-7- and 14-18-month-old groups, parallel with the substantial decrement of the total charge movement or DHPR charge movement measured in the first group compared to latter two groups, supporting that skeletal muscle fibers from aging mice exhibit a significant decline in myoplasmic Ca\(^{2+}\) concentration resulting from a reduction in L-type Ca\(^{2+}\) channel (DHPR)(33).
The endo/ sarcoplasmic reticulum (ER/SR) lumen, which occupies less than 10% of cell volume, stores >90% of intracellular Ca\(^{2+}\) and is pivotal in controlling Ca\(^{2+}\) signaling (34-36). The ER/SR Ca\(^{2+}\) store is pivotal in regulating Ca\(^{2+}\) signaling and maintaining Ca\(^{2+}\) gradients across cellular compartments via multiple ionic channels, receptors and Ca\(^{2+}\) buffer proteins such as calsequistrin in the ER and parvalbumin and calbindinD9K in cytosol. The activity of these channels and receptors is tightly controlled by free [Ca\(^{2+}\)] both in the cytosol and SR lu-
men with a four-order magnitude gradient. Changes in free cytosolic [Ca\(^{2+}\)] modulate proteins such as calmodulin (CaM) and troponin C (TnC), which switch on and off numerous biological processes(37).

Sarcoplasmic Reticulum Ca\(^{2+}\) release in the fast twitch skeletal muscle is very important in regulating skeletal muscle contraction. The ER/SR Ca\(^{2+}\) depletion controls SR Ca\(^{2+}\) release, channel/ryanodine receptor (RyR) activity, and/or SR Ca\(^{2+}\) release termination. It is also essential in determining the role of store Ca\(^{2+}\) depletion in activating store-operated Ca\(^{2+}\) entry (SOCE). Due to the unique feature of high free calcium content in the SR of the skeletal muscle (~ 1 mM) and fast release kinetics (ms), there are several important questions regarding lumen calcium that have yet to be answered. How fast does ER/SR Ca\(^{2+}\) depletion occur in response to cell depolarization? What is the ER/SR Ca\(^{2+}\) buffer capacity? What is the resting SR Ca\(^{2+}\) concentration in muscle cells from young and old species? In addition, understanding the role of calsequestrin and calreticulin in buffering luminal SR free [Ca\(^{2+}\)] and release kinetics is crucial to elucidate the mechanisms underlying ryanodine receptor mutants-related diseases(38).

Studying Ca\(^{2+}\) signaling in this organelle requires highly specific and targeted indicators. A qualified Ca\(^{2+}\) indicator is required to monitor fast ER/SR Ca\(^{2+}\) depletion in response to cell activation, quantifying the role of calsequestrin in buffering luminal Ca\(^{2+}\) and its influence on SR Ca\(^{2+}\) depletion in excitable and non-excitible cells. Additionally, these studies should enable identifying specific Ca\(^{2+}\) pathways involved in different biological states. A Ca\(^{2+}\) indicator to monitor ER/SR Ca\(^{2+}\) concentration with fast release kinetics especially in excitable cells is thus highly desirable(38-41). Unfortunately, there are only a few genetically encoded ER calcium sensor published, and all the \(K_d\)s narrowed around tens of micromolar, while it is well
known that free calcium concentration in SR of skeletal muscle cell is around 1 mM, with extra 20 mM calcium bound by calsequestrin. There is a strong need to design a SR calcium sensor with lower binding affinity which is appropriated to measure SR calcium in the muscle cells or tissues. Ideally, the calcium binding affinity should be around 1 mM or sub-millimolar range, similar to the overall calcium binding affinity of SR calcium buffer protein calsequestrin, which is based on the strategy that the cytosolic calcium indicators such as Fura-2, camelone and GCamp2 exhibit $K_d$ around sub-micromolar range within the same magnitude of $K_d$ as calmodulin.
1.5 Tuning Ca\textsuperscript{2+} binding affinities by rational design.

Understanding the key determinants for calcium binding is essential for designing calcium sensors with different affinities. According to the study of putative calcium binding sites featured by pentagonal bipyramidal geometry structure\cite{42}, two factors can influence the calcium binding affinity, 1) the coordination number of the binding ligands, 2) the geometric arrange-
ment of the negative charged residues in the binding site. The statistic work done by our laboratory and others indicates that calcium has a preference to bind oxygen atoms provided by the carboxyl or hydroxyl groups from the sidechain or mainchain, which can be explained by its hard Lewis acid definition presenting less convalent interaction with a more rigid ionic radius. (43) Compared to other divalent metal ions such as zinc or cooper, the optimal coordination number of calcium is as high as seven.

Our lab has developed the grafting approach for probing site specific Ca$^{2+}$ binding affinity. We have shown that CD2 is an excellent scaffold protein(42, 44-61). It retains the native structure after the insertion of the EF-hand motif both in the absence and presence of Ca$^{2+}$ ions. After optimizing the length of two glycine linkers that connect the Ca$^{2+}$ binding loop and CD2 to provide sufficient freedom for the loop, we have shown that the grafted EF-hand loop retains its native Ca$^{2+}$ binding property using high resolution NMR and $^{15}$N labeled protein(45). Contribution of flanking helices to the metal binding affinity of CaM have been investigated by inserting the EF-loop, the loop with the existing F-helix, and the loop with both EF-helices of Site III of CaM into CD2. In contrast to the largely unfolded structure of the isolated peptide fragment, the inserted flanking helices are partially formed, as revealed by both CD and NMR. Ca$^{2+}$ affinity is enhanced about 3-10 fold when the flanking helices are attached. We have first estimated the intrinsic Ca$^{2+}$ affinities of the four EF-hand loops of CaM (I-IV) by individually grafting them into CD2. EF-loop I exhibits the strongest while EF-loop IV has the weakest binding affinity for Ca$^{2+}$, La$^{3+}$, and Tb$^{3+}$. EF-loops I-IV of CaM has dissociation constants for Ca$^{2+}$ of 34, 245, 185, and 814 µM, respectively. Based on the results, we proposed a charge-ligand-balanced model in which both the number of negatively charged ligand residues and the balanced electrostatic dentate-dentate repulsion by the adjacent charged residues are major de-
terminants for the Ca\(^{2+}\) binding affinities of EF-loops in CaM. Our grafting method provides a new strategy to obtain site-specific Ca\(^{2+}\) binding properties and to estimate the cooperativity and conformational change contributions of coupled EF-hand motifs. We have shown that the contribution of the cooperativity and conformational change to the Ca\(^{2+}\) affinity for the C-terminal is 40\% greater than that for the N-terminal. The same approach will be used to probe the site-specific Ca\(^{2+}\) affinity and kinetic properties of engineered calcium binding proteins and sensors.

Based on the common features of calcium binding sites from detailed structural analysis of calcium binding proteins, our lab has successfully designed a calcium binding site on the surface of a beta-sheet non Ca\(^{2+}\) binding cell adhesion protein CD2 by site-directed mutagenesis of four to five residues to negatively charged. The first generation of the designed Ca\(^{2+}\) binding CD2 variants having five negatively charged residues as the binding ligands, by fluorescent energy transfer between Tb\(^{3+}\) and aromatic residues Tyr81 and buried Trp32, the dissociation binding constant of the designed protein to Tb\(^{3+}\) was measured 21 \(\mu\text{M}\), in comparison to 300 \(\mu\text{M}\) of a native protein gama-crystallin. Later, a more elegant work was completed by designing CD2 variants not rely on sequence or structural similarity between the recipient scaffold and a naturally evolved donor protein. Instead, the strategy takes into account the general local calcium-binding properties such as ligand types, charge, and the geometry of the primary coordination sphere and then identifies a constellation of backbone positions in the recipient scaffold that allows the introduction of the metal-binding site by mutations with potential ligand residues. The designed proteins were filtered using several criteria. First, the calcium should be solvent accessible. Second, the mutations should introduce little or no side-chain steric conflicts with the preexisting atoms. Third, minimal disruption of hydrogen bonding and hydropho-
bic interactions is required. Fourth, three or four negatively charged residues at the primary coordination shell are preferred. The designed calcium-binding site is constructed by ligand residues from three different sequence regions (two anti-parallel beta-sheets and one from a flexible region) with a total of four negatively charged ligands. The metal selectivity of CD2.Ca1 was verified by metal replacement titration, and 1D NMR spectroscopy. Adding 10 mM Ca$^{2+}$ or 0.1 mM La$^{3+}$ can significantly reduced the fluorescent intensity of CD2.Ca1 pre-loaded with 30 µM Tb$^{3+}$, while 10 mM Mg$^{2+}$ or or 100 mM K$^+$ reduced fluorescent intensity only 10-20%, in contrast to 5-10% fluorescent decrement by adding these metals without protein. The metal selectivity further verified by the 1D NMR spectra by observing conformational change induced by Ca$^{2+}$ in the presence of other metal ions. And the Ca$^{2+}$/Mg$^{2+}$ selectivity was verified by adding Ca$^{2+}$ or Mg$^{2+}$ in the presence of 130 mM KCl. Mg$^{2+}$ does not induce conformational change even up to 10 mM, however, the spectra of 1.0 mM Ca$^{2+}$ in the presence of 1.0 mM Mg$^{2+}$ is identical to that of Ca$^{2+}$ loaded form. A consequential work of designed Ca.CD2 was used to investigate the effect of designed Ca$^{2+}$-binding sites on the biological function of CD2. There are different affinities of Ca.CD2-binding to CD48 by surface plasmon resonance in the absence and presence of Ca$^{2+}$. Using CD2 as the control (100%), the binding of Ca.CD2 to the conformation-dependent antibody OX34 is 104 and 106% with Ca$^{2+}$ and EGTA, respectively. The binding to OX55 is 90 and 101% with Ca$^{2+}$ and EGTA, respectively. These results strongly suggest that Ca.CD2 retains its native biological ability to bind to CD48. It is interesting to note that Ca$^{2+}$ binding to Ca.CD2 decreases the binding affinity to CD48 by approximately 15%, although this designed site is located on the surface opposite from the CD48 recognition site. The binding of soluble CD48 results in significant changes in chemical shifts and line broadening of a few residues at the C and C’ strands of domain 1 of CD2, as reported by Driscol and
colleagues. Although L63 is not located at the functional surface, upon binding to CD48, nuclei in this residue exhibit substantial chemical shift changes that are comparable to those of functional surface residues. The Ca$^{2+}$ binding dissociation constant of Ca.CD2 was measured to 1.4 mM by 2D NMR Ca$^{2+}$ titration, while the Tb$^{3+}$ and La$^{3+}$ binding dissociation constant was 6.6 µM and 5.0 µM, respectively. Metal selectivity of Ca.CD2 between divalent and trivalent metal are more than 200 folds\(^{(47)}\). The thermal stability of the designed CD2 variants was also investigated. The T\textsubscript{m} of -5 negative charged residues of CD2 variants decreased to 41 °C in compared to 61 °C of WT CD2, this decrement was partially restored by Ca$^{2+}$ binding, without substantial conformational change of the secondary structure\(^{(62)}\). The local conformational change of protein was investigated by Ca$^{2+}$ and Ln$^{3+}$ titration of CD2.6D79, with similar trend of Tm value changes in the presence and absence of Ca$^{2+}$ \((63)\), with tuned K\textsubscript{d}s ranging from tens of micromolar to millimolar \((47, 52)\). The secondary structure and spatial distribution of the designed Ca$^{2+}$ binding ligands on the surface of CD2 are different from those in traditional EF-hand based a 12-amino acid sequential loop which wraps around a Ca$^{2+}$ ions, but protruding to the outside of the protein with high solvent accessibility. We have further shown that calcium binding affinity can be changed by the ligand types and number of charged residues. Moreover, the thermostability of the designed CD2 variants was significantly enhanced after Ca$^{2+}$ binding investigated by the ITC of the decreased T\textsubscript{m} values, in concordence with the computational simulation results (Yang, W. et. al., 2005, JACS).

In addition to calcium binding affinities and protein folding, metal selectivity is also important. Mg$^{2+}$ concentration in the intracellular environment is maintained at 0.3-0.6 mM\(^{(64)}\) and the concentration of monovalent cations is in the 0.1-0.2 M range. In the resting eukaryotic cell, intracellular Mg$^{2+}$ concentration is about $10^3$-fold higher than that of Ca$^{2+}$ \(10^{-3} \text{ M for Mg}^{2+}\)
and $10^{-6}$ M for Ca$^{2+}$). Hence, calcium binding proteins such as EF-hand proteins are likely to be Mg$^{2+}$ filled\(^{(65-67)}\). Upon activation by the first messages, such as the binding of a hormone or a transmitter, or the passing of a Na$^+$/K$^+$ nerve signal, a dramatic increase in calcium concentration in cells (from $\sim 10^{-7}$ M at resting state to $\sim 10^{-5}$ M at excited state) causes the removal of pre-bound Mg$^{2+}$ of calmodulin and TnC upon calcium-binding. In contrast to the marked structural transitions induced by Ca$^{2+}$ binding, Mg$^{2+}$ binding causes only localized conformational changes within the four Ca$^{2+}$-binding loops of CaM and not able to result in significant structural effects required for the interaction of CaM with target proteins\(^{(67)}\). The selectivity of proteins to Ca$^{2+}$ over Mg$^{2+}$ is defined as a ratio of their association constant, $K_{Ca}/K_{Mg}$. The values of the ratio of $K_{Ca}/K_{Mg}$ for the first two EF-hand motifs of CaM is about $2.5 \times 10^2$\(^{(68)}\). The values of the $K_{Ca}/K_{Mg}$ ratio for motif 3 and 4 of TnC and that of Ca-Mg sites of Parv are much higher, around $4 \times 10^3$. The two motifs of the TnC (N-terminal) are Ca(II)-specific with $K_{Ca}$ about $10^5$ M$^{-1}$. Selectivity against these cations requires the calcium affinity to be superior by several orders of magnitude. In EF-proteins, Ca(II)/Mg(II) exchange appears closely related to physiological processes that involve cell excitation and relaxation, such as muscle contraction\(^{(69, 70)}\). Therefore, in order to specifically detect calcium, biosensors are required to have strong metal selectivity. Currently, the metal selectivity of the genetically encoded calcium sensors has not been determined quantitatively, though a cytosolic Ca$^{2+}$ sensor, TnC-XXL, created by replacing the Mg$^{2+}$ sensitive domain of TnC by a nonsensitive domain, has been found to exhibit improved divalent metal selectivity.

In addition to calcium binding affinities and protein folding, metal selectivity is also important. The designed CD2 variants exhibited $10^2$ metal selectivity between Ca$^{2+}$ and Mg$^{2+}$, supported by the metal replacement titration between Tb$^{3+}$ and Mg$^{2+}$ (citation). We will explore the
Ca$^{2+}$ binding affinities and metal selectivities by fluorescent intensity change and HSQC-NMR chemical shift perturbation with the established methods. The overall Ca$^{2+}$ binding affinity of designed sensors will be obtained by fluorescence spectrophotometer (Photon Technology International, Inc.) with the purified protein samples, while the individual K$_d$ of each ligand will be investigated by chemical shift perturbation of HSQC spectra with NMR 600 MHz in our school as described in the preliminary results. The emitted fluorescent intensity as a function of Ca$^{2+}$ concentration was fitted by equation 1 and 2 with 1:1 binding model(71), where $F$ represents fraction of Ca$^{2+}$-bound protein, $R$ is the detected fluorescence intensity, $[P]_T$ is the total protein concentration and $K_d$ is the dissociate constant. In addition, metal selectivity will be investigated by purposely adding different metal ions such as Zn$^{2+}$, Cu$^{2+}$, Mg$^{2+}$ with various concentrations into protein solution, and examining whether designed sensor can differentiate these metals from calcium according to the fluorescent change. The structural basis of major classes of Mg$^{2+}$ binding proteins, their capability in binding Ca$^{2+}$ and Mg$^{2+}$/Ca$^{2+}$ selectivity are reviewed in Chapter VI.

1.6 Factors contribute to kinetic properties of Ca$^{2+}$ binding.

The dissociation time constants of currently reported sensors are GCaMP1 188 ms (72), TN-L15 860 ms, TN-L15 D107A 580 ms, YC2.3 870 ms, TN-hum TnC 560 ms (73), TN-XL two phases, 142 ms, 867 ms (74), YC 3.6 2900 ms and BRET pair 210 ms.

There are several factors that contribute to the on and off rates of a calcium sensor. In principle, strong calcium binding dictates a great on rate or a small off rate, while weak affinity is related to a large off rate. Among all these reported calcium sensors, calcium release is required from the tightly coupled EF-hand calcium binding sites either in the C-terminal domain of TnC or CaM, which is much slower than calcium release from a single calcium binding site
with a weak affinity as we designed here. In addition, the fluorescence change via direct metal interaction is likely to be faster than indirect interactions via conformational change. In the event of calcium binding induced fluorescence change by calcium binding, it is also important to bypass the slow rate between the ionic states as we observed in G1 (71). Our detailed NMR studies revealed that the fast fluorescent response in CatchER is achieved by calcium induced local conformational change coupled with the slowing down of the exchange rate of the chromophore conformations. This is very different from all the reported calcium sensors based on protein-protein interactions. In order to generate fluorescence signal change upon calcium binding, CaM based FRET sensors such as Cameleon and D1ER depend on several relays of conformational changes. Calcium binding first generates conformational changes in CaM which in turn results in additional conformational change upon binding to its target peptide. Such conformational changes further transfer into relative changes in the orientations and distances between two fluorescent proteins via two flexible linkers. GCaMPs with CaM grafted into cpEGFP depend on the indirect responses due to calcium-dependent binding of CaM to its target peptide. TnC-based FRET pair sensors such as TnL15, TnXL, and TnXXL utilize the C-terminal domain of TnC to sense calcium and in turn transfer into the relative FERT changes in the connected fluorescence pairs. Such multiple levels of conformational relays and flexibilities in linkers make FRET based sensors voluble to the influences of cellular environment in addition to their inherent difficulty in tuning kinetic rates (75) (76).
Additional difficulties and limitations in the tuning kinetics of calcium sensors are originated from multiple coupled calcium binding sites and natural calcium binding proteins used in currently reported sensors (77). The biological function of the CaM and TnC determines the intrinsic kinetics of the calcium binding process. Hence, the up-limit kinetics of signals detected by these indicators can be predicted by the fastest calcium signaling conducted by these proteins. TnC sequentially responds to calcium release from the SR and converts calcium signaling to physical movements during EC coupling with a theoretical delay (75). Further, the multiple calcium binding processes of these two proteins usually become the rate-limited step in addition to their complicated stoichiometric interaction mode as well as the potential in perturbation of calcium signaling network. In contrast, calcium dye and designed calcium binding site have a single coordination is likely to have adjustable kinetics (78) (79) and affinity.

<table>
<thead>
<tr>
<th>Ca$^{2+}$ Indicator</th>
<th>Component</th>
<th>Location</th>
<th>$k_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fura-2</td>
<td>Organic</td>
<td>Cytosol</td>
<td>(4.0 ± 0.2) x 10$^8$</td>
<td>103 ± 7</td>
<td>0.26</td>
</tr>
<tr>
<td>Fluo-3</td>
<td>Organic</td>
<td>Cytosol</td>
<td>(7.1 ± 0.3) x 10$^8$</td>
<td>369 ± 25</td>
<td>0.52</td>
</tr>
<tr>
<td>Mag-fura2</td>
<td>Organic</td>
<td>ER</td>
<td>(7.5 ± 1.2) x 10$^8$</td>
<td>26760 ± 1800</td>
<td>35.7</td>
</tr>
<tr>
<td>D1ER</td>
<td>Protein</td>
<td>ER</td>
<td>3.86 x 10$^6$</td>
<td>256</td>
<td>69</td>
</tr>
<tr>
<td>G1</td>
<td>Protein</td>
<td>ER</td>
<td>Estimated 10$^6$</td>
<td>Estimated 800</td>
<td>800</td>
</tr>
<tr>
<td>Cameleon1/E104Q</td>
<td>Protein</td>
<td>ER</td>
<td>2.4 x 10$^6$</td>
<td>12.9</td>
<td>5.4</td>
</tr>
</tbody>
</table>
The mechanism of kinetical properties of Ca\textsuperscript{2+} binding was reviewed in the introduction part of Chapter 2, including both the synthesized Ca\textsuperscript{2+} dyes and genetically encoded Ca\textsuperscript{2+} indicators, and the experimental methods were summarized in the method part of Chapter 2.

1.7 Criteria of a Ca\textsuperscript{2+} sensor in cells.

The basic criteria of a functional Ca\textsuperscript{2+} sensor require it to 1) specifically recognize Ca\textsuperscript{2+} without being influenced by the other metal ions, especially for the divalent metal ions such as Mg\textsuperscript{2+} and Zn\textsuperscript{2+}, which are in excessive of physiological condition. In addition, it should also exhibit 2) an appropriate binding affinity fit for Ca\textsuperscript{2+} concentration detection, due to the diverse magnitudes of Ca\textsuperscript{2+} concentrations present in different subcellular organelles. 3) The optical signal change caused by the different concentration of Ca\textsuperscript{2+} requires sufficient magnitude for instrument detection and to be easily distinguishable from the noise. Once the above requirements are satisfied, the designed molecule is able to sense the Ca\textsuperscript{2+} concentration in vitro with physiological buffer conditions. An advanced sensor, which can be applied for the living cell imaging, should 4) neither be toxic to cells, nor perturb the original function of cells. 5) With respect to tissue targeting, the designed Ca\textsuperscript{2+} sensor should be specifically delivered to the targeted organelle without branching, and must subsequently be retained at the targets. 6) The detected signal change must represent the immediate Ca\textsuperscript{2+} signaling that occurs as a function of time. Some agonists can induce Ca\textsuperscript{2+} depletion within several macro seconds, so the interaction between Ca\textsuperscript{2+} and the designed sensor is required to be very fast. 7) The designed sensors should be inert to other cellular factors, and resist to pH change and enzymatic reactions under physiological conditions. The protein-based sensor, which can genetically be encoded in specific targeting organelles, exhibits the strong potential to be predominant in further development of Ca\textsuperscript{2+} sensors.
One of the major objectives of this dissertation is to design advanced, genetically encoded Ca\(^{2+}\) biosensors to quantitatively detect Ca\(^{2+}\) signaling in specific subcellular organelles. This will have a significant impact on the understanding of the molecular basis of Ca\(^{2+}\) signaling and homeostasis in cardiac development and diseases (chapters 2-3).
Figure 1.7 Strategies of the major Ca$^{2+}$ indicators. Synthetic organic chemicals based on BAPTA scaffold, e.g. Fura-2, with ratiometric emission wavelength change (first row). Genetically encoded fluorescent indicators are either native Ca$^{2+}$ binding biolumination protein aequorin with single wavelength change (second row), or chimera containing fluorescent proteins and natural Ca$^{2+}$ binding proteins, calmodulin or troponin C. Chimera is either based on fluorescent resonance energy transfer (FRET) by sequentially fusing cyan fluorescent protein (CFP) or mutants, calmodulin and M13 peptide or troponin C, and yellow fluorescent protein (YFP) or mutants with ratiometric emission response to Ca$^{2+}$, or based on single circular permuted green fluorescent protein (cpGFP) flanked with calmodulin and M13 peptide on its N- and C- terminals with single wavelength change.
1.8 Current Ca\textsuperscript{2+} indicators

As shown in Figure 1.7, current calcium sensors can be classified to synthesized organic chemicals based on the BAPTA scaffold, and genetically encoded fluorescent reporters either by native protein aequorin or chimeric proteins containing fluorescent proteins.

Organically synthesized Ca\textsuperscript{2+} sensors. This class of Ca\textsuperscript{2+} sensors was pioneered by Dr. Robert Tsien and evolved from single-wavelength intensity change to ratiometric signal change (78, 80, 81). The majority of these sensors exhibit greater signal dynamic range (e.g. 30-50 fold for fluo-3 (81)) compared with other classes. However, the major barrier of the application to living cells is that they cannot be unambiguously targeted to specific subcellular organelles and easily leak out (82), due to their non-specific membrane-permeable targeting strategy. Thus, the precise cytological location cannot be guaranteed due to limitations associated with both delivery and retention. In addition, it is difficult to load the dyes into denser or thicker tissues, hindering further research with animals or even humans.

Bioluminescent Ca\textsuperscript{2+} indicators are based on Ca\textsuperscript{2+}-binding photoproteins originating from jellyfish which generate luminescence as a result of Ca\textsuperscript{2+}-induced conformational change, but incompatible results have been reported using photoproteins in measuring ER Ca\textsuperscript{2+} concentration compared with other methods (83-86). In addition, even after modifying the Ca\textsuperscript{2+} binding ligands on EF-hands, the Ca\textsuperscript{2+} binding affinity was still beyond the optimal ER measurement range(87, 88). Moreover, a microinjection of cofactor or cosubstrates is required for the luminescence, which is inconvenient for thick tissue measurement.

Calmodulin-based Ca\textsuperscript{2+} sensors are divided into two subgroups. The first is the calmodulin (CaM) associated fluorescent resonance energy transfer (FRET) pair based, initially published by Persechini and Tsien’s laboratory(8, 10, 89). Two kinds of fluorescent pro-
proteins, characterized by significant overlap between the emission wavelength of the donor and the excitation wavelength of the acceptor, were linked by native CaM and a CaM binding peptide from either their N- or C-terminals. The fluorescence resonance can be efficiently transferred from one fluorescent protein to the other upon Ca$^{2+}$ binding to CaM, which has endured a large conformational change and to bring these two fluorescent proteins closer with an appropriate orientation. Unfortunately, the FRET pair-based Ca$^{2+}$ sensor has only a small signal change in the detection of Ca$^{2+}$. A recent development of this kind of sensor has been reported, based on computational design, which has increased the dynamic range around 100-fold (90).

Over the past 30 years, rationally designed optical calcium indicators have tremendously facilitated our understanding of long-term mysterious calcium signaling pathways in living cells with unprecedented high spatial and temporal resolution and further consolidated the significance of calcium in various biological functions. The first modern small-molecule calcium indicator BAPTA was designed based on the scaffold of EGTA exhibiting high metal rejection to magnesium in cytosolic environment, which not only became the prototype of consequential large family of fluorescent calcium dyes with various binding affinities and full palette of excitation and emission spectra, but also triggered the burgeon of designing biosensors to detect diversified cellular signalings in a non-invasive way. Almost two decades after the birth of BAPTA, and thanks to the cutting-edge development of fluorescent protein variants, the first absolutely genetically encoded calcium indicator cameleon was designed by flanking BFP and EGFP as a FRET pair besides a big fusion protein composed by calmodulin and its targeting peptide M13 as the calcium interaction domain. This design conquered the problems of native calcium-sensitive protein Aequorin required laboriously continuous addition
of cofactors during the experiment and opened new area of rational design of genetically encoded biosensors.

Small organic chemical based indicators, or simply called dyes, are advanced in fast kinetics, small size, simple chemical stoichiometry, significant signal response and less perturbation of natural cellular signaling. This results from artificially designed single exogenous binding domain instead of hijacking natural proteins as triggers of fluorescence alternation after experiencing conformational change in response to the molecules of interest as most genetically encoded indicators did. We have witnessed the novel development of dyes as some of them have been successfully targeted to subdomains of receptors, channels, and particular subcellular locations like nucleus mitochondria, plasma membrane and endoplasmic reticulum by several wise strategies during the past decade, which conquered the traditional biases that dyes were suffered from intrinsic inadequacy to reveal cellular signaling of a particular microdomain with high spatial resolution instead of outputing the average of the signals in a bulk volume. It further extended the boundary of applying dyes to the highly heterogeneous biological systems once dominated by genetically encoded indicators. But dyes are still inferior in poor penetration into thick tissues and not genetically heritable preventing them from most intact animal imaging, and long-term observation. The challenges of applying dyes into particular organisms whose cells are easy to transfecDNA but too resistant to load small molecules were acknowledged even in an earlier classic review. (Tsien, Monitoring cell calcium, 1999) In addition, the current efficiency of the conjugation between dyes and targeting molecules especially in cells in situ can still be further improved as high percentage of the dyes remained free, and none persuasive strategy existed to remove non-targeted dyes after they are loaded. Moreover, the targeting strategies are too rigid in comparison to genetically encoded indicator
easily fused to protein of interest by adding peptides. SNAP-tag (a 20 kDa mutant of the DNA repair protein O\textsuperscript{6}-alkylguanine-DNA alkytransferase that reacts specifically with benzylguanine (BG) derives) based targeting for example, requires a protein with 182 amino acids as a linker. This is a size that is unfortunately much bigger than the dye itself, and would cause difficulties of flexibly adjusting the distance between the dyes and the targeted protein, thereby hampering the detection of the signals of microdomains with high spatial and temporal resolution. This would not be a problem with genetically encoded indicators. Here, we are not trying to evoke rivals between small organic chemical-base indicators and genetically encoded indicators, but we frankly point out the advantages and disadvantages of each strategy and try to seek solutions to existing problems in an unbiased way.

Genetically encoded Ca\textsuperscript{2+} biosensors based on fluorescent proteins exhibiting high local specificity have significantly promoted the exploration of subcellular Ca\textsuperscript{2+} signaling with high accuracy and resolution. Moreover, taking the advantage of gene transfection and in situ protein expression, genetically encoded Ca\textsuperscript{2+} biosensors have been applied successfully in various cell types and in animals. However, some Ca\textsuperscript{2+} signals occur within a very short time such as muscle contraction and neural transmission, and even subtle alternation of the signal pattern will result in serious diseases. To differentiate various Ca\textsuperscript{2+} signal patterns is crucial to probe the mechanisms of Ca\textsuperscript{2+} mishandling related diseases on a molecular level, so that fast fluorescent response is required for biosensors to detect these signals in real time, which is also a challenge for current genetically encoded Ca\textsuperscript{2+} biosensors.

The fluorescence change of calmodulin (CaM)-based calcium sensors highly relies on the interaction between calcium bound form calmodulin and M13 peptide (derived from the CaM-binding region of the skeletal muscle myosin light chain kinase (skMLCK) residues 577-602.),
which is a bulk complex with several different binding processes. The calcium binding affinities to C- and N- domain of calmodulin are of different magnitudes. Moreover, holo-form calmodulin and M13 peptide interact will add an additional dissociation constant \( (K_d) \) to the overall binding process, so the apparent \( K_d \) of the sensors does not directly come from the calcium binding, but in a mixture of two \( K_d \)s with different magnitudes from calcium and calmodulin interaction and a sequential \( K_d \) from the calmodulin and M13 peptide interaction. The calmodulin based calcium indicator cannot quantitatively measure the calcium change, as the equation of D1ER binding process involving several constants such as \( K_d1, K_d2 \) and hill coefficients which are difficult to measure in situ. Furthermore, the kinetics of CaM and M13 peptide interaction could not be further accelerated due to complex delay.

1.9 Our strategy to design a genetically encoded fluorescent indicator of Ca\(^{2+}\).

In our laboratory, an EGFP-based Ca\(^{2+}\) sensor was successfully created by grafting an EF-hand motif with a continuous Ca\(^{2+}\) binding site into wild type EGFP as scaffold protein(71). The generated Ca\(^{2+}\) sensor (G1) exhibits a dual 510 nm fluorescence intensity ratiometric change accordingly when excited at 398 and 490 nm was monitored to decide the concentration of Ca\(^{2+}\). Although the dynamic range is relative small (only 10-15% change) in mammalian cell imaging, this work strongly supports our hypothesis that the GFP chromophore can be altered by introducing a Ca\(^{2+}\) induced conformational change. Recently, with exploring the mechanism of fluorescence occurring of EGFP, more and more laboratories have reported the fluorescence sensitive sites on the surface of EGFP and the intention to design small molecular binding sites in these locations for the in vivo detection is growing exponentially. DsRed-based cooper sensors and GFP-based zinc sensors with the site directed mutation on the surface of fluorescent protein have been reported. Illuminated by their successful experience and
encouraged by the solid strength of de novo design calcium binding pocket on the surface of beta-sheet protein of our laboratory, we designed a calcium binding pocket on the surface of EGFP to detect calcium concentration. The advantages of Ca\textsuperscript{2+} sensors by site-directed mutagenesis are listed as follows: 1) Direct design of a Ca\textsuperscript{2+} binding site on the surface of EGFP is supposed to create a bigger dynamic range of the signal change if its distance to chromophore is shorter than the grafting approach. This is because the shortest distance between the surface of GFP to the chromophore is only around 10 Å while Ca\textsuperscript{2+} bound to the grafted EF-hand should crosstalk to the chromophore at more than 30 Å far away. This new strategy may have a more direct influence on the chromophore. 2) We chose EGFP (S65T mutant of wt.GFP) as the scaffold protein, as it is stable, non toxic, and exhibits robust optical fluorescence under physiological conditions (11). The cycle 2 mutations (M153G, V163A)(91) were created in scaffold protein to improve the protein folding efficiency at high temperature, as poor folding will cause not only disqualify the cell imaging due to low fluorescent intensity, but will also cause dysfunction of the Ca\textsuperscript{2+}-binding site. The physiological temperature of mammalian cell is unfavorably high, due to the wtGFP encoded by Aequor Jellyfish inhabitance in the deep cold ocean. 3) Protein with different Ca\textsuperscript{2+} binding affinities can be easily developed by alternating the electrostatic potential of the binding sites originating from the local negatively charged coordination ligands, according to the success of CD2-based Ca\textsuperscript{2+} binding protein design(62). 4) The designed GFP-based Ca\textsuperscript{2+} sensor can specifically target various cellular organelles or tissues by fusing different signal peptides. 5) It can overcome the limitation of currently reported Ca\textsuperscript{2+} sensors based on natural Ca\textsuperscript{2+} binding proteins due to the perturbation of Ca\textsuperscript{2+} signaling(92). Furthermore, we propose to conduct nuclear magnetic resonance analysis to explore the mechanism of particular molecules influencing the chromophore
environment and the chromophore conformational change. This will provide solid theoretical evidence for the development of GFP-based biosensors detecting diverse molecules.

Figure 1.8 Scheme of design strategies of genetically encoded Ca\(^{2+}\) indicators in our lab, and enhanced green fluorescent protein (EGFP, PDB file: 1EMA) was used as a scaffold protein. EF-hand III of calmodulin was inserted into the fluorescent sensitive location of EGFP to create Ca-G1 as the first strategy (top row). Additional Ca\(^{2+}\) binding site was introduced on the surface of beta-sheet Ca-G1 to enhance the fluorescent response as the second strategy (middle row). Negatively charged residues distributed on the vertexes of a pentagon were introduced on the surface of three antiparallel beta sheets of EGFP as Ca\(^{2+}\) binding ligands as the third strategy (bottom row).

1.10 Limitations and challenges in designing Ca\(^{2+}\) sensors.

Currently the reported Ca\(^{2+}\) sensors based on natural Ca\(^{2+}\) binding proteins are limited due to the perturbation of Ca\(^{2+}\) signaling. The drawbacks of this kind of sensors still need to be improved, specifically the bulky size (more than 60k), high requirement of microscopy facility
(dual excitation and emission wavelengths), narrow Ca\textsuperscript{2+} binding affinity range (almost the same as wtCaM), and the risk of perturbing cellular Ca\textsuperscript{2+} signaling and non-specific binding to other small peptides by the functional group CaM or its variants. Another subgroup was created by Nakai and colleagues, who fused Ca\textsuperscript{2+} binding sequence, CaM, and the M13 fragment, into the original N and C terminal of EGFP, but regenerated novel N and C terminal of EGFP in a different location. This circularly permutated EGFP (cpEGFP)\textsuperscript{(93)} exhibited a larger signal dynamic range, but was limited to unquantitatively measuring Ca\textsuperscript{2+} concentration, due to its complex cooperative Ca\textsuperscript{2+} binding feature among its four EF-hands, as the Ca\textsuperscript{2+} binding process cannot be simply described by one association equilibrium equation.

To overcome these limitations, we report the rational design of Ca\textsuperscript{2+} biosensors by engineering a Ca\textsuperscript{2+} binding site into a single enhanced green fluorescent protein (EGFP). These developed Ca\textsuperscript{2+} sensors exhibit a ratiometric fluorescent signal change after binding to Ca\textsuperscript{2+}, with a K\textsubscript{d} value optimal for the measurement of Ca\textsuperscript{2+} in the ER. Metal selectivity of the sensors for Ca\textsuperscript{2+} in comparison with Ln\textsuperscript{3+}, and excessive biological metal ions such as Mg\textsuperscript{2+}, K\textsuperscript{+}, Na\textsuperscript{+} has also been examined. In addition, these developed sensors can be targeted to the ER, and exhibit high potential for living cell imaging. Further, their optical and conformational properties have been investigated using various spectroscopic methods. Moreover, pulsed-field-gradient nuclear magnetic resonance spectroscopy has been applied to probe their oligomeric state in solution and conformational changes of specific ligands due to Ca\textsuperscript{2+} binding were investigated using heteronuclear-labeled proteins with different 2D and 3D NMR techniques.
1.11 Biological functions of thrombin and Structural basis of thrombin substrate specificity.

Thrombin has been researched in various aspects since it was discovered in the 19th century, which is involved in many different processes, for example, cell signaling and memory, but the most important function of it is in blood coagulation. Thrombosis is a common reason to cause the death via venous thromboembolism, myocardial infarction or stroke, which all involve the inappropriate activity of thrombin.

The biological functions of thrombin can be divided to be two major parts: the first one is related with the coagulation cascade, as thrombin converts fibrinogen to fibrin clot and support platelet aggregation. The second role is related with cellular effects, as thrombin is vital for trafficking of inflammatory cells into sites of injury. It also plays a major role in tissue repair by enhancing expression of endothelial cells.

The inhibitors of thrombin are useful in medical applications, as the inappropriate amount of thrombin expression will cause venous thrombosis, inflammatory and fibrotic disorders, neuronal disease and cancer. Anti-thrombin therapies are now widely used in clinics with two major commercial inhibitors: heparin and warfarin.

Thrombin is a trypsin like enzyme and it comes from the chymotrypsin family of serine protease. Similar to trypsin, it has a positively charged amino acid at the P1 position of the cleavage bond. The whole enzyme has a small A-chain and a catalytic B-chain. The catalytic tetrad, which involves His 57, Asp 102, Ser 195 and Ser 214 is present at the bottom of a canyon-like cleft shaping the catalytic pocket. Thrombin’s catalytic activity contains recognition domains, which bind to the substrates, and insertion loops, which regulate the stability of the enzyme(94).
Thrombin interacts substrates with high specificity, and it prefers to cleave Arg-Gly or Arg-Ser in P1'-P1 position. The thrombin optimum recognition sites of the substrate now have a universal standard after more than two decades of research. In the optimized sequence of P4-P3-Pro-Arg-P1'-P2', P3 and P4 should be hydrophobic amino acid and P1' and P2' are nonacidic amino acids according to the study of the natural substrates of thrombin. In 1990s, small peptides which are composed of P4 to P1 sequence and a leaving group which function as a cleavage indicator were composed for the study of optimizing the P4 to P1 sequence(95). Thrombin and interaction models are well studied for the P1' to P3' sequence optimizing \[6\]. Another method for optimizing P1' to P3' is grafting thrombin recognition sites into a protein as a frame and then fixing P4 to P1 sequence but mutate P1' to P3' amino acid randomly to construct the P1' to P3' library and then select the most optimized sequence according to the steady-state kinetic parameters\[7\].

1.12 Our objectives and purpose of designing single EGFP-based thrombin sensors.

In this project, the major purpose is to construct a sensitive thrombin sensor both in vitro and in vivo which has advantages compared to the existing thrombin sensors. Currently, the major thrombin sensors can be divided to be three classes, first, electrode signal changed sensors, for example: DNA aptamer with nano-particles\[8\]; the second one, small peptide with leaving group\[9\]; the third one, the fluorescent resonance energy transfer pairs (FRET)\[10\]. But all of them have limitations. Nano-particles cannot be applied into the living cell image although they are very sensitive to detection of thrombin. The P1' to P3' sequence cannot be optimized for the small peptide with leaving group as all of these sites are occupied by a leaving group. For the FRET pairs, photobleaching is a common problem during the experiment as the structure of the whole protein is well exposed to the solution\[11\]. Another problem which cannot be
ignored is that the proton transfer can happen between different molecules if the distance between the donor and acceptor is appropriate so that the signal change does not come specifically from the proton transfer inside one molecule but also from the molecule and molecule interaction\textsuperscript{11}. In this project, studies of the steady state kinetic parameters were carried out to promote the detection of thrombin activity in living cells with real time.

1.13 Objective and overview of this dissertation.

The objectives of this dissertation are to demonstrate the rational design strategies of novel fluorescent indicators of Ca\textsuperscript{2+} and thrombin by genetically modifying green fluorescent protein (GFP), and structural analysis and determination of designed indicators with high magnetic field nuclear magnetic resonance spectroscopy (NMR) and X-ray crystallography to understand novel fluorescent enhancement of GFP triggered by Ca\textsuperscript{2+} binding. We further demonstrate the wide application of designed indicators in multiple cell lines in understanding unrevealed cellular physiology by using fluorescent microscopes.

The Chapter 2 is mainly focused on designing genetically encoded fluorescent indicator CatchER to study endoplasmic reticulum Ca\textsuperscript{2+} dynamics, including the theories of rationally designing Ca\textsuperscript{2+} binding sites on the surface of beta-sheet proteins assisted with computational algorithms, the experimental study of biochemical and biophysical properties of the designed sensors and measuring real-time endoplasmic reticulum Ca\textsuperscript{2+} dynamics in non-excitable cells with fluorescent microscopes, and the major works has been published recently.

Chapter 3 is the application of CatchER in studying residual sarcoplasmic reticulum Ca\textsuperscript{2+} concentration after Ca\textsuperscript{2+} release in skeletal myofibers from young-adult and old mice mainly contributed by Drs. Zhongmin Wang and Osvaldo Delbono, and this work has been published recently.
Chapter 4 is the structural analysis and determination of CatchER with high nuclear magnetic resonance spectrooscope (NMR) and X-ray crystallography is in Chapter 4 and 5, respectively, and the NMR results have been integrated in the previous publication. Chapter 6 is a review of Mg$^{2+}$ binding sites in proteins, including the major classes of Mg$^{2+}$ binding sites classified by the structural difference, and identifying several key factors to determine Mg$^{2+}$/Ca$^{2+}$ selectivity with binding constants difference up to $10^4$, and this work has been submitted as an invited review to Encyclopedia of Metalloproteins.

Chapter 7 describes designing a novel class of thrombin biosensors with fast ratiometric responses by grafting a short peptide with a thrombin cleavage site into the fluorescent sensitive location of EGFP.

Chapter 8 summarizes major findings and significance of this dissertation.

The DNA sequence of calsequestrin tethered CatchER and commands of NMRpipe, Sparky and chemical shift tables are listed in the Appendix.
1.14 Summary of the protein variants in this dissertation.

Table 1.1 Summary of the protein variants.

<table>
<thead>
<tr>
<th>Variants</th>
<th>Mutations/ Insertation</th>
<th>Purpose</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP cycle 2*</td>
<td>M153T/V163A of EGFP</td>
<td>Increase fluorescence intensity at 37 °C expression.</td>
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<tr>
<td>Ca$$^{2+}$$ indicator</td>
<td></td>
<td></td>
<td>2,3,4,5</td>
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<tr>
<td>D8</td>
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<td>D11(CatchER)</td>
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<td>2,3,4,5</td>
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<td>D12</td>
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<td>Five negatively charged residues as Ca$$^{2+}$$ binding ligands.</td>
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<tr>
<td>Ca-G1</td>
<td>EEEIRE- AFRVFDKDGGNY-ISAELRHVMTNL inserted between E172 and D173 of *</td>
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<td>Calsequestrin 1 fused in the N-terminal of CatchER</td>
<td>Specifically target CatchER in SR lumen</td>
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<td>Ratiometric CatchER</td>
<td>mCherry fused in the N-terminal of CatchER</td>
<td>Dual emission wavelength recording</td>
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<td>Indicator 1</td>
<td>EEEIRE- AFRVFDKDGRGY-ISAAELRHVMNTNL inserted between E172 and D173 of *</td>
<td>N187R of Ca-G1, to create a thrombin cleavage site on the loop of EF-hand III.</td>
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<tr>
<td>Indicator 2</td>
<td>EEEIRE- AFRVFDKDNGYIS-AFNPRGFMTNL inserted between E172 and D173 of *</td>
<td>FNPRGF (194-199) mutation of Ca-G1, to create a thrombin cleavage site on the F-hand of EF-hand III.</td>
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<td>Indicator 3</td>
<td>EEEIRE- AFRVFDKDNGYIS-AFNPRSFMTNL Inserted between E172 and D173 of *</td>
<td>FNPRSf (194-199) mutation of Ca-G1, to create a thrombin cleavage site on the F-hand of EF-hand III.</td>
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<td>Indicator 4</td>
<td>EEEIRE- AFRVFDKDNGYIS-AFTPRGFMTNL Inserted between E172 and D173 of *</td>
<td>FTPRGF (194-199) mutation of Ca-G1, to create a thrombin cleavage site on the F-hand of EF-hand III.</td>
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<td>Indicator 5</td>
<td>EEEIRE- AFRVFDKDNGYIS-AFTPRSFMTNL Inserted between E172 and D173 of *</td>
<td>FTPRSF (194-199) mutation of Ca-G1, to create a thrombin cleavage site on the F-hand of EF-hand III.</td>
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CHAPTER 2: DESIGNING GENETICALLY ENCODED FLUORESCENT INDICATOR TO STUDY ENDOPLASMIC RETICULUM CA\textsuperscript{2+} DYNAMICS

2.1 Overview of designing approaches and challenges of kinetic study.

As discussed in Chapter 1, quantitative analysis of Ca\textsuperscript{2+} fluctuations in the endoplasmic/sarcoplasmic reticulum (ER/SR) is essential to defining the mechanisms of Ca\textsuperscript{2+}-dependent signaling under physiological and pathological conditions. In this chapter, we developed a novel class of genetically encoded indicators by designing a Ca\textsuperscript{2+} binding site in the enhanced green fluorescent protein (EGFP). One of them, CatchER (Calcium sensor for detecting high concentration in the ER), exhibits unprecedented Ca\textsuperscript{2+} release kinetics with an off-rate estimated at around 700 s\textsuperscript{−1} and appropriate Ca\textsuperscript{2+} binding affinity, likely due to local, Ca\textsuperscript{2+}-induced conformational changes around the designed Ca\textsuperscript{2+} binding site and reduced chemical exchange between two chromophore states. CatchER reported considerable differences in ER Ca\textsuperscript{2+} dynamics and concentration among epithelial HeLa, kidney HEK 293, and muscle C2C12 cells, enabling us to monitor SR luminal Ca\textsuperscript{2+} in flexor digitorum brevis (FDB) muscle fibers to determine the mechanism of diminished SR Ca\textsuperscript{2+} release in aging mice. This novel sensor will be invaluable in examining pathogenesis characterized by alterations in Ca\textsuperscript{2+} homeostasis. To achieve our goal to design Ca\textsuperscript{2+} sensor to monitor Ca\textsuperscript{2+} fluctuations in high Ca\textsuperscript{2+} cellular environment requires extensive knowledge about Ca\textsuperscript{2+} binding methods to identify and design Ca\textsuperscript{2+} sites in proteins.

Identification of calcium binding sites in the proteins with significant biological functions, such as calcium sensing receptors, calcium releasing channels, calcium pumps and proteases regulated by calcium concentration can provide the quantitative methodology of calcium and protein interaction measurement and further extend the research boundary for the accurate
definition of the multiple signaling pathways. As shown in Figure 1.8, Ca^{2+} binding sites can be classified as continuous (including EF-hand) and discontinous. In the early ages, the identification was much more relied on the amino acid sequence information and the alignment computational algorithms played an important role to achieve the goal, so the majority of predicted calcium binding sites was limited to be continuous sequences. In 1996, Bertrand and his co-workers (96) identified five calcium binding amino acid sequences which possessed significant sequence homology with EF-hand III loop of calmodulin in N terminal domain of neuronal nico- tinic receptors by the sequence searching software CALM(97) which was designed to search the continuous calcium binding sites. All of these sequences were featured to be rich in hydrophilic and acidic amino acids and involving a terminal glutamate. The site-directed mutation of all the glutamates to glutamines of the five sequences affected the binding affinity and the predicted binding ligands were further proved by this approach. The 5-hydroxytryptamine (5HT_{3}) serotoninergic receptor were turned from calcium insensitive to calcium potentiated by introduction of one of the predicted calcium binding sequence (loop V), which could bolster the accuracy of the previous computational prediction.

Identification can be achieved by sequence comparison with the homological protein with the known calcium binding sites in the absence of x-ray structure. Egmond and his co-workers (98) reported identification of calcium binding site in Staphylococcus hyicus Lipase (SHL) by sequence alignment with other staphylococcal lipases. Sequence alignment was conducted by MacVector package (Oxford Molecular Group). Without X-ray crystal structure of Staphylococcus available, they applied the reported X-ray structure of Pseudomonas glumae lipase sharing about 35% identical amino acid sequence with SHL containing an identified calcium binding site and attempted to predict the putative calcium binding ligands of SHL by the
primary sequence alignment. After comparison with another four Staphylococcus lipases amino acid sequences, they finally located the conserved acidic amino acid sequence and identified the first binding ligand. With the help of site-directly mutation, they predicted the other calcium binding ligands and generated the calcium insensitive staphylococcus hyicus lipase. This sequence homology alignment is largely limited to continuous $\text{Ca}^{2+}$ binding site and it could not directly tell the binding ligands, and less confident when the homology two sequences is low.

As discussed in Chapter 1, advanced prediction programs have been created and applied. Regarding to the vital biological function role of EF-hand protein containing a helix-loop-helix calcium binding motif constituting one of the largest protein families, in 2006, Yang’s group reported a pattern based EF-hand sequence prediction algorithm (99) which was quickly applied in identification of a calcium binding domain in the rubella virus nonstructural protease (Zhou, Journal of Virology, 2007). This pattern based algorithm was a powerful tool to predict and distinguish canonical, pseudo EF-hand and EF-hand like sequence with or without flanking helices by the signature of calcium binding ligands which has been mentioned in the former part. Utilizing this method, a putative EF-hand calcium binding motif within rubella virus was predicted to be highly conserved in the eight genotypes, all involving all of the coordination ligands. This result was further bolstered by the secondary structure prediction by the program PSIPRED (Cuff, Bioinformatics, 1998), JPRED (McGuffin, Bioinformatics, 2000), and PHD (Rost, Nucleic Acids Res., 2004) to demonstrate the helix-loop-helix structure of the predicted calcium binding sequence. Parallel evidence came from the similar prediction results by GG algorithm (Deng, Proteins, 2006) based on the homology model of the rubella virus nonstructural protease generated by the program SWISS-MODEL. The established grafting approach
(Ye, JACS, 2005) was applied to demonstrate the reality and quantity of calcium binding affinity of the predicted loop region by inserting this sequence into CD2. This recombined protein was titrated by calcium trivalent analogs Tb$^{3+}$ and Ln$^{3+}$ to determine the calcium binding affinity. Specific calcium binding ligands were site-directly mutated to test the contribution to the binding affinity. This work demonstrated that the calcium binding loop predicted was required for the stability of rubella virus nonstructural protease under physiological conditions. It is feasible to systematically apply EF-hand sequence prediction methodology by combining utilization of primary sequence prediction algorithm and structure based prediction programs.

The limitation of application of the prediction algorithm based on primary sequence is apparent when applied to discontinuous binding sites which have the binding ligands distantly arranged in the amino acid sequence but close enough sterically to form the binding pockets. The pentagonal bipyramidal oxygen coordination reported (Strynadka, Annu. Rev. Biochem., 1988; Yang, Proteins, 2002) was regarded as the putative calcium binding site geometry. Ligands searching piecemeal was set to be the prediction strategy because a putative bidentate binding ligand appeared in the calcium binding site. The firstly located ligand is the bidentate ligand named anchor, usually Glu for EF-hand while Asp for non-EF-hand. Then a calcium atom was attached to the two oxygen of the carboxyl group in the anchor within the same plane. The geometry of calcium binding pocket was now defined by the coordinate of the anchor and the calcium atom, so that the other ligands were discovered by screening which sidechain had the appropriated angles and distance to fit into the pentagonal bipyramidal geometry. This calcium binding site searching algorithm was called MetalFinder modified from DEZYMER (Hellinga, 1991) and applied to both of calcium binding site identification and design. In 2007, Huang (Huang, JBC, 2007) employed this algorithm to successfully identify three
calcium binding sites in the extracellular domain of calcium sensing receptor. These three calcium binding sites had distinguished features from each others and quite different from the classical EF-hand sequence pattern, which were not able to be detected by the previous sequence-based prediction algorithms. Site one and site two were, to some extent, continuous-sequence binding sites as site one contained five glutamate residues in a 9-residue sequence with three positively charged residues involved located on the surface of an alpha-helices while ligands from site two were dispersed in a 22-residue sequence. Site three was the discontinuous-sequence binding site due to 150-residue distance between the first ligand and the last one. Site-direct mutation, grafting approach, Tb\(^{3+}\) titration, circular dichroism and NMR TOCSY and HSQC were applied to further demonstrate the reality of binding affinity and accuracy of specific binding ligands which matched the results predicted by MetalFinder algorithm. Yang lab has demonstrated the success in identifying multifarious calcium binding sites which are either continuous or discontinuous without being confined by EF-hand pattern based on modeled structure using developed calcium binding site prediction algorithms, including MUG with the potential to visualize Ca\(^{2+}\) binding protein in signal pathways.

**Design:** The rational design of novel proteins initiated a new era of intergradations between profound exploration of protein intrinsic properties and protein functions. It started out as an unsophisticated approach in 1986 and has now evolved into taking advantages of tertiary and quaternary structures, advancing toward creating new sensors, inhibitors, catalysts, clinical agents and pharmaceutical drugs. Dr. Mayo reported an unbiased quantitative design algorithm totally based on the physical properties that determine protein structure and stability and that is not restricted to specific folds or motifs, which could predict and de novo design the hydrophobic core or the hydrophilic surface of protein. It started with the sequence screening
which was achieved by two strategies: the first one was based on the backbone dynamics and the second one was the application of the sidechains' conformations library 'Rotamer' to determine the stability and the final conformation of the backbones. The designed peptides were structurally determined by NMR COSY and the results match the predicted ones.

Dr. Imperiali and her coworkers published the development of the modular peptide scaffold for fluorescent sensing of divalent zinc (Imperiali, JACS, 2003). The signaling component of the chemosensor is the chelation-sensitive fluorophore 8-hydroxy-5-(N, N-dimethylsufonamido)-2-methyquinoline. The synthetic peptides incorporating the signaling chromophore are able to bind zinc with different binding ligands. The applications of these sensors are tested by the fluorescence response and the binding ratio is proved to be 1:1 with verified binding affinity from 10 nM to nearly 1 µM.

In respect to calcium binding motif design, up to now, there are several strategies. I will select three of them for the further discussion.

Dr. Yang and her colleagues systematically and rationally designed calcium binding proteins based on computational algorithms. In 2002, they reported a novel calcium searching program which was based on ligands identified piecemeal according to appropriated angles and distance of sidechain candidators to fit into the putative calcium binding ligands geometry with the shape of pentagonal byprimadary. It could suggest the novel binding ligands by automatically replacing the sidechains to be optimal binding ligands after geometry calculation, which was treated as one of the advantages of this program and different from the other programs specially designed for only prediction algorithms. This program was utilized to designing a series of calcium binding pockets on a non-calcium-binding protein, domain 1 of CD2, which was a beta-sheet dominant protein. In 2003, they published the first generation of CD2-based
calcium binding protein CD2.Ca1 with seven ligands and demonstrated that it selectively bound calcium rather than magnesium. Four of the ligands came from two sequential beta sheets arranged at the vertices of the squares which protrude outside on the same surface. In addition, two spatially close residues are organized within one interval at the terminal of one beta sheet paralleled with another two from the sequential beta sheets connected by a 9-residue loop. The other one came from a sterically close but sequentially distant loop region. It signaled the first successful metalloprotein design with high coordination number on beta sheet protein. In 2005, consequentially in-depth work was reported in the same journal to reveal the ability of controlling the desired structure of scaffold protein during calcium-binding protein design, which plays important role to maintain the host protein biological function. The second generation of CD2 based calcium binding proteins were designed by the same algorithm MetalFinder but were further evaluated and analyzed for their intrinsic coordination properties and protein environment characters with the consideration of protein hydrophobicity and folding conservation by the program PROTEUS to rank the solution accessibility, the number of mutations and charge introduced. NMR HSQC calcium and paramagnetic metal titration clarified the particular binding ligands and cell adhesion investigation demonstrated the conservation of the biological function of the engineering CD2. This paper raised the research of rational metalloprotein design to a higher level, as it represented an attempt to advanced to design biologically significant proteins to control physiological calcium signaling, which had the profound potential for the further application for the creation of new biomaterials, sensors, catalysts, and pharmaceuticals. The clinical practice of designing calcium-binding motifs is definitely heating up nowadays as the functional properties of current generation of MRI contrast agent (Gd_DTPA) which should bind lanthanide with specifically high affinity and selectivity is in ur-
gent need to be improved. Dr. Yang’s novel design of Gd$^{3+}$ binding motif with years of calcium binding motif experience successfully solve the problems of Gd-DTPA such as low relaxation, weak contrast imaging, short blood retention time and lack of targeting to specific sites in the body. (Yang, JACS, 2007) The promising prospect of this contrast agent will further apply to the protein molecular imaging probes to target disease markers and broaden the application of MRI.

2.3 Methods

**Plasmid construction, protein expression, and purification.** Bacterial expression plasmids for EGFP variants D8 to D12 were constructed by site-directed mutagenesis on cycle 2 EGFP (F64L/S65T/M153T/T163A) inserted in the pET28a vector (EMD Biosciences, San Diego, CA) vector between the BamHI and EcoRI restriction enzyme cleavage sites. The DNA sequence of the designed EGFP variants between these two restriction sites were cleaved and inserted into pcDNA3.1+ vector (Invitrogen, Carlsbad, CA). Calreticulin ER targeting sequence (CRsig) MLLSVPLLGLLGLAAAD and ER retention sequence KDEL were added to the N- and C-termini, respectively, to construct the mammalian cell expression plasmids. EGFP variants were bacterially expressed in *Escherichia coli* BL21(DE3) following reported methods (10, 71).

**Characterization of the optical properties of purified EGFP variants.** The absorption spectra of EGFP variants were measured with a Shimadzu UV-1601 spectrophotometer (Kyoto, Japan). The protein concentration was determined at absorption maximum, 280 nm, with the co-efficient 21890 cm$^{-1}$ M$^{-1}$ (71) and fluorescence spectra were determined (Photon Technology International, Inc., Birmingham, NJ).
Cell culture and DNA transfection. C2C12 myoblasts were cultured in Dulbecco’s Modified Eagle’s Medium containing 4.5 g/L glucose (high glucose) with 2 mM L-glutamine plus 1.5 g/L sodium bicarbonate. CatchER plasmid DNA was transfected into these myoblasts by incubating the mixture of DNA and lipofectamine 2000 at a ratio of 1:2 in OPTI solution at 37°C for 4 h. The transfection complex was replaced with fresh Dulbecco’s Modified Eagle’s Medium. Transfected cells were incubated at 30°C and imaged after 48 or 72 h. A similar protocol was applied to HeLa, HEK-293, and BHK cells.

Real-time fluorescence imaging. Real-time cell imaging was conducted on an inverted fluorescence microscope (Leica DMI6000 B) with a cooled EM-CCD camera (Hamamatsu C9100). The single-wavelength sensor was excited at 488 nm by a Xenon lamp (Polychrome V system, TILL PHOTONICS), with an HQ480/20x excitation filter, a 515DCXR dichromatic mirror and a D535/25 emission filter (Chroma Technology Corp). Fura-2 was excited alternatively at 340 nm with an excitation filter D340xv2 and at 380 nm with a D380xv2 filter, a 400DCLP dichromatic mirror, a D510/80 m emission filter. A perfusion system was used for drug application and solution exchange. Cells were imaged at 70% confluence. Intact cell imaging was conducted in Ringer buffer (121 mM NaCl, 2.4 mM K$_2$HPO$_4$, 0.4 mM KH$_2$PO$_4$, 10 mM HEPES) with or without 1.8 mM CaCl$_2$. Cells were permeabilized with 25 mM digitonin for 3 to 5 minutes and imaged in intracellular buffer (125 mM KCl, 25 mM NaCl, 10 mM HEPES, 0.5 mM Na$_2$ATP, 0.2 mM MgCl$_2$, 200 µM CaCl$_2$, 500 µM EGTA. Final free [Ca$^{2+}$]: 100 nM, pH 7.25).

In situ measurement of CatchER's Ca$^{2+}$ dissociation constant. CatchER’s Ca$^{2+}$ dissociation constant ($K_d$) was measured in BHK and C2C12 cells. ER Ca$^{2+}$ in BHK cells was depleted by applying 100 µM histamine and 5 µM thapsigargin in Ringer 0 Ca$^{2+}$ buffer. Cells were permeabilized in 100 µM digitonin in intracellular-like solution containing 140 mM KCl, 10 mM
NaCl, 1 mM MgCl₂, 20 mM Hepes, pH 7.25 (40). Calibration buffers were prepared by adding Ca²⁺ to the intracellular-like solution, reaching final concentrations of 0.05, 0.1, 0.5, 1, 5, and 10 mM, and 200 mM EGTA buffer. F_{min} and F_{max} were determined in 200 mM EGTA and 10 mM Ca²⁺ with no Ca²⁺ ionophore, respectively.

Similar in situ K_d calibration was conducted in C2C12 myoblasts. ER Ca²⁺ of permeabilized cells was depleted in intracellular buffer containing 10 mM IP₃ and 2 mM thapsigargin. For calibration, 1, 3, 10, and 20 mM Ca²⁺ buffers were applied in the presence of 5 mM ionomycin. F_{min} and F_{max} were determined in 3 mM EGTA and 20 mM Ca²⁺, respectively.

The fluorescence was normalized according to the equation:

\[
\frac{[Ca]}{K_d} = \frac{[FCa]}{[F]} = \frac{f - S_F[F]_T}{(S_{FCa} - S_F)([F]_T - [FCa])} = \frac{f - f_{min}}{f_{max} - f}
\]

\[R_f = f_{max}/f_{min}\]

The equation indicates the relationship between the fluorescence intensity change and calcium concentration, provided from Gryniewicz’s published paper, in which f is the fluorescence intensity, S_F is the coefficients unbound form and S_{FCa}, is the coefficient’s bound form.

\[f = \frac{F - F_{min}}{F_{max} - F_{min}}\]

K_d is determined by the Hill-equation.

The K_d value was 1.07 ± 0.26 mM (0.90 ± 0.19 Hill coefficient) in BHK cells and 1.09 ± 0.20 mM (0.94 ± 0.17 Hill coefficient) in C2C12 cells.
Kinetics of CatchER investigated by stopped-flow spectroscopy. The kinetics of fluorescence response to Ca$^{2+}$ of bacterially expressed CatchER were investigated by a Hi-Tech SF-61 stopped-flow spectrofluorimeter (10 mm path length, 2.2 ms deadtime in room temperature) at 22 °C. Fluorescence intensity changes were recorded with a long-pass 455 nm filter with excitation at 395 nm. 10 µM Ca$^{2+}$-free protein solved in 10 mM Tris buffer at pH 7.4 was loaded into one syringe and 40, 70, 100, 400, 600, 1000, 2000, 3000 µM Ca$^{2+}$ with the concentration to be half after mixture ranged from 5 times lower and higher than the apparent $K_d$ (around 100 µM) measured in the same buffer was loaded into the other syringe. At least 6 shots for each concentration were recorded and the last 3 time. 100 µl of protein was mixed with the Ca$^{2+}$ in 1:1 (vol/vol) for each shot. The equations below are applied to fit the fluorescence traces with single exponential change.

$$F = F_\infty - \Delta F \exp(-k_{obs} \cdot t)$$

$$F = F_\infty + \Delta F \exp(-k_{obs} \cdot t)$$

$$k_{obs} \cdot \tau = \ln 2$$

$F$ is the real-time fluorescent intensity; $F_\infty$ is the final fluorescent intensity; $\Delta F$ is the absolute value of fluorescence change between the final plateau and initial stage. $k_{obs}$ is the observed rate constant (s$^{-1}$); $\tau$ is the duration time when half of $\Delta F$ is reached, $\tau = t$, in the condition of $|(F-F_\infty)/\Delta F|=1/2$. Equation (1) or (2) is to fit the results of calcium association or EGTA chelate, respectively. $F_\infty$ is determined by curve fitting with the equation (1) or (2); $\Delta F$ can also be calculated by fitting, however, it is usually smaller than the actual $\Delta F$ measured from the dif-
ference between the \( F_{\infty} \) and the basal fluorescence when the protein mixed with the buffer as a result of underestimation by fitting only the last tail of the whole fluorescence curve with around 30% initial fluorescence lost within the deadtime.

Two approaches were applied to investigate the kinetics. The first one was to mix concentration-fixed calcium-free CatchER with different concentrations of calcium as shown in fig 2 (d). The scheme of the process and kinetic parameters were defined below with 1:1 binding mode.

\[
\text{Ca}^{2+} + P \xrightleftharpoons[k_{-1}]{k_1} \text{CaP}
\]

\[
\frac{d[\text{CaP}]}{dt} = k_1[\text{Ca}^{2+}][P] - k_{-1}[\text{CaP}]
\]

\[
[P] = [P_0] - [\text{CaP}]
\]

\[
\frac{d[\text{CaP}]}{dt} = k_1[\text{Ca}^{2+}][P_0] - [\text{CaP}] - k_{-1}[\text{CaP}]
\]

\[
\frac{d[\text{CaP}]}{dt} = -(k_{-1}[\text{Ca}^{2+}] + k_{-1})[\text{CaP}] + k_1[\text{Ca}^{2+}][P_0]
\]

\[
k_{obs} = k_1[\text{Ca}^{2+}] + k_{-1}
\]

The correlation of \( k_{obs} \) and \( k_{on} \) and \( k_{off} \) were derived from Equation 4) to 8) according to 1:1 binding scheme. \( P \) is the Ca-free protein CatchER; \( P_0 \) is the total protein; \( k_1 \) is \( k_{on} \); \( k_{-1} \) is \( k_{off} \); \( t \) is the time. A gap between the initial fluorescence and the baseline was observed when
CatchER was mixed with calcium, and the amplitude increased as a function of \([\text{Ca}^{2+}]\), revealing around 30~40% of the initial fluorescence increase finished within the deadtime (2.2 ms) at room temperature. The fluorescence continued rising until 40 ms to form a plateau, suggesting the termination of the reaction. The amplitude of the fluorescence was stable in this stage with the longer time observation, suggesting no lagging phase 40 ms after mixing. The calculated \(k_{\text{obs}}\) by equation 1) were 104 ± 22, 74 ± 11, 71 ± 6, 63 ± 5, 59 ± 5 and 60 ± 3 s\(^{-1}\) from low to high \([\text{Ca}^{2+}]\), and the corresponding initial fluorescence intensity \((F_\infty - \Delta F)\) were 0.072, 0.169, 0.233, 0.273, 0.308, 0.386 instead of 0. The amplitude of the lost fluorescence within the dead-time increased at higher \([\text{Ca}^{2+}]\), suggesting the underestimation of the kinetics and partially explained the decreased \(k_{\text{obs}}\) in the function of \([\text{Ca}^{2+}]\). To further investigate whether \(k_{\text{obs}}\) decreased in the function of \([\text{Ca}^{2+}]\), \(k_{\text{obs}}\) was also be calculated by the equation derived from equation 1) or 2), \(k_{\text{obs}} = -\ln |(F - F_\infty)/\Delta F|/t\). The detected initial fluorescence mentioned before was regarded as the real-time fluorescence \(F\) at 2.2 ms. The \(\Delta F\) was the difference between \(F_\infty\) and the basal line 0. \(k_{\text{obs}}\) was calculated as 144, 233, 219, 232, 215, respectively with this method named initial point calculation, in comparison to the previous curve-fitting method. \(k_{\text{obs}}\) maintained in a plateau when \([\text{Ca}^{2+}]\) climbed over 100 µM, featuring a saturated stage, correlated with an approximate 100 µM Kd measured with the same condition. Except for 50 µM \([\text{Ca}^{2+}]\), at which the \(k_{\text{obs}}\) values were relatively similar (104 and 144 s\(^{-1}\)) measured by two methods, big discrepancy both of the values and trend occurred at higher \([\text{Ca}^{2+}]\), suggesting the first method underestimated the kinetics as the loss of fluorescence within the deadtime due to the fast response.

\[
\text{Ca}^{2+} + P \xleftrightarrow[k_1]{k_\text{-1}} \text{CaP}
\]
The second method is to chelate Ca\(^{2+}\)-loaded CatchER by EGTA by the proposed scheme shown above. In addition to the same definition of the parameters as before, E is the EGTA and CaT is the total Ca\(^{2+}\). \(k_2\) and \(k_{-2}\) are \(k_{\text{on}}\) and \(k_{\text{off}}\) of EGTA-Ca, reported as \(5 \times 10^7\) M\(^{-1}\) s\(^{-1}\), and 0.3 s\(^{-1}\). \(k_{\text{obs}}\) for this experiment are contributed by the \(k_{\text{off}}\) value both from CatchER (\(k_{-1}\)) and EGTA (\(k_{-2}\)). As [E] is 100 µM after mixture, 10 times higher than [P] (10 µM), and usually \(k_{\text{on}}\) value for protein based calcium indicator is around \(10^6\) M\(^{-1}\)s\(^{-1}\), much smaller than that of
EGTA ($10^7$ M$^{-1}$s$^{-1}$). We concluded $k_2[E]/(k_1[P]) \gg 1$, and $k_2$ is estimated to be much smaller than $k_1$, the second term of the equation is negligible.

20 µM CatchER pre-mixed with 20 µM Ca$^{2+}$ was loaded into one syringe and either 0.2 mM EGTA was loaded into the other one. 70% fluorescent change finished within the dead-time, fig 2 (e). The kobs fitted by Equation 2) is $10 \pm 2$ s$^{-1}$, but 550 s$^{-1}$ if consider initial fluorescence 0.298 at 2.2 ms. The underestimation is apparent for both Ca$^{2+}$ association and EGTA chelating by curve fitting due to the initial signal lost, however, more data distributed in a longer-time range calculated in the this method than only one point at a particular time was used in initial point calculation advanced in preventing the sporadic errors.

**Apparent pK$_a$ determination by pH profile.** The apparent pK$_a$ of Ca$^{2+}$-free or Ca$^{2+}$-loaded CatchER was determined with bacterially expressed protein by fitting the fluorescence intensity change at 510 nm ($\lambda_{ex} = 488/395$ nm). 5 mM protein was dissolved in different buffers with pH ranging from 4.5 to 9.5 in the presence of either 10 µM EGTA (apo) or 4 mM Ca$^{2+}$ (holo), and the actual pH was determined after measuring fluorescence. The proposed interaction scheme is

$$\text{HP}^+ \rightleftharpoons \text{H}^+ + \text{P}$$
\[ pH = pK_a + \log \frac{[P]}{[HP^+]} \]

\[ f = \frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \]

\[ F_{\text{min}} = [P]_T c_1 \]

\[ F_{\text{max}} = [P]_T c_2 \]

\[ F = ([P]_T - [P]) c_1 + [P] c_2 \]

\[ f = \frac{[P]_T c_1 - [P] c_1 + [P] c_2 - [P]_T c_1}{[P]_T c_2 - [P]_T c_1} = \frac{[P]}{[P]_T} \]

\[ \frac{[P]}{[HP^+]} = \frac{1}{1/f - 1} \]

\[ f = \frac{1}{1 + \exp\left(\frac{pK_a - pH}{c}\right)} \]

\( H^+ \) is the proton; \( P \) is the CatchER protein; \( f \), the normalized \( \Delta F \) change; \( [P]_T \), the total protein concentration; \( c_1 \) or \( c_2 \) is the extinction coefficient of \( HP^+ \) or \( P \) fluorescence, respectively; \( F \) is the real-time fluorescence; \( F_{\text{min}} \), the fluorescence at the lowest pH; \( F_{\text{max}} \), the fluorescence at the highest pH; \( c \) is a constant for adjustment. The value theoretically equals lge. The apparent \( pK_a \), fitted by a single exponential (Equation 11), were 7.59 ± 0.03 and 6.91 ± 0.03 for apo and holo forms excited at 488 nm and 7.14 ± 0.02 and 6.95 ± 0.06 at 395 nm, respectively.
**CatchER:Ca^{2+} stoichiometry studied by the Job Plot.** The stoichiometry of the CatchER and Ca^{2+} interaction was determined at the maximal relative amount of Ca^{2+}-bound CatchER in the Job Plot (106). Ca^{2+}-free and bound [CatchER] were converted to fluorescence intensity following the equation: \( F = S_f \cdot C_f + S_b \cdot C_b \), where \( F \) is the apparent fluorescence intensity; \( S_f \) and \( S_b \) are the coefficients of Ca^{2+} free and bound CatchER, respectively; and \( C_f \) and \( C_b \) are the concentration of Ca^{2+} free and bound CatchER, respectively. The relative amount of Ca^{2+} bound CatchER \((C_b \cdot V, V=1)\) was calculated using the Equation (12). Fluorescence emission \( (\lambda_{ex} = 488/395 \text{ nm}) \) and absorbance spectra were recorded with [CatchER]: 28.7, 23.3, 19.4, 15.1, 11.6 \( \mu \text{M} \) in response to [Ca^{2+}]: 11.3, 16.7, 20.6, 24.9, 28.4 \( \mu \text{M} \), respectively.

\[
\frac{F_{\text{Ca}^{2+}-\text{bound}}}{F_{\text{Ca}^{2+}-\text{free}}} = S_f \cdot C_f + S_b \cdot C_b = S_f (C_f - C_b) + S_b \cdot C_b = 1 + \frac{C_b \cdot (S_b - S_f)}{S_f \cdot C_f}
\]

\[ a = \frac{S_b - S_f}{S_f} \]

\[ \frac{C_b}{C_T} \cdot a = \left( \frac{F_{\text{Ca}^{2+}-\text{bound}}}{F_{\text{Ca}^{2+}-\text{free}}} - 1 \right) \cdot \frac{C_T}{a} \]

**CatchER calibration in FDB fibers.** CatchER fluorescence was transformed into Ca^{2+} concentration according to the equation \([\text{Ca}^{2+}] = K_d (F_{\text{Fmin}})/(F_{\text{Fmax}} - F) \) (107). The \( K_d \) value was calculated in enzymatically dissociated FDB fibers, as reported (108), with some modifications. Fibers from young and old mice expressing CatchER were exposed to 0.01% saponin for ap-
proximately 2 min in a solution containing: 90 mM K-glutamate, 1.02 mM MgCl$_2$, 5 mM NaCl, 10 mM HEPES, 1 mM, BAPTA, 0.323 mM CaCl$_2$, 0.025 BTS, 2% PVP (1 mM free Mg$^{2+}$; 0.0001 mM free Ca$^{2+}$), pH 7.2, adjusted with KOH. The permeabilized myofiber was exposed to 10$^{-6}$ M ionomycin diluted in the previous solution to equilibrate various free Ca$^{2+}$ concentrations among the extracellular space, cytosol, and lumen of the SR. For all solutions, free [Mg$^{2+}$] was set at 1 mM and free [Ca$^{2+}$] was set at concentrations ranging from 10$^{-7}$ to 10$^{-1}$ M by buffering with 1 mM of 1,2-bis(o-aminophenoxy)ethane-n,n,n',n'-tetraacetic acid (BAPTA), as calculated by the Max-Chelator program. CatchER’s $F_{\text{min}}$, $F_{\text{max}}$ were measured in each fiber for which we reported the SR resting Ca$^{2+}$ concentration. CatchER’s $K_d$ measured in FDB fibers from young and old mice was 1.66 ± 0.08 mM and 1.71 ± 0.12 mM, respectively. Fluorescence was recorded after fiber exposure to 0.01% saponin in 100nM Ca$^{2+}$ solution (no ionomycin added), pCa 7 plus ionomycin or pCa 1 plus ionomycin, were considered for $F_{\text{rest}}$, $F_{\text{max}}$, and $F_{\text{min}}$. The $F_{\text{min}}$ value was confirmed by adding 1 mM 4-Chloro-m-Cresol (4-CmC) to fibers incubated in 100 nM Ca$^{2+}$ plus ionomycin. The $F_{\text{cresol}}/F_{\text{Ca+iono}}$ ratio was 1.06 ± 0.007 (n = 5). Fibers were imaged in the x-y mode of the confocal microscope 1 min after solution exchange or until SR fluorescence reached a steady value.

2.4 Results

2.4.1 Design strategy

Based on key determinants for fine-tuning Ca$^{2+}$ binding affinity and Ca$^{2+}$-induced conformational changes and the established chromophore properties of fluorescent proteins, we hypothesized that Ca$^{2+}$ sensors with fast fluorescence response could be better designed by coupling Ca$^{2+}$ binding sites directly to the chromophore rather than relying on stretched protein-protein interaction to modulate chromophore conformation. Our computationally assisted
design is based on the following criteria and considerations: first, it requires four or five oxygen ligand atoms from protein residues (typically, carboxyl groups of D, E, N, Q) situated in the spherical geometry characteristic of natural Ca$^{2+}$ binding proteins (43, 52[Deng, 2006 #2118, 100, 101]); second, appropriate choice of residue charge and type can be chosen to fine-tune Ca$^{2+}$ binding affinity and metal selectivity (62, 63); third, diffusion-limited access of Ca$^{2+}$ to the site requires good solvent accessibility (102); fourth, propagating Ca$^{2+}$-induced, local conformational and electrostatic changes to the chromophore can be achieved by properly locating of the charged ligand residues with respect to it (103, 104); fifth, these changes must occur rapidly—more rapidly than the rate of conversion from a neutral to anionic state ascribed to these chromophores (71, 105); and sixth, the created binding site must not interfere with the chromophore’s synthesis and formation. The EGFP variant with the M153T/V163A mutation (Cycle 2) was chosen as the scaffold protein because of its high fluorescence intensity, folding efficiency, and thermostability (91).

2.4.2 Designing Ca$^{2+}$ binding sites on the surface of beta-sheet protein of EGFP.

Based on the previous findings in the designing calcium binding pockets on the scaffold of CD2 with four negative charged binding ligands and the binding affinity is about 0.2 mM. We directly apply these criteria for designing calcium-binding pocket on EGFP as the fluorescent sensor.

Figure 2.4 and 2.5 show several calcium binding pockets found by computational algorithm based on our defined Ca$^{2+}$ binding geometry and ligand type, In this case, a huge number of designed sites were generated by this program. For our two most important criteria, the mutation should not quench the intensity fluorescence to be invisible and the designed pocket should bind to calcium. To satisfy the first standard, we search the published data which indi-
cates that location can be mutated without disturbing intensity of fluorescence. With the reported copper binding sites, a mutated pocket on the surface of EGFP close to chromophore is discovered. It is the 147, 202, 204 and 223 residues, which are distributed in three beta sheets. According to the excited-state photon transfer schemes show in Fig 2.6, His148, Thr 203, Ser 205 and Glu 222 which are neighboring to 147, 202, 204 and 223 respectively are involved in the proton transfer chain, we choose this site as calcium binding pocket for the purpose of the maximal influence of calcium to the chromophore which will be reflected in the huge dynamic range.

The risk we take to design a binding pocket around this site is that the mutation may disturb the intensity of fluorescence. Although it is reported that this site can be mutated, for their design only two residues are mutated to be negative while the other two are kept to be neutral polar residues while for our design the optimal one is that all of the four residues should be negative charged. Therefore, we designed several mutation types as alternatives.

Among all the designed sites summarized in Figure 2.5, we ranked the two optimal calcium binding pocket candidates shown in Figure 2.7. Both of them include three negatively charged residues located in the point of the angles of a square so that only one residue is necessary to be mutated to be negatively charged in order to create the calcium binding pocket.
Figure 2.4 Selected binding sites on the surface of EGFP by Dezymer.
Figure 2.5 Representative Ca$^{2+}$ binding sites designed by Dezymer on the surface of EGFP, with proposed Ca$^{2+}$ ion located in the center of designed sites. The distance between ligand oxygen to Ca$^{2+}$ is measured by Rasmol with the structural files created by Dezymer.
In addition, we also applied Rasmal to calculate the distance of calcium to ligand oxygen. The optimized distance from calcium to oxygen is 2.4 Å, however, some ligands are not optimal in the binding pockets is not optimal since the sites were not refined to minimize the overall energy. Considering the risk to mutate 147 to be Glu, we also design other mutated pocket to keep the 147 to be Ser as seen in nature.

Figure 2.6 Scheme of GFP excited state proton transfer (ESPT).
The neighboring residues map provides a very direct overall imaging of the residue charge properties. From this map shown in Figure 2.7, it is easy to discover some calcium binding pockets candidates. For example, D21, D19, E17 and E124 generate the two most promising candidates which involve 21, 19, 124 and 126 in one pocket and 19, 17, 124 and 122 in the other pocket as both of these two candidates only need one residue mutation to form a real pocket. Other candidates which are also competitive, including 32, 34, 16, 18 and 19, 17, 28, 30 and 122, 124, 109, 111 and 109, 111, 93, 95, as all of them only need two residues to be mutated to form a real pocket.

**Figure 2.7 Map of EGFP neighboring residues connected by mainchain hydrogen bonds.**
The key to success of designing a sensitive calcium-binding pocket also depends on the dynamic range change and the calcium binding affinity. Improvement of the dynamic range is indicated by enhancement of the influence of calcium on the chromophore fluorescence. The most direct way is to design the calcium-binding pocket close to chromophore which may have the reverse effect of destroying the fluorescence after mutation. One way to solve this problem is to search the published data as the reference. Improving the calcium binding affinity depends on the optimizing the ligand residues. Negatively charged residues such as Glu and Asp are optimal binding residues.

2.4.3 Designing different DNA sequences of EGFP-based calcium sensors and improving Ca$^{2+}$ dynamics of Ca$^{2+}$ sensors developed by grafting.

Dr. Zou in Yang lab developed a Ca$^{2+}$ sensor G1 by grafting an EF-loop at the fluorescence sensitive location of EGFP (between site 172 and 173), shown in Figure 2.8. This sensor has a ratiometric response to Ca$^{2+}$, but small dynamic range. To optimize the dynamic range of calcium detection is to first design the calcium-binding pocket in the sensitive part of the EGFP and second to add more calcium binding pockets on the existing sensors for the multiple interactions.
The site directed mutation may sometime change the absorbance and fluorescence characters of the wild type EGFP. The G1-EGFP increased the absorbance in 398 nm and de-

![Diagram](image_url)

**Figure 2.8** Scheme of Strategy 1. Add additional calcium binding site to enhance calcium response of Ca-G1.

1. Electrostatic map: three negative charged residues closed
2. Close to chromophore: ~ 15 Å (Grafting site > 25 Å)
3. Mimic 6D31 trigger (CD2 mutant)

**Figure 2.9** Scheme of Strategy 2. Designing new calcium binding site on EGFP.

The site directed mutation may sometime change the absorbance and fluorescence characters of the wild type EGFP. The G1-EGFP increased the absorbance in 398 nm and de-
increased the intensity in 490 nm. The same thing happens on D6G1 and G7G1. The mechanism behind them will be explored by measuring the NMR spectra changes.

The multiple calcium binding pockets on the surface have some contribution to increase the dynamic range with analysis D6G1 design. This protein is based on the grafted one with one extra calcium binding pocket on the surface of EGFP. The detection of calcium maintained the fluorescence ratiometric change but the dynamic range is almost doubled, from the original 30 % change to around 60 %. The $K_d$ calculation with the ratio change of dual 510 nm fluorescence change is well fitted by the one to one binding equation. The $K_d$ is very close to the original one. It is supposed that the extra binding pocket on the surface has the similar binding affinity as the grafted site, so the extra sites attract more calcium in the same level to increase the dynamic range.

**2.4.4 Designing Ca$^{2+}$ binding site on the surface of EGFP by site-directed mutagenesis.**

For the other strategy of designing calcium binding pocket in the fluorescence sensitive location of EGFP seems to be more promising as it will dramatically change the fluorescence character of EGFP. D10 has a big single fluorescence intensity change and both wavelength changes are increased with calcium binding. While for the absorbance, it is the ratiometric change. The rationale is that the calcium binding induced the overall EGFP conformational change which makes it more conformationally similar to the wild type.
Figure 2.10 Proposed schematic structure and in vitro optical properties of designed Ca\(^{2+}\) biosensor variants. (A) Truncated structure of wild-type EGFP (1EMA) with the chromophore (CRO) highlighted as cyan spheres. Residues 147, 202, 204, 223, and 225 sidechain, in blue, protruding from the surface in close proximity to the chromophore, were mutated to form the Ca\(^{2+}\) binding ligands. Key residues H147, T203, and E222, involved in proton interaction with the chromophore, were located near the designed Ca\(^{2+}\) binding site. (B) Spatial distribution of the five residues, in blue, that are responsible for Ca\(^{2+}\) chelation. (C) Spatial organization of these residues and their relationship with the chromophore in the EGFP molecule, which shows nonacidic residues. (D-H) Constructs D8, D9, D10, CatchER, and D12 show replacement at residues S147, S202, Q204, F223, and T225, respectively.
2.4.5 Study the optical properties of designed variants by fluorescence and absorption spectroscopy.

Figure 2.11 The optical properties of designed variants investigated by absorption spectroscopes. (A) Absorbance spectra of wild-type EGFP and Ca$^{2+}$ sensors D8 to D12, with normalized absorbance peak at 280 nm. The designed proteins exhibited a major absorbance peak at 398 nm and a lower peak at 490 nm. (B) Absorbance intensity ratio at 395 nm and 488 nm for all designed sensors and wild-type EGFP. The ratio increased with the number of negatively charged residues introduced.
Figure 2.12 The optical properties of designed variants investigated by fluorescence spectroscopes. (A) Change in fluorescence intensity of EGFP variants in response to Ca\(^{2+}\) recorded at 510 nm emission and 488/395 nm excitation with either 10 µM EGTA (black/grey bars) or 5 mM Ca\(^{2+}\) (red/blue bars). The protein concentration of all variants was determined by an extinction coefficient 21890 cm\(^{-1}\) M\(^{-1}\) at 280 nm. EGFP emission maxima 510 nm excited at 488 nm in the presence of 10 µM EGTA was normalized to 1.0. (B) Correlation between the number of negatively charged residues and apparent Ca\(^{2+}\) dissociation constants (K\(_d\)) for D9, D10, and CatchER, measured by fluorescence titration in 10 mM Tris buffer, pH 7.4, in the presence (square) and absence (circle) of 100 mM KCl.
Figure 2.13 Optical characterization of CatchER in vitro. (A) Emission spectra in response to increased Ca$^{2+}$ concentrations. (B) Apparent CatchER Kd determined by fluorescence response in the presence (red) or absence (blue) of 100 mM KCl or by a mainchain chemical shift change of residue Y143 in heteronuclear single quantum coherence (HSQC) spectra in the presence of 10 mM KCl (black). Titration results were fitted to a 1:1 binding mode.

2.4.6 Ca$^{2+}$-induced changes in CatchER’s optical properties.

The model structure of our designed Ca$^{2+}$ sensor, CatchER, was based on the scaffold protein EGFP. The binding site is adjacent to the chromophore (right above the Y66 phenolic oxygen) and next to H148, T203, and E222 (Fig. 1A); its fluorescence sensitivity may be due to hydrogen-bond interaction (19). The X-ray crystal structure shows mutated residue sidechains protruding from the protein surface, providing access to solvents (109). This putative Ca$^{2+}$ binding site is formed by residues 147, 202, 204, 223, and 225, which confer Ca$^{2+}$-preferred geometric properties (Fig. 1B). Five variants were created by introducing charged residues in these positions (Fig. 1D-H).
CatchER (D11) and its variants (D8-D10 and D12) were bacterially expressed and purified using established methods (71, 110). Introducing acidic ligand residues added an absorption maximum at 398 nm at the expense of the 490 nm peak (Fig. 1I). This EGFP feature is associated with predominance of the anionic chromophore. The ratio of absorption maxima 395/488 increases from 0.2 for EGFP with no charged residue to 2.3 for D10 with four acidic residues (Fig. 1J). A fluorescence maximum of 510 nm excited at 488 nm parallels the absorbance maxima (Fig. S1A-L). The geometric arrangement of sidechain types can also alter the optical properties since replacing glutamate with asparate at residue 223 results in a greater 398/490 nm ratio in comparison to CatchER though both of them have the same net charge in this region.

Ca\(^{2+}\) binding to CatchER and its variants D9 and D10 increased absorbance at 490 nm and decreased it at 398 nm (Fig. S1C-E,M), suggesting that Ca\(^{2+}\) binding increases the anionic chromophore. In contrast, a 510 nm emission maximum increased when excited at both 395 and 488 nm (Fig. S1I-K, M). Among all variants, CatchER had the largest fluorescence enhancement (~80%) upon Ca\(^{2+}\) binding (Fig. 1K and Fig. S1N) and attained ~50% of EGFP fluorescence intensity. D8’s fluorescence response is negligible, possibly because it has few ligand residues and low Ca\(^{2+}\) binding affinity. Further mutating S147E at the flexible loop region generates detectable Ca\(^{2+}\)-induced fluorescence enhancement as D9. The metal-binding assisted chromophore formation can be further revealed by a 0.5 unit decrease of the pK\(_a\) of CatchER in the presence of Ca\(^{2+}\). More similar to the apparent pKa value 6.1 of EGFP. Taken together, Ca\(^{2+}\) binding reverses the change of fluorescence properties due to the addition of charged ligand residues with a parallel fluorescent enhancement instead of a ratiometric fluo-
rescence change excited at 490 and 398 nm, suggesting a unique mechanism when combined with both fluorescence recovery and switching of two ionic forms of the chromophore.

2.4.6 Metal binding affinity and selectivity of designed indicators.

Ca\(^{2+}\)-induced chemical shift changes of several residues close to the designed CatchER's Ca\(^{2+}\) binding site (Fig. 3A, C) can also be fitted to a 1:1 binding process, with K\(_d\) values consistent with those determined by fluorescence change. CatchER exhibits the

![Figure 2.14 Fluorescence responses of various physiological molecules—20 mM Na\(^+\), 100 mM K\(^+\), 2 µM Cu\(^{2+}\), 2 µM Zn\(^{2+}\), 1 mM Mg\(^{2+}\), 0.2 mM ATP, 0.1 mM GTP, and 0.1 mM GDP in the presence of 1 mM Ca\(^{2+}\). Values were normalized to 1 mM Ca\(^{2+}\) in the absence of other metals. The fluorescence recorded with emission maxima 510 nm excited at 488 nm.](image-url)
strongest Ca$^{2+}$ binding affinity, with an apparent $K_d$ of 0.18±0.02 mM, while D9 has the weakest, with an apparent $K_d$ of 0.95 ± 0.08 mM in 10 mM Tris pH 7.4 (Fig. 1L). CatchER’s dissociation constant increases to 0.48 ± 0.07 mM in the presence of 100 mM KCl, consistent with Ca$^{2+}$ electrostatic interaction. Na$^+$, K$^+$, Cu$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, ATP, GTP, and GDP cannot compete with Ca$^{2+}$ for binding CatchER (Fig. 2C), which demonstrates its good selectivity.

The metal selectivity of CatchER is supported by several experimental results. In the presence of 1 mM Ca$^{2+}$, adding 1 mM Mg$^{2+}$ cannot further increase the fluorescent intensity of bacterial expressed and purified CatchER. 2) in situ calibration of $K_d$, in the presence of 0.2-1.0 mM Mg$^{2+}$, CatchER expressed in mammalian cells can response to Ca$^{2+}$ titration with measured $K_d$ around 1.0 mM. 3) CatchER can respond to various Ca$^{2+}$ agonists and antagonists in living cells, where the free Mg$^{2+}$ is estimated to be around 1 mM. 4) NMR HSQC titration, the chemical shift change is almost identical of CatchER in salt free (10 mM Tris buffer) and 10 mM KCl buffer. Several residues either adjunct to the designed binding site or sequentially close to the chromophore exhibited major chemical shift change in response to sub millimolar Ca$^{2+}$ in the presence of 10 mM KCl.
Fig. 2.28 The fluorescent emission spectra of competing Ca$^{2+}$ binding between CatchER and alpha-albumin or EGTA, with the excitation at 488 nm.
In addition to the equilibrium dialysis, the Ca\(^{2+}\) binding capability of CatchER was also verified by competition titration. Two Ca\(^{2+}\) chelators, protein based alpha-lactalbumin and synthesis molecule based EGTA with strong binding affinity (\(K_d\) around nM) were used to compete for Ca\(^{2+}\) from Ca\(^{2+}\) loaded CatchER with weak binding affinity (\(K_d\) around hundreds of \(\mu M\)). The fluorescent intensity first gradually increased after adding Ca\(^{2+}\) into apo CatchER, and decreased after adding alpha-lactalbumin or EGTA, respectively, suggesting that the fluorescence intensity change of is induced by the available free Ca\(^{2+}\), but not chelated Ca\(^{2+}\), supporting the conclusion that CatchER can bind Ca\(^{2+}\).

Figure 2.24 The fluorescent intensity change of CatchER in the competition of Ca\(^{2+}\) binding between chelators and CatchER.
2.4.6 Kinetics study of CatchER in response to calcium with stopped-flow technique.

We applied the method described in Figure 2.1, 2.2, and 2.3 to measure the kinetics of CatchER, and all the measured $k_{obs}$ values were narrowed down to around 50 s$^{-1}$.

Table 2.1 Summary of rate constants of CatchER measured from stopped-flow technique. The protein concentration in syringe was set to be 100 µM and the interaction buffer was 10 mM Tris, 100 mM KCl, pH 7.4.

<table>
<thead>
<tr>
<th>[Ca$^{2+}$] mM</th>
<th>$k_{obs}$ (s$^{-1}$)-1</th>
<th>$k_{obs}$ (s$^{-1}$)-2</th>
<th>$k_{obs}$ (s$^{-1}$)-3</th>
<th>Ave (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67.1 ± 13.4</td>
<td>58.0 ± 7.2</td>
<td>57.8 ± 7.8</td>
<td>61.0 ± 5.3</td>
</tr>
<tr>
<td>1.5</td>
<td>63.4 ± 7.3</td>
<td>59.5 ± 9.1</td>
<td></td>
<td>61.5 ± 2.8</td>
</tr>
<tr>
<td>20</td>
<td>56.0 ± 2.6</td>
<td>57.1 ± 3.2</td>
<td></td>
<td>56.6 ± 0.8</td>
</tr>
</tbody>
</table>

Table 2.2 Summary of rate constants of CatchER measured from stopped-flow technique. The protein concentration in syringe was set to be 100 µM and the interaction buffer was 10 mM Tris, pH 7.4

<table>
<thead>
<tr>
<th>[Ca$^{2+}$] mM</th>
<th>$k_{obs}$ (s$^{-1}$)-1</th>
<th>$k_{obs}$ (s$^{-1}$)-2</th>
<th>$k_{obs}$ (s$^{-1}$)-3</th>
<th>Ave (s$^{-1}$)</th>
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<tr>
<td>1</td>
<td>54.4 ± 2.2</td>
<td>53.3 ± 2.1</td>
<td></td>
<td>53.8 ± 0.8</td>
</tr>
<tr>
<td>1.5</td>
<td>52.6 ± 2.5</td>
<td>56.2 ± 1.9</td>
<td></td>
<td>54.4 ± 2.6</td>
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<tr>
<td>2</td>
<td>57.1 ± 1.9</td>
<td>57.0 ± 1.9</td>
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<td>57.1 ± 0.1</td>
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<tr>
<td>4</td>
<td>53.7 ± 1.7</td>
<td>54.3 ± 1.5</td>
<td></td>
<td>54.0 ± 0.4</td>
</tr>
<tr>
<td>20</td>
<td>57.1 ± 1.4</td>
<td>57.1 ± 1.3</td>
<td></td>
<td>57.1 ± 1.3</td>
</tr>
</tbody>
</table>
Table 2.3 Summary of rate constants of CatchER measured from stopped-flow technique. The protein concentration in syringe was set to be 100 µM and the interaction buffer was 10 mM Tris, pH 7.4.

<table>
<thead>
<tr>
<th>[Ca^{2+}] mM</th>
<th>k_{obs} (s^{-1})-1</th>
<th>k_{obs} (s^{-1})-2</th>
<th>k_{obs} (s^{-1})-3</th>
<th>Ave (s^{-1})</th>
</tr>
</thead>
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<td>0.5</td>
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<td>53.4 ± 2.8</td>
<td>64.9 ± 3.3</td>
<td></td>
</tr>
<tr>
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<td>59.2 ± 4.2</td>
<td>56.1 ± 2.9</td>
<td>53.4 ± 3.7</td>
<td>57.2 ± 4.3</td>
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<td>61.5 ± 6.5</td>
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<td>62.4 ± 2.2</td>
<td>65.6 ± 2.3</td>
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<tr>
<td>10</td>
<td>72.0 ± 2.5</td>
<td>65.0 ± 2.1</td>
<td>68.5 ± 5.0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.14 Analysis of rate constants of CatchER interacting with different concentration of calcium measured from the stopped-flow techniques and the fluorescent intensity change fitting.
While there was still very obvious fluorescent intensity change during 1 ms to 10 ms according to the rough data plotted with exponential x-axis, which indicated that calcium response of CatchER occurred within 10 ms. Even the Δ fluorescent intensity change during this short period were also different according to the different calcium concentrations. The kinetic results could not explain the calcium interaction properties of CatchER with the same mechanism of D1ER, however, a reasonable $K_d$ could be fitted by plotting Δ fluorescent intensity change with the function of the calcium concentration. According to the classic study of kinetics, we have two proposed models to elucidate the results.

**Equation derivation**

- $\text{Ca}^{2+} + P \xrightleftharpoons[k_{-1}]{K_1} \text{CaP}$
  
  $\frac{d[\text{CaP}]}{dt} = k_1[\text{Ca}^{2+}][P] - k_{-1}[\text{CaP}]$
  
  $[P] = [P_0] - [\text{CaP}]$

- $\frac{d[\text{CaP}]}{dt} = k_1[\text{Ca}^{2+}][P_0] - [\text{CaP}] - k_{-1}[\text{CaP}]$

- $\frac{d[\text{CaP}]}{dt} = -(k_1[\text{Ca}^{2+}] + k_{-1})[\text{CaP}] + k_1[\text{Ca}^{2+}][P_0]$

- $k_{\text{obs}} = k_1[\text{Ca}^{2+}] + k_{-1} \quad \text{([Ca}^{2+}] \gg [P], \text{[Ca}^{2+}] \text{constant})$

  - $k_{\text{obs}} = k_1 \Rightarrow k_{-1} = k_{\text{off}}$
  - $k_{\text{obs}} = k_1 \Rightarrow k_{\text{off}}$

  - Slope = $k_1 = k_{\text{on}}$

  - If $k_{\text{obs}} = k_{\text{obs1}}$ when $[\text{Ca}^{2+}] = 0$
  - And $k_{\text{obs}} = k_{\text{obs2}}$ when $[\text{Ca}^{2+}] = \infty$
  - And if $k_{\text{obs1}} = k_{\text{obs2}}$

  - $k_1 = 0$ (no binding, or extremely slow binding)
  - $k_{-1}$: Y-intercept

**Example:**

- $k_{\text{on}} = 3.683 \times 10^4 \text{M}^{-1} \text{s}^{-1}$
- $k_{\text{off}} = 256 \text{ s}^{-1}$
- $K_d = 69 \mu\text{M}$

**Figure 2.15** Model I proposed that protein and calcium interaction directly induced fluorescent intensity change and equation derivation. The plot of the example was adopted from (1)

The first model proposed that protein and calcium interaction would directly induced fluorescent intensity change, so that there was a one-step scheme to represent the whole...
process. The free protein concentration could be replaced by the terms of total protein concentration and bound-form protein concentration. Finally, $k_{\text{obs}}$ equals to $k_1[\text{Ca}] + k_1$ with the equation derivation, and the pre-condition that the concentration of calcium added each time was set to be much higher than that of protein. According to this equation, $k_{\text{off}}$ is the value of $Y$ intercept and $k_{\text{on}}$ is the slope. If we hypothesized that $k_{\text{obs}}$ was a constant independent of $[\text{Ca}^{2+}]$, a phenomena observed similarly from our rough data, $k_1$ was apparently to be 0, resulting that there was even no binding process which was in contradiction with our hypothesis.

**Equation derivation**

\[
\text{Ca}^{2+} + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{CaP}^{*} \underset{k_3}{\overset{k_4}{\rightarrow}} \text{CaP}^{*}
\]

\[
\frac{d[CaP^*]}{dt} = k_1[CaP] - k_4[CaP^*]
\]

**Applying the steady-state condition to [CaP]**

\[
k_1[Ca^{2+}][P] + k_4[CaP^*] = (k_2 + k_3)[CaP]
\]

\[
[P] = [P_0] - [CaP] - [CaP^*]
\]

**Substituting [CaP] in equ**

\[
\frac{d[CaP^*]}{dt} + \frac{k_1 k_2 [Ca^{2+}]}{k_3 [Ca^{2+}] + k_3} [CaP^*] = 0
\]

**Imposing [Ca^{2+}] >> [P]**

\[
\frac{d[CaP^*]}{dt} + \alpha[CaP^*] - \beta = 0
\]

\[
[CaP^*] = -\frac{\beta}{\alpha} e^{-\alpha t} + \frac{\beta}{\alpha}
\]

**Substituting [P] with ([P] - [CaP] - [CaP^*])**

\[
[CaP] = \frac{k_1[P_0][Ca^{2+}]}{k_1[Ca^{2+}] + k_1 + k_3} + (k_4 - k_1[Ca^{2+}])[CaP^*]
\]

\[
\alpha = \frac{k_1 k_2 [Ca^{2+}]}{k_1 [Ca^{2+}] + k_2 + k_3}
\]

**Figure 2.16 Model II proposed that an intermediate formation during calcium and protein interaction and equation derivation.**
The second model characterized an intermediate formation during calcium and protein interaction, which could be regarded as the rate-limited step between the calcium binding to protein and final fluorescent change, so two kinetic equilibrations were established. The steady-state condition was applied to the intermediate CaP, the concentration of which was supposed to be a constant during the whole reactions. With the same pre-condition as the first model to impose total concentration of calcium to be much higher than that of protein, the $k_{\text{obs}}$ was a constant in each given condition.

$$\text{Ca}^{2+} + \text{P} \xleftrightarrow[k_2]{k_1} \text{CaP} \xrightarrow[k_3]{k_4} \text{CaP}^*$$

$$\alpha = k_{\text{obs}} = \frac{k_1 k_3 [\text{Ca}^{2+} ] + (k_1 [\text{Ca}^{2+} ] + k_2) k_4}{k_1 [\text{Ca}^{2+} ] + k_1 + k_3}$$

$[\text{Ca}^{2+}] >> [\text{P}]$

If $k_{\text{obs}} = k_{\text{obs}1}$ when $[\text{Ca}^{2+}] = 0$

And $k_{\text{obs}} = k_{\text{obs}2}$ when $[\text{Ca}^{2+}] = \infty$

And if $k_{\text{obs}1} = k_{\text{obs}2}$

$$k_{\text{obs}1} = \frac{k_1 k_4}{k_2 + k_3} \quad k_{\text{obs}2} = k_3 + k_4$$

$$\frac{k_2 k_4}{k_2 + k_3} = k_3 + k_4$$

$$k_1 (k_2 + k_3 + k_4) = 0$$

$k_3 = 0$ (no intermediate formation)

$k_2 + k_3 + k_4 = 0$

$k_{\text{obs}} = a \quad k_{\text{obs}}$ is a constant

$$k_1 k_3 [\text{Ca}^{2+} ] + k_1 k_4 [\text{Ca}^{2+} ] + k_2 k_4$$

$$= a k_1 [\text{Ca}^{2+} ] + a k_2 + a k_3$$

$[\text{Ca}^{2+} ](k_1 k_3 + k_1 k_4 - a k_1)$

$$= a k_2 + a k_3 - k_2 k_4$$

$$k_1 (k_3 + k_4 - a) = 0$$

$$a k_2 + a k_3 - k_2 k_4 = 0$$

$$a = k_3 + k_4$$

$$k_2 (k_2 + k_3 + k_4) = 0$$

$k_3 = 0$ (no intermediate formation)

$k_2 + k_3 + k_4 = 0$

Figure 2.17 The conditional Model II at the concentration of calcium much higher than that of protein equation derivation.
After the $k_{\text{obs}}$ equation was derived, the values were also proposed to be a constant as the experimental results for further analyzing each reaction rate. In order to satisfy all the preconditions, $k_3$ or $k_{\text{on}}$ of CaP from non-excitable to excitable state should be 0, or all of $k_2$, $k_3$ and $k_4$ should be 0, which trapped in a pitfall as the previous results: there were no forward process of the second equilibrium.

All in all, the biosensor CatchER, which can detect Ca$^{2+}$ signaling during muscle contraction in real-time detect Ca$^{2+}$ signaling during muscle contraction exhibited fast response within tens of milliseconds. The kinetics of current Ca$^{2+}$ biosensors can be measured by stopped-flow techniques. The $k_{\text{off}}$ rates detected ranged from tens to hundreds per second. The kinetics of Fura-2 studied by EGTA competition provided a good model to study our sensors. Two interaction modes have been proposed to explain our kinetics results. However, our experiment conditions did not perfectly satisfy the requirement of proposed modes, in which our protein concentrations were higher than optimal. The ignorance of viscosity caused by high protein concentration, which could add an unpredicted but significant rate-limitation step might impair the explanation of our results within reasons.

Quantitatively assessing the speed of fluorescence response is essential to determine the application field of a calcium biosensor. In particular scenarios, especially for electrophysiology such as muscle contraction and neuronal firing, transient calcium fluxes were estimated to occur within milliseconds or microseconds which is different from global calcium change lasting for seconds to hours usually triggered by various agonists or antagonists. An ideal calcium biosensor should intrinsically bind and release calcium within the time scale of calcium fluxing frequencies to report them with high fidelity. One of the initial kinetic studies of Ca$^{2+}$ biosensor was conducted in Ashley’s laboratory by observing fluorescent change of Ca$^{2+}$ dyes.
Fura-2 and Indo-1 with stopped-flow by mixing saturated dye with EGTA or EDTA. After that, stopped-flow kinetic report became a standard for evaluation, though recently, with the development of imaging techniques, more and more kinetic results of calcium indicators are measured in living cells or tissues.

![Figure 2.18 Kinetics study of CatchER with Ca\(^{2+}\) by stopped-flow spectroscope](image)

Figure 2.18 Kinetics study of CatchER with Ca\(^{2+}\) by stopped-flow spectroscope has been conducted at two protein concentrations: 2 µM and 20 µM with the same protocol. For each Ca\(^{2+}\) concentration, curve was averaged by results from three parallel shots. 40-50% of fluorescent intensity has been finished within the deadtime of instrument (2.2 ms).

CatchER expressed in bacteria was collected for kinetic study by stopped-flow techniques. Two approaches were conducted to determine the association and dissociation constants. The first one was to mix concentration-fixed calcium-free CatchER with different concentrations of calcium and record the consequential fluorescence responses. Moreover, the fluorescence baseline as a control was acquired by protein interacted with calcium-free buffer.
As 1:1 binding mode was proposed to CatchER and calcium interaction, the initial fluorescence readouts were fitted by single exponential equation to calculate the \( k_{\text{obs}} \) value. A plot of \( k_{\text{obs}} \) against calcium concentration was fitted by linear function to derive two constants, the \( Y \)-intercept and the slope. The first one indicated the dissociation constant of a biosensor where calcium concentration assumed to be 0, and the later represented the association constant. In addition to the reaction speed, the amplitudes between the plateaus of fluorescence to the baseline were direct proportion to the free calcium concentration within two times of \( K_d \) if the steady-state the free calcium concentration was much higher than the bound one.

\[
k_{\text{obs}} = k_1 [Ca^{2+}] + k_{-1}
\]

\[
\text{When} [Ca^{2+}] = 0 \quad k_{\text{obs}} = k_{-1} = k_{\text{off}}
\]

\[
\text{Slope} = k_1 = k_{\text{on}}
\]

Equation 2.1 Equations derived for pseudo-first order kinetics determination.

From Figure 2.16, we observed a gap between the initial fluorescent intensity and the baseline when CatchER mixed with calcium. The amplitude of the gap is directly proportional to the calcium concentration increment, revealing around 30~40\% of the initial fluorescence increase finished within the deadtime of the instrument, estimated to 2 ms at room temperature. While the fluorescence continued rising until 40 ms forming a plateau, indicating the termination of the reaction. With the longer time data collection, we did not see significant fluorescent alternation after this time point except for photobleaching, verified CATCHER exhibiting fast reaction properties as no lagging phase existing 40 ms after mixed with calcium.
20 μM CatchER pre-mixed with 20 μM Ca$^{2+}$ was loaded into one syringe and either 0.2 mM EGTA or 2 mM EGTA was loaded into the other one. More than 60% of fluorescent change has been finished within the deadtime (2.2 ms). $\Delta F$ between baseline and EGTA chelating results was fitted with single exponential equation, and rough $k_{off}$ value was calculated to be around 112 ± 14 s$^{-1}$.

2.4.7 Determination of calcium and CatchER interaction stoichiometry.

One of the major advantages of our design is to offer a chemically 1:1 binding-mode genetically encoded calcium indicator, which mimics the interaction model of calcium dyes and allows us to quantify calcium concentration and fluorescence intensity with simple equations. It
is superior to calmodulin or troponin C based calcium indicators naturally possessing four calcium binding sites resulting in a complicated stoichiometry reaction. For those indicators a long-last question that haven’t been answered well is the how to simplify the complicated chemical interaction stoichiometry by linear fluorescence readout of calcium concentration. The common solution is to establish a 1:1 indicator to calcium binding mode by modifying the calcium binding sites or calmodulin and peptide interaction surface by site-directed mutagenesis resulting in the ideal linear fluorescence readout in a broad range of calcium concentration. The mutations are mainly focused on one or two calcium binding loops of calmodulin and leave the rests intact, E104Q for example of cameleon to diminish the strong binding phase and uniformize the apparent $K_d$s to be single, the untouched calcium binding loops raise the possibility to chelate calcium silently without provide any fluorescence readout. There is evidence to support the complicated stoichiometry of fluorescence readout and calcium concentration change of the calcium indicator possessing multiple binding domains, especially in a fast reaction, and the percentage of each phase in contribution to the total fluorescence change is not equivalent as some phases are almost negligible, which supports the rationale of silent calcium reaction to indicators. (David Yue and Oliver Griesbeck TnC) Although intense progresses have been made to reduce the cooperativity among multiple binding domains and improve the linear fluorescence response, there are no direct evidences to support the real interaction stoichiometry agrees with the 1:1 binding mode applied for the apparent $K_d$ fitting, leaving a puzzle that after the concentration of these kinds of indicators are determined in situ, whether only one calcium should be considered to interact with one indicator according to the apparent $K_d$ calculation or four which is more plausible for the real chemical reaction, especially in a scenario of the indicator saturated by free calcium. In addition, it is uncertain whether the modified calcium bind-
ing domains of calmodulin can maintain the same chelate coordination to calcium as the wt. One amazing example is that the solved crystal structure of Gcamp2 revealed the uncom-
pleted interaction between the loops of EF-hand and calcium, instead of calcium coordinating
to residues in site 1, 3, 5, 7, and 12 of the loop as a classic EF-hand binding mode, only very
limited residues from the loop and extras from the helixes directly interact to calcium, which is
totally different from the native EF-hand and calcium binding mode. We wonder whether any
hill equation derived to fit native calmodulin and calcium interaction or just a simplified 1:1
binding mode can still be persuasive to fit the fluorescence change of calcium indicators with
modified calcium binding sites, though this apprehension may be released by possible different
conformations of the same proteins between in solutions and in crystals. Unfortunately, few
laboratories analyzed the correlation of stoichiometry between the real chemical interaction
and the apparent fluorescence readout of the genetically encoded calcium indicators. For our
design, we applied the Job’s plot to determine the stoichiometry of CatchER and calcium.
The mechanism of a Job's plot is to determine the ratio between two reactants with a fixed total concentration at which the highest amount of products are yielded. The quantity of two reactants is controlled manually, and the measurements of the absolute products are achieved by converting the fluorescence increment to the concentration of Ca-loaded form protein. Ca-free form protein concentrations were determined by absorbance at 280 nm in the readout with the coefficient of 21890 cm⁻¹ M⁻¹, and calcium concentrations were determined by the original stock. The concentration of Ca-loaded form protein—the product was calculated by
equations derived for single-wavelength fluorescent dye containing different fluorescent coefficients of either Ca-free or Ca-loaded form, as the apparent fluorescent intensity was a mixture of both Ca-free CatchER and Ca-loaded CatchER during the titration. The only requirement of this equation, which was proven to be satisfied for our case was that the fluorescent intensity should linearly relate to the protein concentration, both in Ca-free and Ca-saturated forms, guaranteeing the coefficients are constants. The peak amount of the product reached when the concentration of calcium to protein is 1:1, revealed by the Job’s plot.

Besides the fluorescence response, we also calculate the stoichiometry by absorbance change. Different from single-wavelength fluorescent enhancement, CatchER exhibited ratiometric absorbance alternation, with 488 nm maxima increasing and 395 nm maxima decreasing in response to calcium. It is hypothesized that the intensities of these two peaks directly reveal the population of neutral and anionic forms of chromophore, so one peak rises at the expense of the other if the total amount of chromophore is fixed in a confined environment. Therefore, the absorbance change more directly represents the perturbation of the chromophore populations. Either single-wavelength intensity change or the overall ratiometric change was converted to the amount of products with the same strategy. The stoichiometry calculated by these two strategies maintained 1:1 binding mode, indicating the high conservation of intrinsic optical properties of CatchER.
2.4.8 The pKa measurement of CatchER in the presence and absence of Ca$^{2+}$.

Figure 2.21 The pH stability of CatchER before and after binding Ca$^{2+}$ is investigated by measuring the apparent pKa values based on pH-dependence of the fluorescence intensity. (A) Fluorescence emission intensities at 510 nm were recorded in the presence of 10 µM EGTA (circle) or 4 mM Ca$^{2+}$ (square) with excitation at 488 nm at corresponding pH values. The apparent pKa is calculated with the fitting equations in the Methods to be 7.59 ± 0.03 (EGTA) and 6.91 ± 0.03 (Ca$^{2+}$). (B) The pH-dependence of the fluorescence emission intensities at 510 nm excited at 395 nm were recorded and fitted with the same methods in panel (A). The apparent pKa is 7.14 ± 0.02 (EGTA) and 6.95 ± 0.06 (Ca$^{2+}$).

To further investigate the pH influence to CatchER, we calculated the pK$_a$ both in calcium-free form and calcium-loaded form by plotting the emission maxima at 510 nm in the function of different pH values. Apparent pK$_a$ of with the excitation maxima of 490 nm decreased from 7.6 of the calcium-free form to 6.9 of the calcium-loaded form, while that of the excitation maxima of 398 nm revealed only a slightly change from 7.0 to 6.9 of these two forms, respectively, moreover, the dual emission bands of CatchER exhibiting parallel decrease mode rather than ratiometric change at pH >5 possessed by the wt-EGFP as one band increase in expense of the intensity of the other band with the decrease of pH values, illustrat-
ing the independence of these two emission bands. The higher apo-form pK\textsubscript{a} value with the excitation maxima 490 nm of CatchER is in agreement with the increment of pK\textsubscript{a} value from 4.9 of T-sapphire (Zapata-Hommer, BMC Biotech, 2003) featured with only one predominated 399 nm band to 6.0 of GFP-S65T mutant with single 490 nm band (Kheen, BJ). The chromophore pH sensitivity is reported to be determined by the pKa values of residues at or near the chromophore (Kneen, BJ, 1998), and as we know that the sidechains of Glu222 and Ser205 stabilize the phenolic oxygen of neutral-form chromophore while Thr203 and His148 donate protons to bind deprotonated phenolate of anionic-form chromophore (Brejc et al. 1997), which is in accordance to the indifference of pK\textsubscript{a} values between anionic-form chromophore (6.0, S65T-GFP) and pyrrole NH of histidine (6.0) or similiarity of that between neutral-form chromophore (4.9, T-sapphire) and carboxyl group of glutamate (4.4). For CatchER, the pK\textsubscript{a} values of both bands dramatically increased by more than 1 unit, suggested the different hydrogen-bond network between the sidechains to chromophore. Limited to the unveiled structure of CatchER, we assumed that the alternation of protonated- and deprotonated-form chromophore were determined by more than one single residue as the pK\textsubscript{a} values in apo or holo-form ranged from 6.9 to 7.5 does not agree with that of any single functional group. The proposed mechanism is that neutral chromopore both in calcium-free and calcium-loaded forms, exhibiting two close apparent pK\textsubscript{a} values around 6.9, were significantly stabilized by pyrrole NH of His148, with a pK\textsubscript{a} value 6.0, while anionic chromophore with an apparent pK\textsubscript{a} 7.6 mainly interacted with sidechain amine group of N146 (pK\textsubscript{a} 8.0) in the calcium-free condition, which was replaced by sidechain of His148 due to calcium binding induced conformational change resulting in 0.5 unit decrease of pK\textsubscript{a} in calcium-loaded form. According to the optical study of CatchER, these two fluorescent bands exhibiting independently change, with different mecha-
nisms, but similar $K_d$ within the same magnitude still suggested a simple 1:1 calcium-binding mode to quantitatively construct a relationship between the free calcium concentrations to the fluorescent intensities. Furthermore, fluorescent intensity change was measured to examine the metal selectivity of CatchER in vitro via adding different metal ions in the presence of 1 mM calcium which mimic the physiological calcium concentration in SR. Figure 2.19 reveals that both emission wavelength exhibiting high metal selectivity for the divalent metal ions in the physiological concentration, while slightly quenched by trivalent metal ions with excitation peak at 490 nm but not influencing 398 nm.
2.4.9 Ca\(^{2+}\) binding CatchER investigated by equilibrium dialysis and an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES).

Figure 2.22 Ca\(^{2+}\) binding CatchER investigated by equilibrium dialysis and an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Dialysis tubes contained 6 ml myoglobin (Sigma Chemical Co.) (non-Ca\(^{2+}\)-binding protein), EGFP (non-Ca\(^{2+}\)-binding protein), CatchER and \(\alpha\)-lactalbumin (Sigma Chemical Co.) (Ca\(^{2+}\)-binding protein with reported \(K_d = 10^{-9}\) M (24)) at the concentration of 20 \(\mu\)M determined before dialysis, floating inside a beaker containing 1800 ml 10 mM Tris buffer with Ca\(^{2+}\) at pH 7.4. ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer) is a sensitive method of determining the total concentration of a particular metal in solution with high selectivity. (A) Representative spectra of ICP-OES to determine the total Ca\(^{2+}\) concentration (both bound and unbound) outside the dialysis tube (buffer) and inside the dialysis tube with the samples of myoglobin, EGFP, CatchER and \(\alpha\)-lactalbumin respectively, with maximal intensity at 370.602 nm. Each spectrum is the average of three-time repeats with the error bars, and the amplitude of peak intensity of each sample represents the concentration of Ca\(^{2+}\). (B) The comparison of Ca\(^{2+}\) concentration of each sample determined by ICP-OES. The peak intensities recorded at 396.847, 373.690, 219.779, 370.602, 317.933, 643.907 and 220.861 nm were converted to Ca\(^{2+}\) concentration calibrated by the pre-determined Ca\(^{2+}\) standard linear curve at each wavelength, respectively. The Ca\(^{2+}\) concentration of the buffer outside the dialysis tube was 60.4 ± 0.7 \(\mu\)M (unbound), and inside (both bound and unbound), containing myoglobin, EGFP, CatchER and \(\alpha\)-lactalbumin was 61.5 ± 1.2, 64.5 ± 1.1, 74.6 ± 1.5 and 79.1 ± 1.7 \(\mu\)M (both bound and unbound), respectively.
Several lines of evidence support a simple CatchER-Ca\(^{2+}\) stoichiometry reaction. The Job Plot suggests that Ca\(^{2+}\) forms a 1:1 complex with CatchER, and the fluorescence change in response to Ca\(^{2+}\) titration can be fitted to a 1:1 binding equation. However, these results are based on the quantifying the fluorescence change induced by Ca\(^{2+}\), the classic methods such as radioisotope stain, Isothermal Titration Calorimetry (ITC) or equilibrium dialysis to demonstrate the direct chemical interaction between metals and proteins are not based on optical characterization change. Ca\(^{2+}\) binding CatchER was investigated by equilibrium dialysis and an Inductively Coupled Plasma Optical Emission Spectrometer to determine the Ca\(^{2+}\) concentrations in multiple samples. Figure 2.24 shows the scheme of equilibrium dialysis, which has been widely applied to study the binding affinity between the protein and ligands. A membrane or dialysis tube functioning as the molecular filter only permeabilized to the small ligand but not to the protein. The membrane is used to separate the equilibrium system to two parts, one only containing free ligands and the other one containing both proteins and bound and unbound ligands. The binding affinity of the protein will attract the ligand perfuse to its part. After quantifying the different concentrations of ligands with other methods, usually with ICP-OES to determine the total metal concentration, the binding affinity can be measured. ICP-OES is a sensitive method to determine the total concentration of a particular metal in solution with high accuracy.
Figure 2.23 Representative spectra of ICP-OES. Determined the total Ca$^{2+}$ concentration (both bound and unbound) outside the dialysis tube (buffer) and inside the dialysis tube with the samples of myoglobin, EGFP, CatchER and α-lactalbumin respectively, with maximal intensity at 396.847, 219.779, 370.602, 220.861, 370.602 nm. Each spectrum is the average of three-time repeats with the error bars, and the amplitude of peak intensity of each sample represents the concentration of Ca$^{2+}$. (10 mM Tris buffer, pH 7.4)
Table 2.4 Numerical results of Ca^{2+} concentration in buffer, myoglobin, EGFP, CatchER and alpha-lactalbumin, respectively after equilibrium dialysis measured by ICP-OES at different wavelengths.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>396.847</th>
<th>373.690</th>
<th>219.779</th>
<th>370.602</th>
<th>317.933</th>
<th>643.907</th>
<th>220.861</th>
<th>Avg (uM)</th>
</tr>
</thead>
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<td>Buffer-1</td>
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<td>59.867</td>
<td>60.725</td>
<td>59.743</td>
<td>60.728</td>
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<td>63.307</td>
<td>59.956</td>
<td>61.280</td>
<td>62.178</td>
<td>62.174</td>
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<td>65.517</td>
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<td>66.323</td>
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<td>77.071</td>
<td>76.155</td>
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<td>79.804</td>
<td>77.766</td>
<td>77.949</td>
<td>82.324</td>
<td>79.864</td>
<td>79.090</td>
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<tr>
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<td>55.478</td>
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<td>103.010</td>
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<td>58.523</td>
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<td>60.356</td>
<td>57.778</td>
<td>57.974</td>
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Figure 2.24 Scheme of equilibrium dialysis. The dialysis tube (gray square) floated in buffer inside a beaker (black square) with a stir bar to accelerate the equilibration. The protein (gray cycle) was sealed inside the tube. A fraction of the protein bound Ca$^{2+}$. The unbound Ca$^{2+}$ (black dot) equilibrates between inside and outside of the dialysis tube. Dialysis tubes contained 6 ml myoglobin (Sigma Chemical Co.) (non-Ca$^{2+}$-binding protein), EGFP (non-Ca$^{2+}$-binding protein), CatchER and α-lactalbumin (Sigma Chemical Co.) (Ca$^{2+}$-binding protein with reported $K_d = 10^{-9}$ M)(7) at the concentration of 20 µM determined before dialysis, floating inside a beaker containing 1800 ml 10 mM Tris buffer with Ca$^{2+}$ at pH 7.4. ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer) is a sensitive method of determining the total concentration of a particular metal in solution with high selectivity.

The dialysis tube (gray square) floated in buffer inside a beaker (black square) with a stir bar to accelerate the equilibration. The protein (gray cycle) was sealed inside the tube. A fraction of the protein bound Ca$^{2+}$. The unbound Ca$^{2+}$ (black dot) equilibrates between inside and
outside of the dialysis tube. The dialysis tubes contained 6 ml myoglobin (Sigma Chemical Co.) (non-Ca\(^{2+}\)-binding protein), EGFP (non-Ca\(^{2+}\)-binding protein), CatchER and \(\alpha\)-lactalbumin (Sigma Chemical Co.) (Ca\(^{2+}\)-binding protein with reported \(K_d = 10^{-9}\) M)\cite{Bryant,1984} at the concentration of 20 \(\mu\)M determined before dialysis, floating inside a beaker containing 1800 ml 10 mM Tris buffer with Ca\(^{2+}\) at pH 7.4. ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer) is a sensitive method of determining the total concentration of a particular metal in solution with high selectivity. Representative spectra of ICP-OES to determine the total Ca\(^{2+}\) concentration (both bound and unbound) outside the dialysis tube (buffer) and inside the dialysis tube with the samples of myoglobin, EGFP, CatchER and \(\alpha\)-lactalbumin respectively, with maximal intensity at 370.602 nm. Each spectrum is the average of three trials with the error bars, and the amplitude of peak intensity of each sample represents the concentration of Ca\(^{2+}\). The comparison of Ca\(^{2+}\) concentration of each sample is determined by ICP-OES. The peak intensities recorded at 396.847, 373.690, 219.779, 370.602, 317.933, 643.907 and 220.861 nm were converted to Ca\(^{2+}\) concentration calibrated by the pre-determined Ca\(^{2+}\) standard linear curve at each wavelength, respectively. The Ca\(^{2+}\) concentration of the buffer outside the dialysis tube was 60.4 \(\pm\) 0.7 \(\mu\)M (unbound), and inside (both bound and unbound), containing myoglobin, EGFP, CatchER and \(\alpha\)-lactalbumin was 61.5 \(\pm\) 1.2, 64.5 \(\pm\) 1.1, 74.6 \(\pm\) 1.5 and 79.1 \(\pm\) 1.7 \(\mu\)M (both bound and unbound), respectively.

The equilibrium dialysis experiments using myoglobin (noncalcium-binding protein), EGFP (noncalcium-binding protein), CatchER, and \(\alpha\)-lactalbumin (Ca\(^{2+}\)-binding protein with reported \(K_d = 10^{-9}\) M, \cite{34}) with Ca\(^{2+}\) demonstrate that CatchER binds calcium with weak binding affinity, which correlated with the fluorescent titration results.
Figure 2.25. Spectra of ICP-OES measured calcium concentrations in different dialysis samples in 10 mM Tris buffer, pH 7.4.

Though the different Ca\(^{2+}\) binding capability between EGFP and CatchER is justified, the numerical difference is not significant (approximately 10%, in the previous study). According to a simulation of Ca\(^{2+}\) concentrations between two parts of the equilibrium system, a remarkable difference will be achieved with high-concentration protein providing abundant free occupancy, and low-concentration ligands easily fully bound the protein. In Fig.4 (A), the concentration of CatchER was 200 \(\mu\text{M}\) sealed inside the dialysis tube, as well as 10 \(\mu\text{M}\) free calcium added in the buffer. The total calcium concentration inside the dialysis bag with 200 \(\mu\text{M}\) CatchER is 12 \(\mu\text{M}\), more than 2 times higher than that outside the dialysis bag.
Table 2.5 Numerical results of $\text{Ca}^{2+}$ concentration in buffer, EGFP, CatchER and calmodulin, respectively after equilibrium dialysis measured by ICP-OES at different wavelengths. (old means dialysis for 24 hours; new means dialysis for 48 hours, 10 mM Tris buffer, pH 7.4).

<table>
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<td>74.3</td>
<td>76.2</td>
<td>73.2</td>
<td>75.8</td>
<td>75.2</td>
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<tr>
<td>50 $\mu$M Ca$^{2+}$ buffer new</td>
<td>74.0</td>
<td>75.4</td>
<td>77.3</td>
<td>74.0</td>
<td>75.9</td>
<td>77.9</td>
<td>74.1</td>
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<tr>
<td>EGFP [Ca$^{2+}$] $\mu$M</td>
<td>79.1</td>
<td>79.9</td>
<td>81.1</td>
<td>78.4</td>
<td>82.5</td>
<td>82.9</td>
<td>78.7</td>
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<tr>
<td>CatchER [Ca$^{2+}$] $\mu$M</td>
<td>87.8</td>
<td>87.5</td>
<td>89.0</td>
<td>86.2</td>
<td>92.0</td>
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<td>144.6</td>
<td>149.5</td>
<td>145.0</td>
<td>156.8</td>
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</tbody>
</table>

Table 2.6 Measured calcium concentration by ICP-OES with calcium standard curve 0-20 ppm. (old: dialysis for 24 hours; new: dialysis for 48 hours, 10 mM Tris buffer, pH 7.4).

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>396.847</th>
<th>317.933</th>
<th>219.779</th>
<th>370.602</th>
<th>643.907</th>
<th>220.861</th>
<th>373.690</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 $\mu$M Ca$^{2+}$ buffer old</td>
<td>101.1</td>
<td>76.6</td>
<td>78.6</td>
<td>74.7</td>
<td>72.3</td>
<td>76.8</td>
<td>75.0</td>
</tr>
<tr>
<td>50 $\mu$M Ca$^{2+}$ buffer new</td>
<td>101.9</td>
<td>77.7</td>
<td>79.7</td>
<td>75.5</td>
<td>72.3</td>
<td>79.5</td>
<td>76.0</td>
</tr>
<tr>
<td>EGFP [Ca$^{2+}$] $\mu$M</td>
<td>110.0</td>
<td>82.5</td>
<td>83.7</td>
<td>80.1</td>
<td>78.5</td>
<td>83.9</td>
<td>80.8</td>
</tr>
<tr>
<td>CatchER [Ca$^{2+}$] $\mu$M</td>
<td>123.6</td>
<td>90.4</td>
<td>92.0</td>
<td>88.1</td>
<td>87.3</td>
<td>91.6</td>
<td>88.7</td>
</tr>
<tr>
<td>CaM [Ca$^{2+}$] $\mu$M</td>
<td>213.4</td>
<td>150.6</td>
<td>155.3</td>
<td>148.7</td>
<td>147.7</td>
<td>153.0</td>
<td>149.0</td>
</tr>
</tbody>
</table>
Table 2.7 Recipe of Ca\textsuperscript{2+} standard for ICP-OES experiments.

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+} (ppb/ ppm)</th>
<th>Ca\textsuperscript{2+} (mM/ (\mu)M)</th>
<th>Total Volume (ml)</th>
<th>Volume of Ca\textsuperscript{2+}</th>
<th>Volume of H\textsubscript{2}O (ml)</th>
<th>HNO\textsubscript{3} (70%) (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 ppm</td>
<td>12.5 mM</td>
<td>16 ml</td>
<td>8 ml from 1000 ppm</td>
<td>7.543 ml</td>
<td>0.457 ml</td>
</tr>
<tr>
<td>100 ppm</td>
<td>2.5 mM</td>
<td>15 ml</td>
<td>3 ml from 500 ppm</td>
<td>11.571 ml</td>
<td>0.429 ml</td>
</tr>
<tr>
<td>20 ppm</td>
<td>0.5 mM</td>
<td>15 ml</td>
<td>3 ml from 100 ppm</td>
<td>11.571 ml</td>
<td>0.429 ml</td>
</tr>
<tr>
<td>4 ppm</td>
<td>0.1 mM</td>
<td>15 ml</td>
<td>3 ml from 20 ppm</td>
<td>11.571 ml</td>
<td>0.429 ml</td>
</tr>
<tr>
<td>1 ppm</td>
<td>25 (\mu)M</td>
<td>16 ml</td>
<td>4 ml from 4 ppm</td>
<td>11.571 ml</td>
<td>0.457 ml</td>
</tr>
<tr>
<td>500 ppb</td>
<td>12.5 (\mu)M</td>
<td>12 ml</td>
<td>6 ml from 1 ppm</td>
<td>5.657 ml</td>
<td>0.343 ml</td>
</tr>
<tr>
<td>100 ppb</td>
<td>2.5 (\mu)M</td>
<td>10 ml</td>
<td>2 ml from 500 ppb</td>
<td>7.714 ml</td>
<td>0.286 ml</td>
</tr>
<tr>
<td>Blank</td>
<td>0 (\mu)M</td>
<td>10.5 ml</td>
<td></td>
<td></td>
<td>0.3 ml</td>
</tr>
</tbody>
</table>
Table 2.8 Fitting equations of Ca\textsuperscript{2+} standard calibrations.

<table>
<thead>
<tr>
<th>Range of ppb/ppm</th>
<th>[Ca\textsuperscript{2+}]</th>
<th>Linear fitting Equation</th>
<th>R value</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-500 ppb</td>
<td>12.5 µM</td>
<td>y = 5390.4 + 22.934x</td>
<td>0.99983</td>
</tr>
<tr>
<td>0-1 ppm</td>
<td>25 µM</td>
<td>y = 4097.1 + 32.362x</td>
<td>0.99985</td>
</tr>
<tr>
<td>0-4 ppm</td>
<td>0.1 mM</td>
<td>y = 4124.6 + 32.284x</td>
<td>0.99999</td>
</tr>
<tr>
<td>0-20 ppm</td>
<td>0.5 mM</td>
<td>y = 3900 + 32.508x</td>
<td>1</td>
</tr>
<tr>
<td>0-100 ppm</td>
<td>2.5 mM</td>
<td>y = 6301.3 + 31.865x</td>
<td>0.99999</td>
</tr>
<tr>
<td>0-500 ppm</td>
<td>12.5 mM</td>
<td>y = 55378 + 28.703x</td>
<td>0.99976</td>
</tr>
</tbody>
</table>
Fig. 2.26 Correlation of ICP-OES readout intensities of spectra peaks at individual wavelength with standard Ca\(^{2+}\) concentration fitted with linear equations.
The calcium concentration of each sample is calculated with the standard curve fitted with linear equation. The concentrations of the standards are exponentially distributed, due to the sequential dilution. At the final concentration, 2% HNO₃ was added in each standard and sample for completely dissolving the precipitate. Ca²⁺ concentration from low µM to 12.5 mM is directly proportional to the peak intensity at 396.847, 317.933, 219.779, 370.602, 643.907, 220.861 and 373.690 nm, respectively, suggesting the high accuracy of the ICP-OES with a broad linear measurement range. The Ca²⁺ concentration is calculated with individual fitting equation at different wavelength.

Fig. 2.27 Spectra of ICP-OES measured calcium concentration in different dialysis samples.

EGFP: 14.9 uM
CatchER: 15.5 uM
CaM: 15 uM
Ca²⁺ buffer: 50 uM (0.5 ml 0.1 M Ca²⁺ standard was added in 1 L Tris buffer)

\[
K_d = \frac{[Ca^{2+}]_{free} [CatchER]_{free}}{[Ca \cdot CatchER]}
\]

\[
K_d = \frac{80.4 \times [15.5 - (88.3 - 80.4)]}{(88.3 - 80.4)}
\]

\[
K_d = 77.3 \mu M
\]
2.4.10 Co-localization of CatchER and DsRed2-ER with confocal microscope.

Figure 2.25 Localization of CatchER expressed in the ER of HEK-293 and C2C12 cells. Colocalization of CatchER and DsRed2-ER (BD Biosciences Clontech) in HEK-293 (A) and C2C12 (B) cells. CatchER (green) and DsRed2-ER (red) were transiently co-transfected and expressed in two cell lines for confocal microscopy imaging. The overlay imaging shows the colocalization of CatchER corresponding to ER-tracker DsRed2-ER.
The location of CatchER inside the cells was verified by co-localization imaging
CatchER and commercial genetically encoded ER tracker DsRed2-ER by wide field fluorescence microscope and confocal microscope in HEK-293 cells and C2C12 cells. The DNA of CatchER and DsRed2-ER at the same concentration was co-transfected into the mammalian cells, and cultured at 30°C for 36-72 hours for fluorescent protein expression and folding. The fluorescent intensity was first tested with a wide-field inverted fluorescence microscope, with the scheme of the setup and main parameters shown in Fig.6. (Leica DMI6000 B) with a cooled EM-CCD camera (Hamamatsu C9100). The single-wavelength sensor was excited at 488 nm by a Xenon lamp (Polychrome V system, TILL PHOTONICS), with an HQ480/20x excitation filter, a 515DCXR dichromatic mirror and a D535/25 emission filter (Chroma Technology Corp). With 63x objective magnification, it is sufficient to see the subcellular morphorage lighten up by fluorescent protein of HEK293 cells. The high density of ER structures labeled by DsRed2-ER between the nucleus and plasma-membrane was well correlated with the fluorescence imaging by CatchER within the same cells, suggesting the co-localization of DsRed2-ER and CatchER. High-resolution imaging was taken with confocal microscope to further verify the location of CatchER within the ER lumen.
2.4.11 In situ measurement of calcium binding affinity of CatchER expressed in endoplasmic reticulum of BHK and C2C12 cells.

Figure 2.26 In situ determination of $K_d$ and endoplasmic reticulum $Ca^{2+}$ dynamics of HeLa and HEK293 cells. (A) In situ determination of $K_d$ in ER of C2C12 myoblast cells. 1-5 correspond to 1, 3, 10, and 20 mM $Ca^{2+}$ and 3 mM EGTA, respectively. (B) $K_d$ determination in BHK cells. 1-7 represent 0.05, 0.1, 0.5, 1, 5, and 10 mM $Ca^{2+}$ and 200 $\mu$M EGTA. CatchER fluorescent signals of transfected permeabilized cells after equilibration with various extracellular $Ca^{2+}$ concentrations excited at 488 nm. (C) $K_d$ calculation with a 1:1 binding mode.
A passive equilibration protocol described by Dr. Pozzan and Filippin was applied *in situ* to measure calcium binding affinity of our sensor expressed in endoplasmic reticulum of BHK cells, which were transfected with CatchER DNA. First, histamine and thapsigargin were applied coordinately to deplete calcium from endoplasmic reticulum to cytoplasm, in which histamine functioned as an agonist to the histamine-receptors to stimulate calcium release from internal store while thapsigargin inhibited the activation of ATPase in the SERCA pumps, resulting in the blockage of cytoplasm calcium uptaken into ER. Then the cells were permeated by high concentration of digitonin, causing a massive drop of fluorescence intensity, which were contributed by both of calcium depletion from ER and the fluorescent protein loss. Intracellular buffer with 200 µM EGTA was perfused extracellularly to chelate the trace amount of calcium in ER. Sequentially, extracellular calcium concentration was increased gradually from 0.05 mM to 10 mM with a four-minute equilibrium for each step. The fluorescent intensity at 510 nm excited both by 488 nm and 395 nm of CatchER rose companied with the calcium concentration increment, which correlated with the *in vitro* calcium titration results. A fluorescent intensity plateau was formed for each step, assumed to be the sign of fully equilibrium of calcium concentration extracellularly and inside ER. The average value of each plateau was calculated respectively and fitted for the $K_d$ calculation by 1:1 binding model. To further verify the fluorescent intensity change really caused by calcium binding but not pH fluctuation, extracellular calcium concentration was further changed directly from 0 to 5 mM twice, and the fluorescent intensity reached the same level as the previous one, which indicated that the calcium response of our sensor was repeatable and reliable. Moreover, BHK cells loaded with Mag-fura-2 regarded as a positive control and the same type of cells transfected with wt mCherry as a negative control were conducted with the same protocol to further demonstrate
the fluorescent intensity changes came from the calcium response. The positive control exhibited the fluorescent intensity change according to the calcium concentration change, while the negative control did not reveal any change after treated with digitonin, though both of them showed a big decrease of fluorescence after treated with 100 µM digitonin, which was regarded as the loss of fluorescent protein or dye through the permeated membrane induced by digitonin. Fig 5 revealed the calculation results of in situ $K_d$s around 0.8 ~ 1.3 mM, to which belongs the free calcium concentration in SR.

We transiently expressed CatchER in SR of C2C12 myoblast cells, rather than in myotubes which would significantly influence the fluorescence of probes during contraction, (Rudolf et al. 2006, JCB) to measure the $K_d$ in situ after the sarcolemma was permeabilized by digitonin, which is more persuasive for in vivo calibration as several reported results suggested the different calcium binding affinities between in vitro and in vivo of the same sensor. The fluorescent intensity of CatchER was temperate-sensitive as adequate fluorescent emission could be detected after 48 hours expression at 30 °C, but very dim at 37 °C, possibly due to inefficient protein folding at higher temperature as similar phena was observed for cpEGFP. C2C12 myoblast cells were cultured DMEM medium containing 20% fetal bovine serum to avoid differentiation, and ready for the imaging experiment once reaching 70% confluency with maintained spindle shape. Localization of CatchER in SR was achieved by fusing ER targeting sequence of calreticulin (CRsig) in N terminal and ER retention sequence, KDEL in C terminal, which is a strategy widely applied to deliver fluorescent proteins to ER or SR of mammalian cells. Intact myoblast cells were initially cultured in Ringer buffer containing 2.3 mM Ca$^{2+}$ to maintain good shape and resting status with fully filled internal calcium store, exhibiting strong emission intensity of expressed CatchER which was easily to be differentiated from untrans-
fected cells by microscope due to the saturation by high concentration of free calcium in SR. Cells were permeabilized by applying 100 µM digitonin extracellularly, after replacing the extracellular buffer with intracellular buffer containing 100 nM Ca\(^{2+}\) by perfusion system. 50\% of CatchER emission intensity was eliminated upon three-minute treatment of digitonin, with excitation maxima at both 395 nm and 488 nm, possible due to the passive leakage of free calcium from SR lumen to cytosol resulting in calcium dissociating from CatchER. But similar phenomena were observed for a calcium-insensitive protein mCherry with the same protocol, suggested that partial SR targeted FPs could leak into the cytosol due to the protease digestion of the KDEL ER retention signal peptide. The cell shape maintained as original according to the bright field imaging, as digitonin allows selective permeabilization of sarcoplasma, indicating that the subcellular organelles are protected well inside the cells. Furthermore, 1 µM thapsigargin was applied to block SERCA pumps, by inhibiting ATPase tagged on the surface of this pump from cytosolic side, to prevent cytosolic ATP degradation induced pH change, and dysfunction SERCA pump to avoid calcium active loading from cytosol. Fluorescent intensity of CatchER in situ in response of calcium was quantitatively calibrated via equilibrating internal and external SR calcium concentration by passive loading buffer containing high calcium concentration from extracellular. Extracellular calcium concentration was increased gradually from 0.5 mM to 20 mM with a four-minute equilibrium for each step, with the aid of 5 µM ionomycin to accelerate the cation passive delivery. The fluorescent intensity at 510 nm excited both by 488 nm and 395 nm of CatchER rose accompanied with the calcium concentration increment, which correlated with the in vitro calcium titration results. A fluorescent intensity plateau was formed for each step, assumed to be the sign of fully equilibrium of calcium concentration extracellularly and inside ER. During each application of calcium buffer, fluorescent intensity kept
increasing initially and then stayed in a plateau representing the equilibration of the internal and external calcium concentration, and finally forming stepping curve correlated with the increment of calcium concentration. \( F_{\text{min}} \) was determined in the presence of 1 mM EGTA, while \( F_{\text{max}} \) was measured when adding 20 mM calcium extracellular. To avoid the pH influence caused by calcium interaction with EGTA to produce free protons, before or after EGTA application, 0 calcium intracellular buffer without EGTA was applied to wash away free EGTA, to prevent high concentration of EGTA and calcium interaction to change the pH of the buffer. The \( K_d \) value was calculated by fitting average value of each plateau with 1:1 binding mode with single wavelength calibration equation, and the final results were around 1 mM for both wavelengths. Similar results with the same protocol were obtained of BHK cells.

2.4.12 Resting ER \( \text{Ca}^{2+} \) concentration in HeLa, HEK-293 and C2C12 cells calibrated by CatchER.

CatchER was fused with the calreticulin signal peptide and KDEL at the scaffold EGFP N- or C-terminus, respectively, to target it to the ER (Fig. 4C). Confocal microscopy of CatchER and the ER-tracker DsRed2-ER colocalized in HEK-293 and C2C12 cells further confirm CatchER’s targeting specificity to the ER (Fig. S6).

To determine CatchER’s \( \text{Ca}^{2+} \) binding affinity, we exposed permeabilized C2C12 myoblasts to increasing \( \text{Ca}^{2+} \) concentrations as described (40, 111). CatchER’s \( K_d \) was 1.07 ± 0.26 mM in BHK cells and 1.09 ± 0.20 mM in C2C12 cells (Fig. S7C). The fluorescence intensity at the end of the experiment was fully recovered to the value prior to calibration (Fig. S7A,B), which demonstrates that CatchER was not washed out in permeabilized BHK and C2C12 cells, further supporting its targeting to, and retention in the ER. A discrepancy in the \( K_d \) measured in test tubes and \textit{in situ} is usual for both synthetic and genetically encoded indi-
cators (27). The resting ER Ca\textsuperscript{2+} concentration in HeLa, HEK293, and C2C12 cells was: 396 ± 13.2 (n = 7), 742 ± 134 (n = 5), and 813 ± 88.6 µM (n = 11), respectively, in agreement with reported ER Ca\textsuperscript{2+} concentrations of 100-900 µM using several Cameleon-based ER sensors (1, 112-114).
2.4.13 Ionomycin induced ER Ca\textsuperscript{2+} depletion and reloading.

Figure 2.27 Ca\textsuperscript{2+} concentration change in BHK cells detected by CatchER in the presence of 10 µM ionomycin. (A) Representative fluorescent imagines of CatchER expressed BHK cell in response extracellular Ca\textsuperscript{2+} change in the presence of 10 µM ionomycin. (B) Real-time fluorescent spectra of CatchER expressed BHK cell in response to extracellular Ca\textsuperscript{2+} change in the presence of 10 µM ionomycin, correlated to (A).
2.4.14 InsP₃ induced ER Ca²⁺ release in permeabilized BHK cells monitored by mag-fura2.

Figure 2.28 ER calcium depletion of BHK living cells monitored by Mag-fura-2. A) 340/380 fluorescent intensity ratio change profile of ER calcium depletion. B) Fluorescent imaging of BHK cells loaded with mag-fura2 without drug treatment. C) Fluorescent imaging of BHK cells loaded with mag-fura2 treated with 5 µM InP3.
The initial attempt to measure ER Ca\(^{2+}\) dynamics was achieved using the Ca\(^{2+}\) dye Mag-fura-2 in plasma-membrane permeabilized live cells. Mag-fura-2 with \(K_d = 60 \ \mu M\) measured in test tube can detect subcellular calcium dynamics in hundreds of \(\mu M\) to mM calcium environment without being influenced by cytosolic calcium change which is only 0.1% concentration of internal calcium store. Mag-fura-2 is initially a magnesium biosensor created by Dr. Tsien. New avenue was paved by Dr. Hofer to directly monitor ER calcium concentration change with this dye. We used the similar protocol depicted in Dr. Hofer's paper to detect calcium depletion from ER in BHK living cells. Pic B showed the fluorescence imaging of BHK cells loaded with Mag-fura-2 excited by both of 340 nm and 380 nm. Different color ratio indicated the heterogeneity of calcium concentration in the subcellular compartments. BHK living cells were first immersed in the Ringer buffer with 1 mM Ca\(^{2+}\), and then stimulated with high concentration of KCl similar to cytosolic condition. KCl rinse solution was changed by the permeabilization solution containing 1 \(\mu M\) digitonin with perfusion system. In this stage, fluorescence intensity of each single wavelength reduced dramatically, and the nucleus and cytosolic appeared to be nonfluorescent as the dyes retaining in the cytosolic were leaking out through the pores in the plasmatic membrane caused by digitonin permeabilization, but the ratiometric change increased apparently as the quench rate for each wavelength was not the same. Intracellular buffer with high concentration of KCl was applied to balance the cytosolic condition as the cells were permeabilized. The ratio of fluorescent intensity dropped sharply as an indicator for the calcium concentration decrease when 5 \(\mu M\) InP\(_3\) was applied, regarded as the inducer for the IP\(_3\) receptor to stimulate the calcium release from the ER store. This procedure was terminalized by change back to intracellular buffer, and the calcium was refilling. 100 nM Thapsigargin was sequentially used to block the SERCA pump which could uptake free calcium from cytosol-
lic into ER, so that fluorescent intensity ratio didn’t increase when intracellular buffer was perfused after InP3 was applied for the second time to release calcium from ER. And finally, the rest free calcium in ER was depleted by 10 µM ionomycin exhibiting a small drop of fluorescent intensity ratio.

2.4.15 InsP₃ induced ER Ca²⁺ release in intact and permeabilized C2C12 cells measured by CatchER.

![Figure 2.29](image)

Figure 2.29 C2C12 myoblast endoplasmic reticulum Ca²⁺ dynamics monitored with CatchER. (A) Two representative fluorescence responses, evoked by 100 µM ATP (pH 7.0) twice (arrow), and separated by Ringer buffer washout (triangle), to intact myoblasts without extracellular Ca²⁺ or EGTA. (B) The same batch of cells was permeabilized with 25 µM digitonin in intracellular buffer applied for 3 minutes and sequentially treated with IP₃, intracellular buffer washout, thapsigargin, IP₃ (arrow), washout (triangle), and ionomycin (arrow).

C2C12 cells exhibiting P2U-purinergic receptors in sarcoplasm, and InsP3 receptors, SERCA pumps on SR membrane which could be responsible to ATP, IP₃ and thapsigargin to
induce calcium release from SR to cytosol, respectively, are commonly used to examine the
calcium signaling induced by various agonists and antagonists of muscle cells. We chose
C2C12 myoblast cells to avoid the artificially fluctuation of fluorescence caused by myotube
cells contraction. The initial drop of fluorescence in panel H of figure 8 was caused by slightly
cell shape change due to buffer change. A big drop was observed and lasted for 100 seconds
when 100 uM ATP was applied to induced calcium release from SR. ATP initially interacted
with P2U-purinergic receptors in sarcoplasma of intact cells, which induced signaling molecule
InsP3 producing in cytosol after cascade reactions. Then high dose of InsP3 bound to InsP3
receptors resulting in calcium release from SR. As the extracellular buffer maintained to be low
calcium concentration, SR could not load calcium from cytosol until the extracellular buffer was
changed with 2.3 mM calcium mimicking the physiological condition, so there was a refill state
and stopped once perfused with 0 mM calcium buffer. But the 0 calcium extracellular buffer
was changed earlier than it should, the SR was not fully refilled as the signal amplitude was
not as high as initial. This process was repeated to verify the ATP response was not sporadic,
and another similar signaling was observed. Further, we permeabilized the cell with digitonin
and apply agonists to directly interact with receptors on the SR membranes. 10 µM IP₃ induced
a fast and big drop in the initial step and followed by a plateau which indicated the depletion of
calcium from SR to cytosol. Intracellular buffer containing 100 nM calcium was perfused extracelular to move high concentration of IP₃ and provided cytosolic conditions for SR calcium refilling. The fluorescent intensity slowly recovered to the original level within 400 seconds, followed by a gradually decrease stage as 1uM thapsigargin was applied to block the ATPase of SERCA pumps, so that the calcium could not refill into the SR, but only release from IP₃ receptors or Rynadine receptors. Additional 10 µM IP₃ was added to further accelerate the depletion
of SR calcium and the fluorescent intensity went back to the baseline as original, quantitatively indicating the SR calcium level was the same as only applied IP₃ in the initial. When another intracellular buffer was added, no refill state indicated the SRECA pumps were fully blocked by the thapsigargin. Another 5 \( \mu \)M of the ionomycin was added into the cellular, no further fluorescent intensity change monitored, indicating the fully depletion of SR calcium with free calcium to be around several micromolars. An alternative explanation is the binding affinity of CatchER was too weak, so that CatchER could not sense the trace amount of calcium change in comparison to Mag-fura-2. Ionomycin was acidic, but during this experiment, we did not observe any fluorescent change, indicating the pH is stable for the experiment.
2.4.16 ER Ca\textsuperscript{2+} release triggered by thapsigargin inhibition of SERCA pump.

Figure 2.30 Cytosolic Ca\textsuperscript{2+} signaling monitored by Fura-2 in HeLa cells. (A) A representative imaging of fura-2 loaded Hela cells. (B) 2 µM thapsigargin induced cytosolic Ca\textsuperscript{2+} elevation in the presence of extracellular 1 mM Ca\textsuperscript{2+}. 
Figure 2.31 ER Ca\(^{2+}\) depleted by thapsigargin of HeLa cells detected by CatchER. Real-time fluorescent response of CatchER expressed HeLa cells to 2 µM thapsigargin in the presence of 1 mM extracellular Ca\(^{2+}\), followed by adding 5 µM ionomycin for extracellular Ca\(^{2+}\) influx to ER.
Figure 2.32 ER Ca\(^{2+}\) depleted by thapsigargin of HEK293 cells detected by CatchER. Real-time fluorescent response of CatchER expressed HEK293 cells to 2 \(\mu\)M thapsigargin, followed by adding 5 \(\mu\)M ionomycin in the presence of 5 mM EGTA for complete ER Ca\(^{2+}\) depletion to calibrate \(F_{\text{min}}\). 50 mM extracellular Ca\(^{2+}\) was added to obtain \(F_{\text{max}}\), and finally washed by 0 mM extracellular Ca\(^{2+}\) to re-measure \(F_{\text{min}}\).
Ryanodine receptor type I plays a major role for the calcium depletion from SR to cytosol of skeletal muscle cells, so that the dysfunction or impair of this receptor will largely affect the Ca$^{2+}$ handling in physiological condition, which consequentially causes various diseases related to muscle contraction.

The quantitative study of calcium handling by inducing ryanodine receptor activation with chemicals or E-C coupling has been an important and standard topic to assess the healthy of the muscles. Most work has been focused on cytosolic calcium signaling perturbation after chemical induction. But as the complicated calcium buffer system in the cytosol and robust calcium channels and receptors in the plasma-membrane, the calcium signaling detected in the cytosol was patterned to be transient change, which hindered the further quantita-
tively analysis of the persistence of the ryanodine activation and how much calcium could be depleted by this type of receptors.

4-CmC is an abbreviation of 4-chloro-m-cresol, which is a burgeoning but very promising drug to identify muscle diseases for the biopsies from the patients according to its high selectivity and specificity to distinguish RyR I and RyR III in comparison to caffeine. The biological function of this drug is to active ryanodine receptor I from cytosol to induce calcium release from this receptor.

The intact C2C12 myoblast cells were applied with 4-CmC after 48-hour culture since transfected with CatchER. The fluorescent signaling dropped and stayed in a plateau as long as 4-cmc applied, and the fluorescent intensity recovered to the initial level once 4-cmc was removed by ringer buffer rinsing. These phenomena indicated that the 4-cmc persistently activate ryanodine receptor to deplete calcium as long as it physically interacted with this receptor, which could not directly observed by real-time calcium signaling from the cytosol, and it will be explained in the following paragraph. In addition, the level of calcium depletion is in a 4-CmC dose-dependent way which can be apparently observed from the plots according to the bottom of each wave. The previous study reported by Dr. Delbono supported this observation. In their work, adult mouse skeletal muscle was applied with different concentrations of 4-cmc and the corresponding forces produced by muscle contraction were in relative ratio to the dose applied, which can be elucidated by our working model that 4-cmc induce Ca^{2+} depletion from SR in a dose-dependent manner, which increased the cytosolic calcium concentration also in a dose-dependent way. The cytosolic calcium concentration change is the source to decide the forces can be produced by the muscle.
A more well-controlled experiment was conducted with the same protocol, and photo-bleach and cell shape change were avoided to allow the basal fluorescent intensity to be constant, representing the calcium concentration maintained to be the same during the rest condition. The fluorescent intensity change also occurred in a dose-dependent manner, which indicated the previous results were repeatable. An interesting phenomena was observed as a deep and sharp drop would form closely after application of high doses of 4-cmc, and fluorescent-intensity curves maintained oscillating with gradual reduced amplitude until reaching the balanced plateau. The rationale is that 4-cmc would induce an initial fast depletion of Ca\(^{2+}\) from SR, and maintain cytosol Ca\(^{2+}\) to be extremely high which could produce an intensive muscle contraction, but high-dose calcium retaining in cytosol would be toxic, so that the SERCA pump would restore the Ca from cytosol into ER and produce that initial narrow wave.

Moreover, an in vitro titration of purified CatchER and 4-CmC was conducted in order to exclude the influence to the fluorescent intensity change from non-specific interaction between protein and 4-CmC. No fluorescent intensity change occurred when directly adding the same amount of 4-CmC as the live cell imaging into the purified protein, revealing that the fluorescent change originated from 4-CmC induced calcium depleting instead of non-specific interaction between 4-CmC and CatchER.

For the further verification of the previous study, another two experiments were conducted with either negative or positive control. For the first one, with a coined name “traffic light of SR calcium” exhibited dual color alternation in corresponding to calcium concentration change inside SR of C2C12 skeletal muscle myoblasts. mCherry with ER tags was co-expressed with CatchER, resulting a initial combination of green and red color merged in the same location of the cells. When 4-CmC was applied, the co-transfected cell turned to be red
and the intensity was apparently to be more intense by higher dose of 4-CmC, characterizing as an alarm of low calcium concentration, but diminished after the drug was washed out. Actually, the red fluorescence originated from mCherry should have maintained to be a constant during the whole experiment, as mCherry could not response Ca\(^{2+}\) change, but only CatchER could, the intensity of which regularly changed in response to calcium signaling. From the fluorescent intensity curves results, the CatchER behaved as prediction, but mCherry curve kept linearly decrease during the whole experiment, unbiased to the application of 4-CmC, revealing that mCherry has been photo-bleached during the experiment instead of specific response to 4-CmC, which further verified the specificity of CatchER response to 4-CmC induced calcium depletion.

The positive control was operated with a separated batch of C2C12 cells loaded with Fura-2 in cytosol. The same amount of 4-CmC was applied with the same protocol and transient calcium increase in cytosol was observed according to Fura-2 ratiometric fluorescent change, proving that 4-CmC could efficiently depleting Ca\(^{2+}\) from SR of C2C12 myoblast cells.
Figure 2.34 (A) 4-CmC evoked Ca\textsuperscript{2+} release in the absence and presence of thapsigargin. (B) 4-CmC evoked cytosolic Ca\textsuperscript{2+} changes detected by Fura-2.
2.4.18 ATP and Histamine induced transient ER $\text{Ca}^{2+}$ release measured by Fura-2 and CatchER.

Figure 2.35 Representative ER $\text{Ca}^{2+}$ signaling detected by CatchER in HeLa cells triggered by ATP (A) and histamine (B) and corresponding cytosolic $\text{Ca}^{2+}$ signaling measured with Fura-2 in separated cells (C-D) using the same protocol.
2.4.19 CPA reversible inhibition and thapsigargin irreversible inhibition of SERCA pump.

Figure 2.36 Reversible Ca^{2+} release triggered by 15 µM CPA in HEK293 cells.
Figure 2.37 Quantification of irreversible ER Ca\(^{2+}\) release in HEK293 cells induced by 2 µM thapsigargin in the presence of 1 mM extracellular Ca\(^{2+}\). \(F_{\text{min}}\) and \(F_{\text{max}}\) were determined by adding 5 mM EGTA and 50 mM Ca\(^{2+}\), respectively, to the intact cells in the presence of 5 µM ionomycin (n=6).
Figure 2.38 Comparison of ER Ca\textsuperscript{2+} depletion by ATP, 4-CmC and ionomycin of C2C12 myoblast cells detected by CatchER. (A)-(E) Representative fluorescent images of CatchER transfected C2C12 myoblast cells. (F). Real-time fluorescent response to ATP and 4-CmC induced Ca\textsuperscript{2+} release from ER. (G). Comparison of Ca\textsuperscript{2+} release amounts from ER induced by different drugs.
2.4.20 SR Ca\(^{2+}\) release in adult and aging skeletal muscle.

Figure 2.39 SR and cytosolic Ca\(^{2+}\) transients recorded in CatchER-expressing mouse FDB fibers loaded with Rhod-2/EGTA. (A) Normalized SR and cytosolic fluorescence transients elicited by 100-ms command pulses at various voltages in FDB fibers under patch-clamp. Normalized Rhod-2/EGTA and CatchER fluorescence, recorded in the same fiber, were plotted arbitrarily as positive (increased cytosolic Ca\(^{2+}\) concentration) and negative (decreased SR Ca\(^{2+}\) concentration) signals, respectively, to compare their relative amplitude and voltage dependence. Data points were fitted to a Boltzmann equation of the form: 

\[ F = \frac{F_{\text{max}}}{1 + \exp(V_{F1/2} - V_m)/K} \]

where \(F_{\text{max}}\) is the maximal fluorescence; \(V_{F1/2}\) is the fluorescence half-activation potential; \(V_m\) is the membrane potential; and \(K\) is the steepness of the curve. \(V_{F1/2}\) and \(K\) values were -14.7 mV and 8.7, respectively, for Rhod-2 and -15.1 mV and 7.9, respectively, for CatchER (\(n = 7\)). The amplitude of the signal was measured from onset (average of the first 10 points immediately before applying the command pulse) to peak (Rhod-2) or nadir (CatchER). A steep increase in Ca\(^{2+}\) flux into the cytosolic compartment (Rhod-2) in response to 100-ms pulses (at various voltages, -30 to +40 mV) was observed concomitant with a decrease in the SR lumen (CatchER). The fluorescence changes were voltage-dependent, reaching a plateau at about +20 mV. (B) Representative Rhod-2/EGTA (top) and CatchER (bottom) intensity profiles and their confocal line-scans in response to 100-ms/40mV pulses are displayed.
To test the in vivo Ca\textsuperscript{2+} sensing capability, we first recorded mouse muscle SR Ca\textsuperscript{2+} release 3-4 weeks after in vivo electroporation of CatchER into FDB myofibers. The voltage-dependent maximal increase or decrease in fluorescence occurred upon Ca\textsuperscript{2+} binding to Rhod-2 or dissociation from CatchER (Fig. 5A,B), respectively (see Supporting Information).
CatchER detects SR Ca\textsuperscript{2+} depletion in response to an action potential (Fig. 5C). It enabled the first direct demonstration that resting SR Ca\textsuperscript{2+} concentrations are similar in young and old mice, although, SR Ca\textsuperscript{2+} release diminishes significantly in old mice (Fig. 5D). Impaired SR Ca\textsuperscript{2+} release but preserved SR Ca\textsuperscript{2+} content supports the excitation-contraction uncoupling mechanism in aging muscle fibers (33, 115, 116).
2.4.21 Targeting CatchER to microdomain of receptors.

ZM-5 is a short, 22-amino acid peptide from the fifth transmembrane helices of ryanodine receptor. ZM-5 N-terminal fused EGFP was hypothesized to face toward the cytosol when this construct was expressed in mammalian cells. The protein expression location of this construct was verified by the real-time fluorescent imaging. We co-expressed ZM-5 tethered EGFP (green) and cytosol-mCherry (red) in C2C12 cells (Figure 2.41 (A)), and the overlapped two-channel fluorescent imaging showed that cytosol-mCherry located in both cytosol and nu-

Figure 2.41 Localization of ZM5-EGFP in C2C12 myoblast cells by fluorescence imaging. (A) Representative fluorescent images of C2C12 myoblast cells co-expressed ZM5-EGFP and cytosol mCherry. (B) The real-time fluorescent imaging response of C2C12 myoblast cells co-expressed ZM5-EGFP and cytosol mCherry. 25 μM digitonin was applied to permeabilize the cells, and trypsin was applied to digest membrane-anchored protein, sequentially.
ucleus with bright red fluorescence co-existed in these two places, while ZM5-EGFP was not in nucleus. 25 µM digitonin was applied to permeabilize the plasmembrane, and a fast and large decay of the red fluorescence in cytosol was immediately observed, suggesting that cytosol-mCherry was washed away when the plasmembrane was permeabilized. A continuing decrease of red intensity in nucleus was observed with a final completed wash of all the subcellular organelles, indicating that the tag-less mCherry was not anchored on any membranes. The expression pattern of ZM-5-EGFP was similar to the ER-retention-EGFP, and the green intensity maintained during the digitonin permeabilization, supporting that ZM5-EGFP was anchored on the membranes of subcellular organelles. However, the orientation of the EGFP, whether it faces toward cytosol or lumen of ER, was challenging to determine. We tested the solvent accessibility of ZM5-EGFP in cytosol by performing protease digestion to the EGFP, and partial ZM5 tethered EGFP was digested by protease as a minor decrease in green fluorescent intensity was observed during the addition of trypsin. This did not rule out the possibility that ZM5-EGFP faces toward both cytosol and lumen of ER.
The fluorescent imaging of HeLa expressed ZM5 tethered CatchER with x100 magnification clearly showed an ER morphology, as the fluorescent intensity close to the nucleus was higher than the edge of the cells, verifying that ZM-5 tethered CatchER mainly anchored on the membranes of ER.

Figure 2.42 Representative fluorescent imaging of HeLa-expressed ZM-5-CatchER. The images were collected at 488 nm excitation and with x100 magnification.
The in situ $K_d$ of ZM5-CatchER was determined in C2C12 myoblast cells with a fluorescence microscopy. 25 µM digitonin was used to permeabilize the plasma membrane, and 20 mM, 0 mM, 0.1 mM, 0.5 mM and 1.0 mM Ca$^{2+}$ were sequentially perfused in the dish to calibrate the fluorescent intensity at each Ca$^{2+}$ concentration (Figure 2.43 (A)), with a fluorescent plateau formed at the equilibrium. Fluorescent intensity curves from two representative regions were fitted with 1:1 binding mode, due to high noise during the plateau, the $K_d$ was fitted to 1.4 and 2.7 mM, respectively. However, the $K_d$ obtained from these experiments still agreed with the 1 mM $K_d$ of CatchER with ER retention sequence.
The 4-CmC induced Ca$^{2+}$ release from the lumen of ER through ryanodine receptor to cytosol was also studied by ZM5-CatchER in C2C12 cells. Interestingly, both the increment and decrement of the fluorescent intensity was observed for the ZM-5 tethered CatchER. The increment of fluorescence could represent the elevation of cytosolic Ca$^{2+}$ concentration, while the decrement could suggest the release of ER lumen Ca$^{2+}$. There is a high possibility that the membrane anchored CatchER faced toward both cytosol and lumen of ER, correlating to the first experiment that trypsin could only digest partial of ZM-5 tethered EGFP, as not all the EGFP was exposed to trypsin in cytosol. Our ZM-5 sequence did not contain C-terminal Flag sequence, which is possible to impair the targeting specificity.

Figure 2.44 Fluorescent imaging of ZM5-CatchER in response to 4-CmC in C2C12 myoblast cells. (A) Representative fluorescent imaging of C2C12 expressed CatchER. (B) The real-time fluorescent imaging curves of ZM5-CatchER in response to 500 $\mu$M 4-CmC.
2.4.22 Constructing the lentivirus transfection system of CatchER and preliminary results of Ca\(^{2+}\) imaging.

![Diagram of the experimental procedure of packaging viral particles]

Figure 2.45 The experimental procedure of packaging viral particles. One vector containing the DNA of interest and other two helper vectors were cotransfected into the host cells 293 FT to produce viral particles. The supernatant of the 293 FT cell culture containing the viral particles was collected to determine the titer. Add the viral supernatant was added to the cells of interest for protein expression. Adopted from Life Technologies.
Figure 2.46 The DNA map of the FUGW vector. CatchER was inserted between BamHI and EcoRV.
Figure 2.47 Lentivirus expression of CatchER in HEK-293-T cells and C2C12 cells. (A) Fluorescence imaging of lentivirus expressed CatchER in C2C12 cells with the excitation 488 nm. (B) The bright field of the morphology of C2C12 cells in the (A). (C) Fluorescence imaging of lentivirus expressed CatchER in HEK-293T cells with the excitation 488 nm. (D) The bright field of the morphology of HEK-293T cells in the (C).
CatchER was inserted in Lentivirus FUGW vector between BamHI and EcoRV restriction enzyme cleavage sites (Fig. X.2). 5 ug FUGW plasmid was co-transfected with another two help vectors (psPAX2 3.3 ug and pMD2.G 1.7 ug) by lipofectamine-2000 (10 ul) into the host cells HEK-293T in 6 cm dish for packing lentivirus. The supernatant of the cell culture media was collected 48 hours after DNA transfection, and filtered with 0.48 µM membrane to get rid of cell pellets. The filtered supernatent was added into the medium of cells of interest, and culture for 72 hours in 30 °C for protein expression (Figure 2.45).

Figure 2.47 shows the fluorescent image of the lentivirus expressing CatchER in HEK-293T and C2C12 cells, and bright field images. The images were collected at excitation 488 nm, with emission maximum at 510 nm. The basal fluorescent intensity is much lower than the transient transfection with DNA plasmid. The possible pitfall is the low titer of the lentivirus for
mammalian cell transfection or expression. One of the suggested approaches is to super-centrifuge (30000~50000 r/min for 3 hours) the collected supernatant from host cells to concentrate the lentivirus to desired concentration.

The real-time fluorescent imaging curves of CatchER were shown in Figure 2.48. In the presence of 10 mM extracellular Ca$^{2+}$, the addition of 10 µM ionomycin would result in a transient increment of fluorescent intensity, followed by a fast spontaneous decay. A further decay would be observed when 0 mM extracellular Ca$^{2+}$ buffer was applied. Though these results correlated with the theoretical influx and extrude of ER Ca$^{2+}$ in the presence of 10 mM and 0 mM extracellular Ca$^{2+}$ delivered by high dose of ionomycin, the low basal fluorescent intensity and heterogeneous CatchER expression pattern (Figure 2.48(B)) did not rule out the high auto-fluorescence background in these results, and the morphology change of the cell may contribute to the artificial fluorescence response. The lentivirus transfection and protein expression condition still need to be improved.

2.4.23 Constructing the ratiometric CatchER

A ratiometric sensor requires two different wavelengths of either excitation or emission, with a fluorescent response to the analyte. For a single fluorescent protein based sensor, 1) directly create two excitation or emission peak, respond to the analyte with the same binding affinity and kinetics, which is directly correlated with the population of anionic/neural form chromophore change. However, the dynamic range will be a challenge for this strategy, and the kinetics will be limited to the internal vibration of polar residues close to the chromophore. 2) directly fuse another analyte inert fluorescent protein to generate additional excitation/emission spectra which does not overlap with previous spectra. With this approach, one-wavelength changes, another wavelength does not change. The basal fluorescent intensity of
these two fluorophore should be equal or similar. The advantages of this strategy is that there are broad choices of the wavelength of excitation and emission spectra of the inert fluorophore to stratify the instrumental or experimental requirement, however, the total molecular weight of the fusion protein will be enlarged twice, limiting the application of fusion protein in some cases. We directly fused a red fluorescent protein mCherry to CatchER to create a ratiometric Ca$^{2+}$ sensor.

2.5 Discussion

2.5.1 Kindle fluorescence change mechanism by designing of metal binding instead of protein-protein interaction.

In this study we have shown that optical properties of EGFP can be modulated by designing a calcium binding site via electrostatic perturbation and further changed upon calcium binding. NMR studies further revealed that such optical property change with ratiometric shift of ionic states is associated with the local unfolding/folding at the designed site coupled with changes (solvent accessibility) near the chromophore especially around oxygen of Tyr66 of chromophore which was originally well sealed beneath beta strands 10 and 11.

The rational introduction of acidic residues in the dimerization surface as Ca$^{2+}$ binding ligands shifts the major absorbance peak of EGFP from 488 nm to 395 nm by two factors. First, introducing charged ligand residues results in the alteration of the well-balanced native charge surface. Second, addition of electrostatic repulsions among clustered negatively charged residues in a restricted region located at two beta strands (10 and 11) contributes to the dynamic properties and packing of chromophore Tyr66. This is supported by the order of the absorbance 395/488 ratio CatchER < D10 < D9 < D8 following the trend of five, four, three and two negatively charged residues. In addition, Ca$^{2+}$ binding provides a direct and specific
neutralization allowing the recovery of the native like environment as well as the repacking of
the chromophore near Tyr66 as supported parallel increase of fluorescence and appearance of
NMR splitting of Gln69. This is different from pH induced fluorescence change without a par-
ticular proton binding pocket and often with global perturbation. Third, ligand types also play
roles in the repulsion density that is important in perturbation of the chromophore as well as
coordination required for Ca\textsuperscript{2+} binding. Charge effects by Glu with a longer sidechain and more
space distribution is different from that by Asp. D12 has greater perturbation in optical proper-
ties with a greater absorbance ratio 395/488 than CatchER although both CatchER and D12
have a total of five negatively charged residues with the only difference being in replacement
of Phe225 by Glu in CatchER and Asp in D12. Results from electrostatic interaction calcula-
tions and geometric analysis are consistent with the experimental results. D12 also has less
Ca\textsuperscript{2+} binding affinity and calcium induced fluorescence restoration than CatchER which is also
likely due to a Ca\textsuperscript{2+} binding geometry.

Structural determination not only explains the mechanisms of current sensors but also
provides a theoretical background for their further improvement. X-ray structural analysis of
GCaMP2 (117, 118) have assisted our understanding of triggering the fluorescence mecha-
nism in the atomic level and further facilitated rational design to accelerate the birth of
GCaMP3 with advanced signal to noise ratio (118, 119). On the other hand, high-field nuclear
magnetic resonance spectroscopy is superior in providing dynamic and conformational
changes and protein folding in solution (47). Our high resolution NMR studies provide several
evidence to support our design and proposed kindle fluorescence change mechanism. First,
CatchER maintains beta-barrel structure and dynamic properties supported by the similarity to
the w.t EGFP in both relaxation of properties and the C\alpha chemical shift change index. On the
other hand, the local conformation of EGFP at the designed site and near the chromophore was perturbed by the introduction of acidic residues. Second, Ca$^{2+}$ binding also induces local conformational change instead of global folding. The binding affinity measured by 2D NMR chemical shift changes of mainchain amides of several individual residues close to the designed binding site is in the same order of magnitude as measured by fluorescence change. The NMR study also eliminates the involvement of the residues, hydrogen-bonding with the chromophore at the opposite side of the beta barrel. Third, Ca$^{2+}$ binding also kindles local changes around the chromophore and slowdown of the exchange rates of Gln69 presumably related to chromophore dynamics (Kindling fluorescence by metal binding).

The mechanism of our designed sensor is different from that of GCaMP although CatchER exhibits a similar fluorescence enhancement excited at 488 nm in response to calcium compared to GCaMP. First, the scaffold protein of CatchER is EGFP instead of circular permutated EGFP with a new N-terminal at position 144(cpEGFP-144) with different optical properties. cpEGFP-144 exhibits a major absorbance maximum at 395 nm with a protonation at neutral pH. GCaMP created by fusing M13 peptide and CaM at the N- and C-terminals, respectively, also maintain these optical properties (72). Ca$^{2+}$-dependent fluorescence enhancement of GCaMP2 and its variants originate from Ca$^{2+}$ binding to the grafted CaM and the attendant interaction with the M13 peptide that destabilizes protonated fluorophore and restores deprotonated chromophore structure in cpEGFP (117, 118).

**2.5.2 Implications in developing calcium sensors with desired metal binding properties for the high calcium environment.**

In principle, a single calcium binding site with a $K_d$ close to the cellular environment is desired for a calcium sensor with quantification capability. Unfortunately, current CaM and TnC
based calcium indicators use natural calcium signaling proteins with two or four coupled calcium binding sites with a cooperative binding process. Their $k_d$s are locked at their functional region either submicromolar for the C-terminal domain of CaM or TnC or uM for the N-terminal domain of CaM. The formation of M13 complex usually locked the affinity in a narrower $K_d$ region with a strong cooperativity. This leaves a little opportunity to tune affinity 100-1000 fold weaker to be suitable for measuring the high calcium concentration in the SR/ER (120). Further mutations at the calcium binding sites or the protein-protein interface often result in disruption in cooperativity with multiple binding processes that pose difficulties in quantification (10) (1).

2.5.3 Implications for kinetic properties of calcium sensors.

CatchER exhibits a fast off rate that is finished within the dead time of the stopped flow instrument (2.2 ms) and its on rate of <28 ms. To our knowledge, CatchER has the fastest off rate among all the reported sensors.

Quantitative assessment of how fast Ca$^{2+}$ biosensor response is based on both in vitro and in vivo kinetics study. One of the initial kinetic studies of Ca$^{2+}$ biosensors was conducted in Ashley’s laboratory by observing the fluorescent change of Ca$^{2+}$ dyes fura-2 and indo-1 with stopped-flow by mixing saturated dye with EGTA or EDTA.
According to the proposed schemes to calculate fluorescence change, the concentrations of dye saturated with Ca\(^{2+}\) were set to be relatively low in comparison of chelators, which allows the concentration of free chelator nearly to be constant during the competition. The steady-state condition was applied to free [Ca\(^{2+}\)] due to negligible free [Ca\(^{2+}\)] during the experiment as the total concentration of dye and chelator is much higher than total [Ca\(^{2+}\)]. With the equation derivation, \(k_{\text{obs}}\) was contributed by kinetic rates of both dye and chelator. A plot of \(k_{\text{obs}}\) against chelator concentration was fitted by hyperbolic function extended with a saturation plateau. The Y-intercept where chelator concentration is assumed to be 0 indicated the

Figure 2.1 Kinetic study of Fura-2 in response to Ca\(^{2+}\) by stopped flow. (A) The structure of fura-2 and one calcium atom docking in the binding site. (B) Fura-2 is the structure analogy of EGTA with the same calcium binding site but extended with chromophores. (C) Fluorescent change of fura-2 in response to calcium. (D) Fluorescent response of calcium saturated fura-2 mixed with EGTA during the kinetic study by stopped-flow technique. (E) Rate constants fitted exponentials to the calcium saturated fura-2 fluorescence changes on mixing with different EDTA and EGTA (chamber concentration).
dissociation rate of chelator, and kobs value in the plateau where chelator concentration assumed to be infinity indicated the dissociation rate of dye.

$$\text{EGTA (E) competes with fura-2 (F) for Ca}^{2+}$$

$$\text{Ca}^{2+} + F \xrightarrow{k_{1}} CaF \quad (1)$$

$$\text{Ca}^{2+} + E \xrightarrow{k_{2}} CaE \quad (2)$$

**The rate of EGTA-Ca}^{2+} \text{ complex formation}**

$$\frac{d[\text{CaE}]}{dt} = k_{2} \cdot [\text{Ca}^{2+}] \cdot [E] - k_{-2} \cdot [\text{CaE}] \quad (3)$$

**The rate of free Ca}^{2+} \text{ change}**

$$\frac{d[\text{Ca}^{2+}]}{dt} = -k_{1} \cdot [\text{Ca}^{2+}] \cdot [F] + k_{-1} \cdot [\text{CaF}] - k_{2} \cdot [\text{Ca}^{2+}] \cdot [E] + k_{-2} \cdot [\text{CaE}] \quad (4)$$

**The rate of free Ca}^{2+} \text{ change equals to 0}**

$$k_{1} \cdot [\text{Ca}^{2+}] \cdot [F] + k_{2} \cdot [\text{Ca}^{2+}] \cdot [E] = k_{-1} \cdot [\text{CaF}] + k_{-2} \cdot [\text{CaE}] \quad (5)$$

**Substituting equ (5) into equ (3)**

$$\frac{d[\text{CaE}]}{dt} = \frac{k_{2} \cdot [\text{Ca}^{2+}] \cdot [F] + k_{-2} \cdot [\text{CaE}]}{k_{1} \cdot [F] + k_{2} \cdot [E]} \cdot [E] - k_{-2} \cdot [\text{CaE}] \quad (6)$$

**Set parameter \([Ca] \text{ indicating total [Ca}^{2+}]\)**

$$[\text{Ca}_{0}] = [\text{CaF}] + [\text{CaE}]$$

**Substituting [CaF] by ([Ca]−[CaE])**

$$\frac{d[\text{Ca}]}{dt} = \frac{k_{1} \cdot [\text{Ca}^{-1}] \cdot [\text{Ca}]}{k_{1} \cdot [F] + k_{2} \cdot [E]}$$

The kinetic rate of EGTA is highly pH.

**Figure 2.2 The proposed scheme of chemical reaction kinetics of the calcium saturated Fura-2 mixing with calcium chelators.**

The Ca\(^{2+}\) dissociation rate (k\(_{\text{off}}\)) of Fura-2 was directly measured for the first time to be 84 s\(^{-1}\) in the given condition, which is in correspondence with later reports by Tsien or others with different methods. The Ca\(^{2+}\) association rate (k\(_{\text{on}}\)) of Fura-2 was also obtained from analyzing the plot instead of calculating from known K\(_{\text{d}}\), but relied on the k\(_{\text{on}}\) value of chelator reported from other independent study. Unfortunately, the kinetic rate of EGTA is highly pH,
buffer and temperature dependent, which hamper the accuracy of dye $k_{on}$ value measurement by this method. However the $k_{on}$ value of Fura-2 reported to be $2.5 \times 10^8$ M$^{-1}$s$^{-1}$ in this paper matched well with the later studies. But a big challenge of stopped-flow to measure too fast responses was also pointed out as the majority of fluorescent change finished within the dead time of instrument when mixing Fura-2 with Mg$^{2+}$, so the up-limited time constant detected is the dead time of the instrument usually 1 to 2 ms, providing a maximal $k_{obs}$ to be 500 to 1000 s$^{-1}$, which is still higher than $k_{off}$ value of most calcium dyes, in except for Mag-fura-2 the $k_{off}$ value of which was measured by temperature jump to be over 20000 s$^{-1}$, characterizing tricarboxyl group as the single calcium binding site and exhibiting 2 order weaker binding affinity than Fura-2.

![Figure 2.3 Analysis of rate constants of calcium bound Fura-2 interacting with different concentration of EGTA measured from the stopped-flow techniques and the equation derivation.](image)

When $k_{obs} = k_{1}/2$, $k_1[F] = k_3[E]$

According to the plot

$[E][F] = 4.9$

Assume $k_2 = 5 \times 10^5$ M$^{-1}$s$^{-1}$

$k_1 = 2.5 \times 10^8$ M$^{-1}$s$^{-1}$

The estimation of $k_1$ for fura-2 is dependent on the constants assigned to EGTA or EDTA which, in turn, are very pH dependent. It is clear however that $k_1$ is in excess of $10^9$ M$^{-1}$s$^{-1}$ and probably approaches the diffusion-controlled limit. The pH independence of fura-2 thermodynamics and its kinetics around neutrality contribute much to its usefulness as an accurate intracellular Ca$^{2+}$ indicator.
The kinetics of genetically encoded calcium biosensors was explored in the similar way by stopped-flow technique. The very initial FRET pair based genetically encoded calcium biosensor hijacked the natural calcium binding protein calmodulin and one of its targeting peptides M13 (synthesized peptide with the same sequence as skeletal muscle myosin light chain kinase) and the $k_{on}$ and $k_{off}$ was measured to be $2.4 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and $12.9 \text{s}^{-1}$, respectively. An improved camelone with weaker binding affinity appropriate to measure free calcium signaling in endoplasmic reticulum of non-excitable cells was reported to exhibit faster $k_{on}$ and $k_{off}$ rate around $3.683 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and $256 \text{s}^{-1}$, respectively by modifying the calmodulin and M13 peptide interaction surface. But the experiment design was different from previous Fura-2 measurement. Instead of chelating Ca$^{2+}$ from the saturated calcium dyes, calcium indicator was mixed with calcium directly in stopped-flow. The advantage of this experiment was that fewer parameters were involved in the equation as it did not require to consider the $k_{on}$ and $k_{off}$ values of the chelators, but with a simplifier equation direct to measure the kinetics of the calcium indicator. But the limitation is also obvious, according to the proposed equations, the very initial $k_{obs}$ measured was already bigger than the $k_{off}$ value, which challenged the instrument to have a especially small dead-end time during the measurement of fast indicator, and the $k_{obs}$ had a direct ratio with the concentration of calcium which meant that the $k_{obs}$ would become even bigger at high calcium concentration. The reason of that was the Y intercept represented the $k_{off}$ value and the slope was the $k_{on}$ value, and $k_{on}$ was always bigger than 0.

### 2.5.4 Implications in SR/ER calcium and calcium release.

One of the major achievements of CatchER is that it can quantitatively measure the Ca$^{2+}$ release from the SR of FDB with shorter than 100 ms pulse stimulation and even faithfully detect the signals during single action potential (0.5 ms) stimulation which has never been re-
ported by other indicators, though the recovery rate is not comparable to the signals detected by Rhod-2 in the cytosol simultaneously. Since disputes were raised for the inconsistent calcium signaling kinetics observed between cytosol and SR of previous observation, a highly targeted SR calcium biosensor characterized by fast dissociation constant without tethering calsqustrin can effectively elucidate the influence of calsqustrin buffering capacity to the SR calcium signaling during short pulse stimulation (Cheng, 2008 #79)(Launikonis, 2006 #78). Moreover, it can differentiate subtle differences in kinetics and amplitude of calcium signaling during EC coupling between diseased, aging and healthy states. It facilitates elucidation of the disease mechanisms by quantifying the activity of particular receptors or channels (Jimenez-Moreno, 2008 #82)(Renganathan, 1997 #81)(Delbono, 1995 #80).

2.5.5 Theoretical calculation of the diffusion-control limit $k_{on}$ value, and estimation of the $k_{off}$ value of the sensor with submilimolar $K_d$.

Usually the molecule-molecule interaction is diffusion controlled, without considering the charge-charge attraction or repulsion. However, from the hypothesis of the enzymatic reaction, Kirkwood proposed that the diffusion-controlled reaction will be greater or smaller than the calculated constant, if the enzyme and substrate exhibit the opposite or the same charges. However, the high salt environment will shield the charge-charge interaction and reduce the influence.

There are several diffusion control models, for example, Alberty-Hammes-Eigen model (AHE model) described the upper limits of the second-order rate constant between enzyme and substrate; however, Chou model was one order of magnitude larger than those estimated through the former.
Protein folding kinetics were studied experimentally by stopped-flow technique, and they could also be investigated by computational simulation.

The Einstein-Stokes’ equation is shown below:

\[ D = \frac{kT}{(6\pi \eta r)} \]

\( r \) is the radius of the molecule (the structure is proposed as spherical particle); \( \eta \) is the viscosity; \( D \) is the diffusion constant; \( k \) is the Boltzmann’s constant; \( T \) is the temperature.

The diffusion control limit rate constant was calculated with the equation below:

\[ A_{\text{diff}} = 4\pi (r_m+r_n)(D_m+D_n)N_A \times 10^3 \text{M}^{-1}\text{s}^{-1} \]

For a particular chemical, the diffusion control limit rate constant can be estimated by the interaction diffusion rate of two same molecules, with the equation as below:

\[ A_{\text{diff}} = 4\pi \times 2r \times 2D \times N_A \times 10^3 \text{M}^{-1}\text{s}^{-1} \]

\( D \) was replaced by the Einstein-Stokes’ equation:

\[ A_{\text{diff}} = 4\pi \times 2r \times 2D \times N_A \times 10^3 \text{M}^{-1}\text{s}^{-1} = \frac{8kT}{(3\eta)} \times 10^3 \text{M}^{-1}\text{s}^{-1} \]

So for small molecules with spherical morphology, the diffusion control limit is unrelated to the radius of the molecule, but related to the temperature and viscosity of the environment, and usually the estimated uplimit diffusion rate constant is \( 7 \times 10^9 \text{M}^{-1}\text{s}^{-1} \).

For a \( \text{Ca}^{2+} \) sensor with 1:1 (sensor: \( \text{Ca}^{2+} \)) binding stoichiometry, the dissociation constant can be calculated with the equation below:

\[ K_d = \frac{k_{\text{off}}}{k_{\text{on}}} \]

If the \( k_{\text{on}} \) value is close to the uplimit (\( 10^8 \text{M} \)), and the \( k_{\text{off}} \) value is around 1000 \( \text{s}^{-1} \), the \( K_d \) is estimated to \( 10^{-5} \text{M} \).
2.5.6 The reported rate constant in cell and how the crowding condition in cell influences the kinetic properties?

The diffusion coefficient of Ca$^{2+}$ ion in water is $6 \times 10^{-6}$ cm$^2$/s (Robinson and Stokes, 1955), which is lower than the diffusion rate in the cytoplasm (in the absence of buffers). The reported rate of movement of barium in the cytoplasm of neurons of marine gastropod, Archidoris montereyensis (Connor et. al. 1981) was close to $2 \times 10^{-6}$ cm$^2$/s. In cells, the diffusion coefficient is not as big as in the solution due to the viscosity change. From the experimental results, the buffer effect also influences the diffusion coefficient.

Moreover, the molecular size also influences the diffusion rate constant in the cell, which is different from the theoretical calculation. For the latter, the molecule-molecule interaction distance is estimated to two times of the molecular radius, and the terms for the radius of the molecule in the equation cancel out. However, the terms for radius cannot be eliminated in the cell as the interaction distance is much longer than two times the molecular radius.

The figure below adopted from a reported paper showing how the molecular size influences the mobility in buffer, cytosol and nucleus.
In addition to the traditional methods used to measure the mobility in cell, using advanced fluorescent imaging techniques, such as fluorescence recovery after photobleaching (FRAP) can also investigate the protein mobility in situ.

The influence of the kinetics by buffering proteins in cell can be investigated by studying the in situ kinetics of CatchER in SR calcium buffering protein calsequestrin knockout mice. The estimated Ca$^{2+}$ association and dissociation rate constants measured in KO mice should be larger than those measured in wt mice.
2.5.7 Calculating the Gibbs free energy change of Ca\(^{2+}\) binding CatchER, and comparing the binding energy changes with other Ca\(^{2+}\) binding proteins.

The binding dissociation constant between Ca\(^{2+}\) and CatchER was measured experimentally, and the Gibbs free energy change was calculated by the equation. The K\(_d\) of CatchER was \(1.8 \times 10^{-4}\) M, determined by the fluorescent titration at the room temperature (T=298 K).

The Gibbs free energy change can be calculated with the equation below:

\[
\Delta G^\circ = -RT\ln K_d
\]

\(\Delta G^\circ\) is the Gibbs free energy change; R is the gas constant; T is the temperature; K\(_d\) is the dissociation constant.

To calculate the Gibbs free energy change of CatchER, the dissociation constant was added into the equation. \(\Delta G^\circ = -RT\ln K_d = -(8.314 \times 10^{-3} \text{ kJ/mol}) \times 298 \times \ln (1.8 \times 10^{-4}) = 21.4\) kJ/mol= 5.1 kcal/mol (dissociation unfavorable). This is the theoretical result.

There are reported Gibbs free energy change values for calcium binding proteins. For calmodulin, the binding affinity was determined by the Trp fluorescence titration.

For calmodulin binding Ca\(^{2+}\)

Site 1 with K\(_d\) = \(0.16 \times 10^{-6}\) M

\(\Delta G^\circ = -RT\ln K_d = -(8.314 \times 10^{-3} \text{ kJ/mol}) \times 298 \times \ln (0.16 \times 10^{-6}) = 38.8\) kJ/mol (dissociation unfavorable)

Site 2 with K\(_d\) = \(0.58 \times 10^{-6}\) M

\(\Delta G^\circ = 35.6\) kJ/mol (dissociation unfavorable)

Site 3 with K\(_d\) = \(4.12 \times 10^{-6}\) M

\(\Delta G^\circ = 30.7\) kJ/mol (dissociation unfavorable)

Site 4 with K\(_d\) = \(9.49 \times 10^{-6}\) M
\[ \Delta G^\circ = 28.7 \text{ kJ/mol} \]

For calmodulin binding Mg\(^{2+}\)
Site 1 with \(K_d = 44.0 \times 10^{-6} \text{ M}\)
\[ \Delta G^\circ = 24.9 \text{ kJ/mol} \]
Site 2 with \(K_d = 740.0 \times 10^{-6} \text{ M}\)
\[ \Delta G^\circ = 17.9 \text{ kJ/mol} \]
Site 3 with \(K_d = 740.0 \times 10^{-6} \text{ M}\)
\[ \Delta G^\circ = 17.9 \text{ kJ/mol} \]
Site 4 with \(K_d = 2000.0 \times 10^{-6} \text{ M}\)
\[ \Delta G^\circ = 15.4 \text{ kJ/mol} \]

The Gibbs free energy changes of Ca\(^{2+}\) binding to CatchER is smaller than that of Ca\(^{2+}\) binding to calmodulin EF-hand, similar to the Mg\(^{2+}\) binding to calmodulin EF-hand.

The electrostatic interaction energy was calculated from the Coulomb’s law with the equation below:

\[ E = \frac{Q_1 Q_2}{(4 \pi \varepsilon_0 r)} \]

\(\varepsilon_0\) is the vacuum permittivity: \(8.854187817 \times 10^{-12} \text{ A}^2 \text{s}^4 \text{ kg}^{-1} \text{ m}^{-3}\) or \(C^2 \text{ N}^{-1} \text{ m}^{-2}\) or \(F \text{ m}^{-1}\).

However, the electrostatic interaction energy calculation is based on a point to point interaction, instead of a point to a cavity interaction. The electrostatic distribution based on the geometry of designed calcium binding site is relatively challenging to calculate. Moreover, for a high affinity binding site, like the EF-hand I and II of calmodulin, other factors will contribute more to the binding energy rather than to the electrostatic interaction, as the number of the
charged residues between high-affinity EF-hand and low-affinity EF-hand is the same or similar, while the binding affinities are more than 10 fold difference.

2.5.8 **Comparing the kinetic parameters between CatchER and other Ca$^{2+}$ indicators.**

The in vitro study shows that for Ca$^{2+}$ dyes with affinity around $10^{-7}$ M, the $k_{on}$ value is $10^8$ - $10^9$ M$^{-1}$s$^{-1}$, the $k_{off}$ is 100 - 370 s$^{-1}$ (calcium-green-1, fura-2, fluo-3) (Mank, M. et. al.), and the Cameleon-1/E104Q $k_{on}$=$2.4 \times 10^6$ M$^{-1}$s$^{-1}$, $k_{off}$=12.9 s$^{-1}$.

The in situ study of the kinetics of calmodulin-based single GFP Ca$^{2+}$ sensor GCaMP-2 in utero electroporated acute cortical slice shows a $k_{on}$= $8 \times 10^5$ M$^{-1}$s$^{-1}$, $k_{off}$ = 1 s$^{-1}$, and $K_d$ =1250 nM (Hires, S.A., et. al. 2008).

The troponin C based FRET pair sensor TN-XL reported the presynaptic Ca$^{2+}$ signals at the Drosophila larval neuromuscular junction after stimulation of the motoneuron at 40 Hz with a rise of 430 ms and a decay of 240 ms. However, the N-terminus of chicken skeletal and cardiac muscle troponin C with $k_{on}$ of $10^8$ M$^{-1}$s$^{-1}$ and $k_{off}$ of 500-800 s$^{-1}$ and the off rate of the high affinity C-terminal lobe is 1 to 2 orders of magnitude slower. In comparison to the calmodulin based FRET pair Ca$^{2+}$ sensor, the kinetics of single calmodulin measured by the Ca$^{2+}$ induced conformational change of C-terminus and N-terminus are 490 µs and 20 ms, respectively (Mank, M. et. al., 2008).
Table 2.8 The major kinetic parameters of Ca\textsuperscript{2+} indicators in Drosophila with 40 Hz stimulation.

<table>
<thead>
<tr>
<th>Ca\textsuperscript{2+} sensor</th>
<th>Rise (s)</th>
<th>Decay (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YC3.3</td>
<td>1.41</td>
<td>1.05</td>
</tr>
<tr>
<td>YC3.6</td>
<td>0.82</td>
<td>0.73</td>
</tr>
<tr>
<td>YC2.6</td>
<td></td>
<td>5.24</td>
</tr>
<tr>
<td>D3cpv</td>
<td>0.68</td>
<td>1.96</td>
</tr>
<tr>
<td>TN-L15</td>
<td>0.81</td>
<td>1.49</td>
</tr>
<tr>
<td>TN-XL</td>
<td>0.59</td>
<td>0.20</td>
</tr>
<tr>
<td>GCaMP1.6</td>
<td>1.38</td>
<td>0.45</td>
</tr>
<tr>
<td>GCaMP2</td>
<td>0.63</td>
<td>0.38</td>
</tr>
<tr>
<td>OGB-1</td>
<td>0.24</td>
<td>0.38</td>
</tr>
</tbody>
</table>

The difference between in vitro and ex vivo affinity is possibly due to endogenous protein interference, incomplete exchange of pipette solution and cytosol, saturation of the calcium buffer (Neher, 1998), or internal calcium store release.

In conclusion, the in situ kinetic parameters of both genetically encoded and synthesized Ca\textsuperscript{2+} indicators are slower than those measured in vitro. The kinetics of endogeneous Ca\textsuperscript{2+} binding proteins (CaM, TnC) are much faster than that of proteins fused in FRET pair. CatchER exhibited the fastest $k_{\text{off}}$ rate among all the reported genetically encoded Ca\textsuperscript{2+} sensors, and the in situ on rate correlated with the Ca\textsuperscript{2+} uptake rate into SR based on the activity of SERCA pumps.
2.5.9 Further increasing the binding affinity of CatchER.

The metal binding affinity is correlated with the charged residues, which has been demonstrated and reported. We further mutate an additional negatively charged residue close to the designed binding site (N149E-CatchER), and the Ca\(^{2+}\) binding affinity was reduced to one third in comparison to CatchER, and the mutant preserved 100% fluorescent intensity enhancement in Ca\(^{2+}\) saturated form compared to that in Ca\(^{2+}\) free form, with a similar dynamic range to CatchER measured with bacterial expressed and purified protein in 10 mM Tris buffer, pH 7.4 (Figure 2.50).

The dissociation constant of N149E-CatchER measured by fluorescent titration was 78 ± 5 \(\mu\text{M}\) with excitation at 491 nm. The intensity of emission maximal 510 nm was fitted with 1:1 binding mode, which is around 43\% of \(K_d\) value of CatchER. These experimental results further demonstrated that the binding affinities of CatchER variants could be turned by adjusting the numbers of charged residues of the designed Ca\(^{2+}\) binding site.
Figure 2.50 The $K_d$ of S149E-CatchER was determined by fluorescence titration. The fluorescent emission spectra of N149E-CatchER in response to 0-5 mM Ca$^{2+}$ excited at 491 nm (A) and the emission spectra recorded in the same condition excited at 396 nm (B). The protein concentration is 2 µM, in 10 mM Tris buffer, pH 7.4. (provided by Florence Reddish)
2.5.10 The metal selectivity between Ca$^{2+}$ and Mg$^{2+}$ of CatchER.

The in situ calibration $K_d$ of CatchER in C2C12 cells was measured to be $1.1 \pm 0.2$ mM in the presence of 0.25 mM Mg$^{2+}$, and 1.66 mM in FDB muscle fibers in the presence of 1 mM Mg$^{2+}$. Though the discrepancy of $K_d$ was plausibly due to different buffer systems, it is highly possible that the high Mg$^{2+}$ concentration would compete with the endogenous Ca$^{2+}$ and increase the apparent dissociation constant of CatchER to Ca$^{2+}$ in cells.

To improve the metal selectivity between Mg$^{2+}$ to Ca$^{2+}$, the first strategy is to increase the number of negatively charged residues in the designed Ca$^{2+}$ binding site, as discrepancy of the $K_d$ of designed variants (D9, D10 and CatchER) was decreased in the absence and presence of high concentration of K$^+$, suggesting that the monovalent and divalent metal selectivity can be improved by this strategy. This is also possibly helpful for improving the selectivity of Ca$^{2+}$ and Mg$^{2+}$.

In addition, T203I-CatchER also exhibited improved metal selectivity between Ca$^{2+}$ and Mg$^{2+}$. The original rationale to mutate T203 to I is to reduce the pK$_a$, as T203I-GFP is called sapphire gfp with perfect pK$_a$ around 4.8, (Kneen, M., Farinas, J., Li, Y., and Verkman, A.S. 1998. Green fluorescent protein as a noninvasive intracellular pH indicator. Biophys. J. 74:1591-1599.). but the 488 nm peak was eliminated and only 395 nm peak maintained, so sapphire-gfp is not bright. However, in the presence of 5 negatively charged residues functioning as the Ca$^{2+}$ binding pocket, T203I-CatchER seems not to exhibit tremendous advantages in pK$_a$ value. The pK$_a$ of T203I-CatchER was only roughly measured, but not as low as 4.8, still above 6. 203I is a residue that faces toward the chromophore, but is not solvent accessible. However, this variant shows bigger dynamic change in the presence of 1 mM Mg$^{2+}$ in comparison to CatchER in vitro titration, but as the major big peak of T203I-CatchER is 395 nm, so it is
not as bright as CatchER with the exciation at 488 nm.

The rationale of T to I is to change the hydrophilic residue to a hydrophobic residue. Fewer hydrophilic residues closer to the Ca\textsuperscript{2+} binding sites (without changing the binding pocket itself) may help to eliminate the non-specific sticking or attracting of other divalent metals. Additionally Ile is a residue with a bulkly sidechain that can function as the electron donor, which can influence the hydrogen network with the chromophore. This may relate to the rational design of Ca\textsuperscript{2+} dyes as the introduction of electrophilic or other functional group can change the optical properties of dyes or the metal binding affinity.

Overall, the rational design scope may not restrict the surface charge or the binding ligands themselves. The neighboring residues or the residues involved in the hydrogen networking with the chromophore can also be altered, like the BAPTA-based Ca\textsuperscript{2+} dyes. The core binding sites are maintained for all the family. Only extending or adding functional groups to the chromophore can dramatically change the optical properties, kinetics and binding affinity and selectivity of the sensors.

### 2.6 Conclusion

In conclusion, we have designed a novel type of genetically encoded calcium indicators by mutating native residues of EGFP as calcium binding ligands instead of hijacking natural calcium-binding proteins and using calcium binding motif. Ca\textsuperscript{2+}-binding results in a parallel increase of fluorescence at 510 nm when excited at both 395 and 488. By introducing Ca\textsuperscript{2+}-binding ligand residues, we have shown that Ca\textsuperscript{2+}-binding affinity and chromophore properties of CatchER can be concurrently altered. Using various spectroscopic, kinetic and structural methods, we have shown that the designed Ca\textsuperscript{2+} sensor CatchER has several unique merits.
including a new kindle mechanism for tuning fluorescence of FPs by Ca$^{2+}$-induced fluorescence change, tunable and optimal metal binding affinity without complicated cooperative binding by coupled calcium binding sites, the unprecedented off rate, and minimized perturbation of Ca$^{2+}$ signaling network and small size.

CatchER allows us to reveal several important findings that are related to the ER/SR release. First, the free calcium in the SR at resting state is homogeneous instead of heterogeneous as previously reported where the highly varied basal ratio of cameleon based FRET pair indicator expressed in the SR possibly due to variation in basal signals as reported (40, 121). Second, the SR calcium concentrations varies from 800 µM (HEK) to 400 µM (HeLa), which are greater than the reported values under similar conditions by D1ER (1), possibly due to the difference in calcium binding affinity of the sensors. Third, calcium release is directly proportional to the basal calcium concentration. Fourth, fast dissociation kinetics allows us to quantify the buffering capacity of casqustrin. In our study, we observed the SR release even at single action potential stimuli (0.5 ms) instead of a delayed calcium release postulated to relate to calsqustrin (40). The temporal SR calcium decrease is highly correlated to the cytosol calcium increment.

The intrinsic low calcium binding affinity and low protein expression level in situ introduce less perturbation of the natural calcium signaling and eliminate the buffering capacity brought by the biosensors. As the smallest size of all the genetically encoded calcium indicators, CatchER with kinetic fidelity and fast protein diffusion in situ provides a further opportunity for tagging to multiple channels in the membrane with the least perturbation. Furthermore, as a genetically encoded calcium indicator, CatchER allows for long-term observation and high targeting specificity.
2.7 References


CHAPTER 3: RESIDUAL SARCOPLASMIC RETICULUM Ca^{2+} CONCENTRATION AFTER Ca^{2+} RELEASE IN SKELETAL MYOFIBERS FROM YOUNG ADULT AND OLD MICE

3.1 Abstract

Contrasting information suggests either almost complete depletion of sarcoplasmic reticulum (SR) Ca^{2+} or significant residual Ca^{2+} concentration after prolonged depolarization of the skeletal muscle fiber. The primary obstacle to resolving this controversy is the lack of genetically encoded Ca^{2+} indicators targeted to the SR that exhibit low-Ca^{2+} affinity, a fast biosensor: Ca^{2+} off-rate reaction, and can be expressed in myofibers from adult and older adult mammalian species. This work used the recently designed low-affinity Ca^{2+} sensor (K_d=1.66 mM in the myofiber) CatchER (calcium sensor for detecting high concentrations in the ER) targeted to the SR, to investigate whether prolonged skeletal muscle fiber depolarization significantly alters residual SR Ca^{2+} with aging. We found CatchER a proper tool to investigate SR Ca^{2+} depletion in young adult and older adult mice, consistently tracking SR luminal Ca^{2+} release in response to brief and repetitive stimulation. We evoked SR Ca^{2+} release in whole-cell voltage-clamped flexor digitorum brevis muscle fibers from young and old FVB mice and tested the maximal SR Ca^{2+} release by directly activating the ryanodine receptor (RyR1) with 4-chloro-m-cresol in the same myofibers. Here, we report for the first time that the Ca^{2+} remaining in the SR after prolonged depolarization (2 s) in myofibers from aging (~220 µM) was larger than young (~132 µM) mice. These experiments indicate that SR Ca^{2+} is far from fully depleted under physiological conditions throughout life, and support the concept of excitation–contraction uncoupling in functional senescent myofibers.
3.2 Introduction

Measuring SR residual Ca\(^{2+}\) concentration in depolarized skeletal muscle fiber is essential to understanding the influence of luminal SR Ca\(^{2+}\) on SR Ca\(^{2+}\) release and force deterioration with aging. Developing reliable molecular tools to detect Ca\(^{2+}\) kinetics in intracellular organelles is imperative to determining its role in cell signaling, physiological responses to training and exercise, and the pathogenesis of muscular dystrophy, central core disease, malignant hyperthermia, and fatigue syndromes \cite{27, 44}. The SR is the main source of Ca\(^{2+}\) for skeletal muscle contraction, but the role of its intricate terminal cisternae/longitudinal architecture and Ca\(^{2+}\) buffering by endogenous Ca\(^{2+}\)-binding proteins, such as calsequestrin and calreticulin, in intracellular Ca\(^{2+}\) homeostasis is poorly understood. Particularly, the elevated SR Ca\(^{2+}\) concentration requires a low-affinity biosensor to reliably monitor Ca\(^{2+}\) concentration and kinetics. Modifications in Ca\(^{2+}\) concentration are the signal that regulates a number of functions in excitable and nonexcitable cells \cite{15, 16, 36}. However, loading the skeletal muscle SR with Ca\(^{2+}\)-sensitive indicators presents technical difficulties that seriously hamper our ability to determine Ca\(^{2+}\) concentration and release kinetics and to answer questions relevant to muscle physiology and pathology. Assessing residual SR Ca\(^{2+}\) in excitable cells is relevant for the study of luminal Ca\(^{2+}\) buffer capacity, Ca\(^{2+}\)-dependent regulation of IP\(_3\)R and RyR function, store-operated Ca\(^{2+}\) entry, termination of SR Ca\(^{2+}\) release, and age-dependent decline in SR Ca\(^{2+}\) release \cite{12}. Therefore, measuring Ca\(^{2+}\) movements using engineered protein targeted to the endoplasmic/sarcoplasmic reticulum (ER/SR) is increasingly important \cite{4, 12, 19, 28, 34}. Previous studies have shown that decreased expression of the Cav1.1 subunit diminishes SR Ca\(^{2+}\) release in muscle fibers from aging mice \cite{11, 12}, a process known as excitation-contraction un-
coupling (ECU) \[3\]. ECU is partially due to increased Cavβ1a expression with aging \[35\]. Recently, we estimated SR Ca\(^{2+}\) depletion in response to 4-chloro-m-cresol (4-CmC) or myofiber depolarization in myofibers from young adult mice electroporated with the Ca\(^{2+}\) biosensor D1ER \[12\]. We concluded that significant depletion of SR Ca\(^{2+}\) under physiological conditions is unlikely in young mice \[12\]. However, quantifying SR Ca\(^{2+}\) concentration after prolonged myofiber depolarization was not possible due to limitations in calibrating D1ER in the myofiber as described previously\[28\]. Quantifying residual SR Ca\(^{2+}\) requires measuring its resting concentration, release, and content in the same fiber using CatchER as the Ca\(^{2+}\) biosensor. In Chapter 2, we reported the novel enhanced green fluorescence protein-derived biosensor CatchER can monitor Ca\(^{2+}\) concentration and dynamics in high Ca\(^{2+}\) concentration compartments, such as the SR. It exhibits relatively low affinity in the skeletal muscle, as its K\(_d\), calibrated in the myofiber, is 1.66 mM, and an unprecedented off-rate at \(\sim 700 \text{ s}^{-1}\), which makes it one of the more advanced and useful tools for examining changes in SR Ca\(^{2+}\) concentration in response to pulses both as prolonged as those applied to elicit muscle tetanus and as short as an action potential \[34\]. In this chapter, we show that CatchER exhibits a consistent response to muscle fiber stimulation, which allows us to quantify SR Ca\(^{2+}\) content, the rate of Ca\(^{2+}\) flux in the SR lumen, and the residual SR Ca\(^{2+}\) concentration in response to prolonged electrical stimulation beyond physiological demands in voltage-clamped adult mouse flexor digitorum brevis (FDB) muscle fibers. Maximal SR Ca\(^{2+}\) depletion was also examined by bypassing the physiological Cav1.1/ryanodine receptor-1 (RyR1) coupling to stimulate the RyR1 directly. For this application, we used the specific RyR1 agonist 4-chloro-m-cresol based on the reproducibility of its action and the lack of any reported effect on SR Ca\(^{2+}\) refilling. We report a significant larger residual SR Ca\(^{2+}\) concentration in fibers from old compared to young adult mice,
finding that supports the concept of excitation–contraction uncoupling in senescent mice. The most work in this chapter was performed by Dr. Zhong-min Wang in Dr. Osvaldo Delbono’s group. I performed the analysis of in situ Ca\textsuperscript{2+} release and recover rates in 3.5.X.

### 3.3 Materials and methods

*Flexor digitorum brevis muscle fibers.* Single skeletal muscle fibers from the FDB were obtained from 3- to 5-month-old C57BL6 mice raised in the Wake Forest University School of Medicine (WFUSM) Animal Research Program. Mice were killed by cervical dislocation following isoflurane anesthesia. For aging studies, we used young (3–5 months) and old (22–25 months) FVB (Freund virus B) mice from the WFUSM colony. Animal handling followed a protocol approved by the WFUSM Animal Care and Use Committee.

*FDB fiber electroporation and SR Ca\textsuperscript{2+} fluorescence recording.* The pcDNA3.1 plasmid carrying the CatchER biosensor was electroporated into the FDB muscle as described [6]. FDB fibers were enzymatically dissociated and recorded 2–3 weeks after electroporation [34]. Dissociated FDB fibers were voltage-clamped in the whole-cell configuration of the patch-clamp technique [9], according to procedures specific for muscle fibers [39]. Potential voltage-errors associated with whole-cell recording in large cells were minimized by selecting small FDB fibers and adequately compensating for whole cell capacitance transients. The pipette electrode was filled with the following solution (mM): 130 Cs-aspartate, 2 MgCl\textsubscript{2}, 0.2 or 20 Cs2EGTA as specified below, 10 HEPES, 5 Na-ATP, and 0.5 GTP; pH was adjusted to 7.3 with CsOH. High EGTA was used to reproduce experimental conditions in a series of studies on FDB muscle fibers in which SR Ca\textsuperscript{2+} release was recorded in the cytosol [12, 34, 41]. The electrode resistance ranged from 450 to 650 kΩ. The external solution contained (in mM): 100 TEA-OH (tetraethylammonium hydroxide), 50 Na\textsubscript{2}SO\textsubscript{4}, 2 MgSO\textsubscript{4}, 2 CaSO\textsubscript{4}, 2 3-4DAP, and 5
Na-HEPES. Solution pH was adjusted to 7.3 with CH$_4$SO$_3$ [12, 39]. These solutions ensured good control of resting leakage currents, and the preparation was preserved throughout the experiment. Methanesulphonic acid is the counterion to TEA. Drift and noise imposed by silver-chloride wires were corrected by standard methods. All experiments were recorded at room temperature (21–22°C). To permeate the myofibers, we used a solution containing 90 mM K-glutamate, 1.02 mM MgCl$_2$, 5 mM NaCl, 10 mM HEPES, 1 mM 1,2-bis(o-aminophenoxy)ethane-n,n,n’,n'-tetraacetic acid (BAPTA), 0.323 mM, CaCl$_2$, 0.025 N-benzyl-p-toluene sulphonamide, 2% (vol/vol) poly (N-vinyl-2-pyrrolidone) (1 mM free Mg$^{2+}$, 0.0001 mM free Ca$^{2+}$), pH 7.2, adjusted with KOH [43]. Free [Ca$^{2+}$] and [Mg$^{2+}$] in solution were calculated by the Max-Chelator program. Fluorescence was recorded using an Axiovert 200 microscope with a 20°—/0.75 (Zeiss, Oberkochen, Germany) and a Radiance 2100 (Bio-Rad, Zeiss) confocal system. Fibers were imaged through a C-Apochromat 40°— water immersion objective (NA 1.2, Zeiss) or a 20°— Fluar (NA 0.75) using a krypton–argon laser at 488-nm excitation wavelength. CatchER’s fluorescence emission was measured at 528 ± 25 nm wavelength. For most experiments, the laser was attenuated to 6–12% with a neutral density filter. Fibers were imaged in line-scan (x–t) mode. The fiber was always oriented parallel to the x scan direction. Line-scan images were acquired with 256 pixels (0.236 mm/pixel) in the x- and 512 pixels (0.833 ms/pixel) in the t-direction. For image acquisition, we used LaserSharp 2000 software (Bio-Rad, Zeiss). Cytosolic Ca$^{2+}$ transients were recorded using 5 µM Rhod-2 AM (Invitrogen, Carlsbad, CA) loaded for 30 min on CatchER-expressing FDB fibers. Fibers were excited at 568 nm using a krypton laser and recorded at 600 nm. SR Ca$^{2+}$ release and cytosolic Ca$^{2+}$ transients were recorded sequentially (20 s interval). No differences in the amplitude and kinetics of SR Ca$^{2+}$ release monitored with CatchER were apparent in Rhod-2-loaded and unloaded
fibers (data not shown). CatchER calibration in mouse FDB muscle fibers was reported in detail previously (Supporting Information in [34]). Statistic Values are given as mean ± SEM with the number of observations (n). Statistical analysis was performed using the Student’s unpaired t test and the Mann–Whitney rank-sum test when values were not normally distributed. P<0.05 was considered significant.

3.4 Results

3.4.1 RyR1 mediates SR Ca$^{2+}$ release recorded with CatchER.

To verify that RyR1 mediates the Ca$^{2+}$ release detected with CatchER, we exposed fibers to the channel blocker ryanodine. Nadir SR Ca$^{2+}$ fluorescence decreased with time after applying 5 µM ryanodine extracellularly. Figure 3.1 illustrates CatchER fluorescence in response to a series of 20 mV/100 ms pulses delivered before (a) and after (b, c) applying ryanodine to fibers from young adult mice. The amplitude of CatchER fluorescence 28 and 55 min after applying the drug compared to the amplitude of the signal at time zero was 45 ± 4% and 0.3 ± 0.02%(n=3). During ryanodine application, basal ΔF/F did not change significantly; it was 1.61 ± 0.27 and 1.49 ± 0.28 at times 0 and 55 min, respectively. Since RyR1 is the only RyR isoform expressed in adult skeletal muscle, we conclude that it mediates decreases in SR Ca$^{2+}$ in response to sarcolemmal depolarization [2, 22, 26]. Declines in CatchER fluorescence, in response to the agonist 4-CmC, support this conclusion (see below). The slow time scale of the effect indicates slow diffusion of the agent. The kinetics of ryanodine binding to its receptor may be a contributing factor, as reported [12]. Control experiments in which ryanodine was omitted exhibited a maximal decline of 20 ± 2.3% (n=5) in CatchER’s ΔF/F at the end of 60 min.
Like D1ER [12], CatchER displays a fluorescence signal larger in the presence of 20 mM than in 0.2 mM cytosolic EGTA; despite EGTA’s Ca\textsuperscript{2+} buffer capacity, SR Ca\textsuperscript{2+} recovery is not altered. Figure 3.2a shows the time course of CatchER fluorescence in response to 20 mV/100 ms command pulses recorded in 0.2 (black trace) and 20 mM (red trace) EGTA in fibers from young adult mice. CatchER signal in 0.2 mM EGTA was scaled up to overlap with its recording in 20 mM EGTA to compare their kinetics. High cytosolic EGTA concentration (20 mM)
mM) does not seem to prevent SR Ca\textsuperscript{2+} recovery and elicits average fluorescence signals 2.6 times larger than those recorded in 0.2 mM EGTA, which is statistically significant (Figure 3.2b). Why SR Ca\textsuperscript{2+} depletion is more pronounced with high EGTA concentration is not obvious. Perhaps in the low EGTA solution, Ca\textsuperscript{2+} inactivates RyR1. To examine whether strong cytosolic Ca\textsuperscript{2+} chelation modifies SR Ca\textsuperscript{2+} uptake, we analyzed SR Ca\textsuperscript{2+} recovery 2.5 s after the end of the depolarizing pulse. Figure 3.2c shows that SR Ca\textsuperscript{2+} recovery in 0.2 and 20 mM EGTA did not differ significantly. In addition, 38 fibers recorded with 20 mM EGTA responded to electrical stimulation, while two out of 19 in 0.2 mM EGTA did not. Differences in Ca\textsuperscript{2+} recovery beyond c in the two experimental conditions were not statistically significant. Based on these results, we used a pipette solution containing 20 mM EGTA for the remaining experiments.
Figure 3.2 High cytosolic EGTA concentration increases CatchER transient amplitude but does not affect SR Ca\textsuperscript{2+} recovery. a SR Ca\textsuperscript{2+} transients recorded in electroporated FDB fibers and recorded in whole-cell patch-clamp using 0.2 (black trace) or 20 mM (red trace) EGTA in the pipette solution. b CatchER $\Delta F/F$–[EGTA] relationship. CatchER fluorescence at nadir of the response (b) is significantly larger in 20 than in 0.2 mM EGTA ($p<0.001$, n=11 in 0.2 mM EGTA; n=13 in 20 mM EGTA). c SR Ca\textsuperscript{2+} recovery is expressed as $(F_c-F_b)/(F_a-F_b)$, where $a$ is the average of the basal fluorescence points, and $b$ and $c$ are CatchER fluorescence at nadir and 2 s after repolarization starts, respectively. SR Ca\textsuperscript{2+} recovery is independent of cytosolic [EGTA] ($p>0.05$)
3.4.3 Brief and prolonged SR Ca\(^{2+}\) release tracked with CatchER.

Previously reported Ca\(^{2+}\) biosensors targeted to the SR have been used to track slow changes in luminal SR Ca\(^{2+}\) [1, 12, 28, 32, 33, 42, 44]. We demonstrated that CatchER detected SR Ca\(^{2+}\) in response to a single action potential [44]. Here, we examined whether it could detect a variety of pulses from brief repetitive to a single prolonged stimulation under voltage-clamp. Figure 3.3 shows changes in CatchER’s fluorescence in response to various command pulses to 20 mV. These data indicate that CatchER responds adequately to pulses of different duration (a, 10 ms; e, 100 ms), frequency (b, 10; c, 3.3; d, 1.6; and f, 5 Hz), or duration and frequency (g, 50 ms pulse at 3.3 Hz) and can thus monitor SR Ca\(^{2+}\) dynamics under a variety of physiological conditions. Figure 3.3 shows that SR Ca\(^{2+}\) is incompletely recovered within the recording time, regardless of the pulse applied to the cell. We rule out cell damage as a potential mechanism because all myofibers included in this study maintained membrane electrical properties within 10% of the original values throughout the experiment. Photobleaching partially explains the slow fluorescence recovery. Figure 3.3h shows a typical change in CatchER fluorescence in response to a 30 mV/100 ms pulse (raw trace). Correcting for photobleaching (corrected trace) partially speeds recovery (21 ± 3.2%; n=17 fibers), or perhaps calsequestrin binds Ca\(^{2+}\) with higher affinity than CatchER [10], delaying recovery of the biosensor’s fluorescence. We also cannot rule out the influence of the CatchER: Ca\(^{2+}\) on-rate reaction.
Figure 3.3 CatchER tracks SR Ca\(^{2+}\) release in response to single or repetitive prolonged stimulation. Transient changes in CatchER’s fluorescence response to various 20-mV command pulses: a a 10-ms pulse, b five 10-ms pulses at 10 Hz, c a 10-ms pulse at 3.3 Hz, d a 10-ms pulse at 1.6 Hz, e, a 100-ms pulse, f 100-ms pulse at 5 Hz, and g 50-ms pulse at 3.3 Hz. h CatchER’s fluorescence response to a 30 mV/100 ms pulse (raw trace, black) and after correcting for photobleaching (corrected trace, red).
3.4.4 Voltage-dependent CatchER luminal signal and cytoplasmic Ca\(^{2+}\) transients.

Figure 3.4 shows young adult mouse FDB fibers expressing CatchER loaded with 5 µM Rhod-2-AM and depolarized with 100 (A, B) or 500 (C, D) ms command pulses from Vh0~80mV to 20mV applied in the whole-cell configuration of patch-clamp. Panels A and C correspond to Rhod2 while B and D to CatchER. Top panels (a) show images in line-scan mode with vertical and horizontal axes corresponding to space and time, respectively. Fluorescence increases (A, C) or decreases (B, D) depend on whether cytosolic Rhod-2 or SR CatchER fluorescence was recorded, respectively. The use of 20 mM EGTA in the internal (pipette) solution provided a dual benefit: it prevented Rhod-2 saturation and the movement of artifacts during fiber depolarization. Middle panels (b) display time-dependent changes in the Ca\(^{2+}\) indicator’s fluorescence. The decreased fluorescence signal indicates that Ca\(^{2+}\) dissociates from CatchER upon fiber depolarization. The rate of fluorescence signal rise for CatchER and Rhod-2 is similar, while the cytosolic fluorescence decay is faster than the luminal Ca\(^{2+}\) recovery. The rising phase of the Rhod-2 signal was fitted to a double-exponential function with time constants of 2 ± 0.11 and 139 ± 15 ms and 5 ± 0.31 and 246 ± 39 ms in response to 100 and 500 ms pulses, respectively. The decay phase also shows two time constants of 103 ± 17 and 1,132 ± 168 ms and 163 ± 19 and 1,193 ± 145 (n=7), respectively. The CatchER rising and recovery phases were fitted with a single time constant of 26 ± 3.5 and 19 ± 2.3 ms and 1,315 ± 147 and 2,512 ± 323 (n=12) in response to 100 and 500 ms pulses, respectively. Differences in Ca\(^{2+}\) affinity and Ca\(^{2+}\) buffer capacity of SR and cytoplasm can explain differences in the time course of CatchER and Rhod-2 fluorescence. Figure 3.4c illustrates the Ca\(^{2+}\) currents in real-time to compare the time course of the electrical and optical signals. The time-to-peak (in ms) of the Rhod-2 signal were 98 ± 4 and 113 ± 8, while for CatchER, the values were 91 ± 5.
and 139 ± 23 in response to 100 and 500 ms pulses, respectively. Values are mean ± SEM for 14 fibers expressing CatchER and loaded with Rhod-2. Apparent differences between time-to-peak of Rhod-2 and CatchER signals were not statistically significant.
Figure 3.4 SR and cytosolic Ca\(^{2+}\) transients recorded in the same FDB fibers. Ca\(^{2+}\) transients were recorded in response to a command pulse (20 mV and 100-ms [A, B] or 500-ms [C, D] duration [Vh0−80 mV]). a shows images in confocal line-scan mode with vertical and horizontal axes corresponding to space and time, respectively; b Ca\(^{2+}\) transients' fluorescence profile measured with Rhod-2 (a, c) or CatchER (b, d); c Ca\(^{2+}\) currents. Dashed and dotted lines indicate basal fluorescence before pulse application and isoelectric current, respectively. \(\Delta F/F\) for Ab, Bb, Cb, and Db were 6.65, 0.26, 6.7, and 0.28, respectively. The rising phase of the Rhod-2 signal was fitted to an exponential function with two time constants of 3 and 146 ms and 4 and 246 ms in response to 100 and 500 ms pulses, respectively. The decay phase also shows two time constants of 114 and 1,025 ms and 159 and 1,246, respectively. Time to peak is 98 and 111 ms, respectively. The CatchER rising and recovery phases were fitted with a single time constant of 31 and 23 ms and 1,214 and 2,733 in response to 100 and 500 ms pulses, respectively. Time to peak is 100 and 127 ms, respectively.
3.4.5 CatchER’s fluorescence amplitude increases with the duration of sarcolemmal depolarization.

We recently showed that CatchER’s fluorescence decreases with sarcolemmal depolarization, and the fluorescence–membrane voltage relationship follows a Boltzmann equation in fibers from young and old mice [34]. To further determine whether CatchER responds to SR Ca$^{2+}$ release as a function of pulse duration, we voltage-clamped 12 fibers from young adult mice at −80 mV and stimulated them with 20 mV command pulses of increasing duration (10, 25, 50, and 100 ms; Figure 3.5a, b). As shown in Figure 3.5c, CatchER’s fluorescence elicited by progressively longer pulses showed a graded increase in signal amplitude, reaching a plateau for pulses longer than 100 ms (250 and 500 ms).
Figure 3.5 CatchER’s peak Ca\(^{2+}\) transient depends on pulse duration. A. Various pulse durations (10, 25, 50, and 100 ms) tested in FDB fibers, voltage-clamped at -80 mV (holding potential), and pulsed to 20 mV. The pulse waveform is depicted in the top panel. B. Maximal CatchER transient relationship to pulse duration. Data points represent the mean ± SEM of 12 fibers.
3.4.6 Ca\(^{2+}\) remaining in the SR after prolonged depolarization differs in myofibers from young and old mice.

Figure 3.6a shows SR Ca\(^{2+}\) fluorescence in response to a 20 mV/100 ms pulse as depicted on the top trace in a fiber from a young adult mouse. After ~2 min, the same fiber was exposed to 1 mM 4-CmC for the whole recording period, as indicated on the top bar, to bypass the excitation-SR Ca\(^{2+}\) release process and elicit SR Ca\(^{2+}\) release by acting directly on RyR1 (protocol 1). CatchER fluorescence seems to reach a deeper nadir than the response to the electrical pulse. The ratio between the peak response to electrical stimulation (b) and 4-CmC (c) was 0.44 ± 0.09 (n=6). To investigate whether a remaining Ca\(^{2+}\) pool persists in the SR after 4-CmC application, electrical stimulation was prolonged to 500 ms, and 15 µM cyclopiazonic acid were added with 1 mM 4-CmC to block SERCA-mediated SR Ca\(^{2+}\) uptake in addition to agonist-dependent SR Ca\(^{2+}\) release (protocol 2). Increased pulse duration and the combination of the two pharmacological agents resulted in a similar SR Ca\(^{2+}\) depletion (data not shown). Similarly, Figure 3.6b shows SR Ca\(^{2+}\) fluorescence in response to a 20 mV/100 ms pulse in a fiber from an old mouse. While CatchER’s response to the electrical pulse is smaller, its response to 4-CmC does not differ significantly from that obtained in young fibers [34]. The ratio between the nadir in response to electrical stimulation (b) and 4-CmC (c) was 0.21 ± 0.07 (n=7). Figure 3.6c shows the response of myofibers from young (left) and old (right) mice to a 2 s/20 mV train of 3 ms pulses at 150 Hz. The b/c ratio was 0.41±0.08 (n=15 fibers) and 0.24 ± 0.06 (n=17 fibers), respectively. These experiments indicate that a Ca\(^{2+}\) pool remains in the SR lumen after myofiber depolarization through prolonged activation of the excitation-SR Ca\(^{2+}\) release mechanism, consistent with declining voltage-gated SR Ca\(^{2+}\) release with aging [11]. To further examine the residual luminal Ca\(^{2+}\) in the SR after prolonged Ca\(^{2+}\)
release, we applied an alternative technique. After SR Ca\(^{2+}\) release was measured FDB fibers from young and old mice expressing CatchER were exposed to 0.01% saponin for 2 min in permeabilization solution (see “Materials and methods”). The permeabilized myofiber was exposed to 10\(^{-6}\) M ionomycin diluted in the previous solution to equilibrate among the extracellular space, cytosol, and lumen of the SR. Free [Mg\(^{2+}\)] was set at 1 mM, and free [Ca\(^{2+}\)] was set at 10\(^{-7}\) mM with BAPTA. Although apparently more stringent, this technique did not promote a significant difference in residual Ca\(^{2+}\) compared to the 4-CmC protocols. The Ca\(^{2+}\) concentration remaining in the SR after Ca\(^{2+}\) release in response to prolonged repetitive electrical stimulation (2 s) was (in µM): 132 ± 29 and 220 ± 37 in myofibers from young (n=9 fibers) and old (n=7 fibers) mice, respectively. As resting SR Ca\(^{2+}\) concentration did not differ in myofibers from young and old mice [34], our data are consistent with a larger SR luminal Ca\(^{2+}\) depletion in fibers from young mice or an impaired voltage-gated SR Ca\(^{2+}\) release in aging mice [11].
Figure 3.6 Maximal SR Ca\(^{2+}\) depletion evoked by 4-CmC. a CatchER fluorescence transient (bottom trace) in response to 20 mV/100 ms command pulse (top trace) in a voltage-clamped myofiber from a young adult mouse. The same fiber was exposed to 1 mM 4-CmC with ~2-min interval. The dashed line indicates the basal fluorescence (a), while the nadir of the response to electrical stimulation or 4-CmC application is shown in (b) and (c), respectively. b CatchER fluorescence transient in response to a 20 mV/100 ms command pulse in a voltage-clamped myofiber from an old mouse. The same fiber was exposed to 1 mM 4-CmC at ~2-min intervals. c CatchER fluorescence (bottom) in response to repetitive stimulation with 2-ms pulses at 150 Hz for 2 s at 20 mV (top) in myofibers from young (left) and old (right) mice.
3.5 Discussion

The main findings of this work are: (1) CatchER can track changes in SR Ca\(^{2+}\) lumen in response to pulses varying in amplitude, duration, and frequency; (2) photobleaching partially accounts for CatchER’s slow fluorescence recovery; (3) SR Ca\(^{2+}\) release monitored with CatchER is solely mediated by RyR1; (4) SR luminal Ca\(^{2+}\) release measured with CatchER mirrors the cytosolic Ca\(^{2+}\) transient, measured with Rhod-2; and (5) the fraction of residual SR Ca\(^{2+}\) is larger in myofibers from old than young mice, supporting excitation–contraction uncoupling in senescent myofibers.

3.5.1 CatchER is a low-affinity Ca\(^{2+}\) biosensor suitable for measuring luminal SR Ca\(^{2+}\).

CatchER allowed us to monitor changes in Ca\(^{2+}\) concentration dynamics in a practically unexplored environment--skeletal muscle SR. We recently described the structure and optical properties of these genetically encoded Ca\(^{2+}\) indicators (GECI) and the mechanism of its response to Ca\(^{2+}\) [34]. Previous attempts to measure SR Ca\(^{2+}\) in amphibian and mammalian species have been discussed elsewhere [12]. Although CatchER’s single-excitation/single-emission optical spectrum is limited compared to the single-excitation/ dual emission D1ER [12, 18, 28], it has a number of advantages, including responsiveness, reproducibility of the signal, and low Ca\(^{2+}\) affinity. These properties allowed us to measure high SR luminal Ca\(^{2+}\) concentrations and their dynamics in a considerable number of myofibers after FDB muscle in vivo electroporation. Difficulties in reliably calibrating D1ER, the most commonly used biosensor for Ca\(^{2+}\) analysis in the muscle, have been reported [12, 28]; however, its ratiometric signal recorded in muscle fiber provided useful information about relative SR Ca\(^{2+}\) changes [12]. Relative changes in citrine/CFP ratio, rather than SR Ca\(^{2+}\) concentrations, have been reported using D1ER in arterial smooth muscle [42], HEK-293 cells expressing RyR2 [13], and vascular
endothelial cells [21]. Here, we were able to quantify residual SR Ca$^{2+}$ concentration after SR Ca$^{2+}$ released in response to fiber electrical and pharmacological stimulation in young and old mice, which was impractical with previous GECI, representing a net improvement over previous approaches. Also, CatchER expression by electroporation and recording without previous prolonged loading procedures make it a promising tool to investigate the role of SR Ca$^{2+}$ movements under a variety of physiological and disease conditions.

3.5.2 The concentration of residual SR Ca$^{2+}$ after Ca$^{2+}$ release in skeletal myofibers from senescent mice is unknown.

A series of reports estimated SR Ca$^{2+}$ content and depletion using cytosolic synthetic Ca$^{2+}$ indicators in young rodents. In voltage-controlled SR Ca$^{2+}$ release in mouse FDB fibers, a 100-ms voltage pulse that maximally activates Ca$^{2+}$ release, according to the authors, reduces the initial SR content about 80% [37]. Note that they estimated an SR Ca$^{2+}$ content of 3 mM, consistent with previous reports [8] [7, 17, 20, 24, 30]. However, more recent works using engineered Ca$^{2+}$ biosensors or Fluo-5N targeted to the SR showed values in the micromolar range [28, 34, 43]. We have shown that SR Ca$^{2+}$ depletion is unlikely, even in response to prolonged depolarization using the biosensor D1ER, which was later confirmed using D4cpv [19, 32]. Using CatchER, we recorded a resting SR Ca$^{2+}$ concentration of 512 and 573 µM in myofibers from young and old mice, respectively[34]. Some studies have reported an SR Ca$^{2+}$ concentration of ~1 mM in frog muscle fibers [31, 38], while a three- to fourfold lower value (~308 µM) was noted using the Ca$^{2+}$ biosensor D1ER [28]. We found higher basal SR Ca$^{2+}$ concentration than that reported in mouse tibialis anterior muscle [28]. This difference may be explained by difficulties in measuring D1ER’s R$_{max}$ [28] or saturation due to multiple binding sites, as its reported dissociation constant is ~60 µM [18]. Indirect observations suggest that
SR Ca\(^{2+}\) depletion does not limit its release in muscle fibers from aging mice \[^{11}\]; however, no direct measure of residual Ca\(^{2+}\) content after prolonged depolarization was reported. That SR Ca\(^{2+}\) release decreases with aging would account for increased residual SR Ca\(^{2+}\). Measurements of the residual [Ca\(^{2+}\)]/ resting [Ca\(^{2+}\)] relationship required the development of a low-affinity biosensor targeted to the SR. Calibrations in FDB fiber showed that CatchER’s K\(_d\) is 1.66 mM, which make it an excellent instrument to address this question. Recently, SR Ca\(^{2+}\) concentration, fractional Ca\(^{2+}\) release, and resting SR Ca\(^{2+}\) concentration were recorded in FDB fibers from young adult mice using the synthetic Ca\(^{2+}\) indicator Fluo-5N \[^{43}\]. In contrast with the results reported here, the authors reported 88% SR Ca\(^{2+}\) depletion in response to 10 s/50 Hz tetanic stimulation. Although they used long pulses, the reported level of depletion is larger than that recorded with D1ER or CatchER; luminal Ca\(^{2+}\) depletion seems to plateau when measured with these biosensors, possibly due to an equilibrium in SR Ca\(^{2+}\) release and uptake. The Fluo-5N SR-loading method allowed the first quantitative estimates of static content and dynamic changes in SR Ca\(^{2+}\) concentration, but the prolonged dye-loading process limits its use in physiological recordings. We demonstrated previously that resting SR Ca\(^{2+}\) concentration does not differ in fibers from young and old mice; however, SR residual Ca\(^{2+}\) was not quantified in old mice. Here, for the first time, we report that residual Ca\(^{2+}\) increases with aging, which fully supports our theory that SR Ca\(^{2+}\) availability does not limit SR Ca\(^{2+}\) release.

3.5.3 Residual SR Ca\(^{2+}\) after prolonged myofiber depolarization.

We demonstrated previously using confocal microscopy that CatchER expresses in the myofiber SR \[^{34}\]. Here, we show that blocking RyR1 results in complete ablation of CatchER’s fluorescence signal. We rule out the possibility that CatchER is located in the cytosol because,
in addition to the morphological evidence, basal fluorescence is intense, which indicates that the biosensor is exposed to high $\text{Ca}^{2+}$ concentration. Thus, exposed to high $\text{Ca}^{2+}$ concentrations, CatchER signal decreases in response to sarcolemmal depolarization. In contrast, the cytosolic compartment of FDB fibers is strongly buffered, and basal free $\text{Ca}^{2+}$ is below 100 nM [12], preventing significant CatchER fluorescence under resting conditions. Whether SR $\text{Ca}^{2+}$ recovery in response to fiber excitation fully represents SR $\text{Ca}^{2+}$ uptake from the cytosol, or the signal is influenced by $\text{Ca}^{2+}$ redistribution between bound and unbound conformations of the biosensor, calsequestrin’s buffer capacity, or $\text{Ca}^{2+}$ diffusion from the longitudinal to the terminal cisternae SR is not known. CatchER measurements in a calsequestrin knockout mouse model together with a more refined optical detector, such as a two-photon or a spot scanning confocal microscope [5], in stretched muscle fibers might answer these questions. Previous studies assessed SR $\text{Ca}^{2+}$ in amphibian and mammalian skeletal and cardiac muscle SR [12, 14, 23, 28, 29]. The use of cameleon $\text{Ca}^{2+}$ sensors represented an advance over previous methods. First, they can be selectively targeted to intracellular organelles in intact cells or tissues in vivo or in vitro. Second, their ratiometric property minimizes confounding factors, such as mechanical artifacts and probe concentration. In this work, we found a significant residual SR $\text{Ca}^{2+}$ after prolonged myofiber depolarization, and we recorded a larger SR $\text{Ca}^{2+}$ depletion in myofibers from young compared to old mice. Instead of single pulse, we used a more physiological repetitive stimulation. A train of pulses was sustained until CatchER’s signal reached a plateau. Under these experimental conditions, a more significant residual $\text{Ca}^{2+}$ was recorded in the SR from old mice. Residual SR $\text{Ca}^{2+}$ was quantified based on converting CatchER’s signal into $\text{Ca}^{2+}$ concentration according to published methods [34, 43]. These experiments support the conclusion that CatchER is suitable for measuring $\text{Ca}^{2+}$ in a high concentration cellular envi-
ronment; that residual SR Ca\(^{2+}\) concentration is significant beyond physiological demands; and that it is higher in myofibers from old compared to young adult mice. CatchER’s relatively low affinity measured in mouse FDB myofibers [34] makes it appropriate for detecting SR Ca\(^{2+}\) concentration and dynamics. The myofiber sustains SR release in response to repetitive stimulation probably due to efficient SR Ca\(^{2+}\) pump-mediated Ca\(^{2+}\) uptake. Differences in residual SR Ca\(^{2+}\) in response to sarcolemmal depolarization in myofibers from young and old mice reflect excitation–contraction uncoupling, the molecular substrate of which is the age-dependent decline in Cav1.1 expression and a larger number of uncoupled RyR1 [25, 40]. We propose that releasing a larger residual Ca\(^{2+}\) pool in myofibers by increasing Cav1.1 expression will improve myofiber-specific force and power with aging.
3.5.4 *In situ determination of kinetic parameters of CatchER.*

**Figure 3.7** The kinetic parameters of CatchER measured in mouse flexor digitorum brevis (FDB) fibers. (A) SR fluorescence transients elicited by -10 mV, 0 mV, 10 mV and 20 mV command pulses, respectively, in FDB fibers under patch-clamp. (B) Observed rates of dissociation at various voltages in FDB fitted with single exponential equation. (C) Observed rates of association at various voltages in FDB fitted with single exponential equation.
The $k_{\text{obs-d}}$ and $k_{\text{obs-a}}$ values of CatchER in live cells were fitted from the results of voltage induced SR calcium release in adult skeletal muscle FDB. The command pulses at various voltages ranging from -10 mV to 20 mV were under a patch-clamp. All the observed rates were fitted with a single exponential equation. The average $k_{\text{obs}}$ value measured with stopped-flow during the Ca$^{2+}$ association experiments was 63 s$^{-1}$ (Chapter 2, Table 2.1-2.3).
The observed rate of dissociation and observed rate of association in cells are limited by the activation rate of the ryanodine receptors and SERCA pump respectively, which are not exactly the same as the $K_{obs}$ values measured by stopped-flow techniques.

The SERCA pump activation and kinetics in skeletal muscle will be discussed in the next section.

### 3.5.5 Expected $Ca^{2+}$ association rate constant to CatchER in situ based on the activity of SERCA pumps.

![Mechanism of Ca$^{2+}$ uptake to ER/SR by SERCA pump (Ca$^{2+}$-ATPase).](image)

$E_1$, enzyme form with cytoplasmically facing high affinity Ca$^{2+}$ sites; $E_2$, enzyme form with low affinity for Ca$^{2+}$; $E_1$-$P$(Ca$^2_2$), phosphoenzyme with high energy phosphoryl group (transferable to ADP) and occluded calcium ions (shown in parentheses); $E_2$-$P$, phosphoenzyme with low energy phosphoryl group and lumenally facing low affinity Ca$^{2+}$ sites. Adopted from Dode, L. et. al. 2003, JBC.
The Ca\(^{2+}\) refill to the SR is mainly rate-controlled by the SERCA pump (Ca\(^{2+}\)-ATPase) activation. SERCA pumps have several isoforms, and each isoform will experience a multiple-step conformational change during the SR calcium refill.

Skeletal muscle FDB contains more than 90% fast twitch oxidative-glycolytic fibers\(^{(123)}\), and both SERCA1a and SERCA2a expressed in fast-twitch fibers (EDL). From the results of Western blots, the amount of SERCA1a (46 ± 4.3) was higher than SERCA2a (0.51 ± 0.07)\(^{(124)}\). Since the SERCA pumps function as the major pump of SR Ca\(^{2+}\) entry, the activity and kinetics of SERCA1a and SERCA2a will determine the Ca\(^{2+}\) refill rate to the SR.

The ATP-driven Ca\(^{2+}\) uptake rate of SERCA1a and SERCA2a were reported to increase in the function of resting Ca\(^{2+}\) concentration ranging from 0.01-10 \(\mu\)M\(^{(2)}\)
The maximum turnover rates of Ca\(^{2+}\) uptake activity were 18 s\(^{-1}\) and 20 s\(^{-1}\), respectively (2), for SERCA1a and SERCA2a, suggesting that the Ca\(^{2+}\) uptake rate measured by ER/SR Ca\(^{2+}\) indicators should have not exceeded these values. Moreover, these values were measured at high Ca\(^{2+}\) concentration (10 \(\mu\)M); however, cytosolic Ca\(^{2+}\) concentration was rarely higher than 1 \(\mu\)M, even during high voltage stimulation. In the resting state ([Ca\(^{2+}\)]\(_{c}\)= 100 nM), the Ca\(^{2+}\) transport activity was around 3 s\(^{-1}\) for SERCA1a and 5 s\(^{-1}\) for SERCA2a, which agreed with the measured \(k_{obs-a}\) (around 0.6~1.2 s\(^{-1}\)) by CatchER.

Figure 3.10 Ca\(^{2+}\)-dependence of the rate of ATP-driven Ca\(^{2+}\) uptake activity. Ca\(^{2+}\)-uptake was measured at 27 °C after 10 min incubation in the medium, in the presence of 1 mM EGTA and various concentration of \(^{45}\)CaCl\(_2\) to produce the indicated free Ca2+ concentrations. The molecular Ca\(^{2+}\) transport activity was calculated as the ratio between the rate of Ca\(^{2+}\)-uptake/mg protein (\(\mu\)moles Ca\(^{2+}\) transported per second per mg microsomal protein) and the active site concentration determined by phosphorylation with \(^{32}\)P-ATP at 0 °C (\(\mu\)moles enzyme per mg microsomal protein). The lines show the best fits of the Hill equation to the data. Adopted from (2).
### 3.6 References


CHAPTER 4: STRUCTURAL ANALYSIS AND DETERMINATION OF DESIGNED INDICATOR CATCHER BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR).

4.1 Introductions

Nuclear magnetic resonance (NMR) spectroscopy is an advanced tool for studying molecular dynamics, protein ligand interaction, and relaxation properties of protein in solution. Experiments such as heteronuclear single quantum coherence (HSQC) correlates the nitrogen atom of an NHx group with the directly attached proton. Each signal in a HSQC spectrum represents a proton that is bound to a nitrogen atom. The spectrum contains the signals of the HN protons in the protein backbone. Since there is only one backbone HN per amino acid, each HSQC signal represents one single amino acid. The HSQC also contains signals from the NH2 groups of the side chains of Asn and Gln and of the aromatic HN protons of Trp and His. A HSQC has no diagonal like a homonuclear spectrum because different nuclei are observed during T1 and T2. (Horst Joachim Schirra's PPS2 project)

There are two main ways to study the self-diffusion coefficients, one is the tracer-diffusion or intradiffusion coefficients and the other is pulsed-field gradient (PFG) NMR. (Figure 4.1) However, these two methods report molecular motion in different time scales. The relaxation method is sensitive to rotational diffusion, whereas the PFG method measures translational diffusion. In a typical solution phase experiment, the relaxation measures the motion occurring within picosecond to nanosecond time scale, which is the motion time scale of the re-orientational correlation of the nucleus. In PFG diffusion measurements, on the other hand, motion is measured over millisecond to second time scale.

The first NMR study of GFP was reported in 2002 with 19F labeled tryptophan of W66 of the chromophore and W57 of CFP without complete assignment of residues. It reported to es-
tablish an NMR structural analysis method with special labeling of the CFP chromophore using \textsuperscript{19}F labeling and to investigate the unrevealed structural basis of photoisomerization, photo-rotation, and complex on-off blinking and switching behavior which cannot be explained by the three state model of the chromophore proposed by the X-ray crystal structure study. As the chromophore is deeply buried inside the GFP beta barrel without solvent accessibility, its assignment by \textsuperscript{1}H-\textsuperscript{13}C-\textsuperscript{15}N methods was extremely difficult. To overcome this major barrier, \textsuperscript{19}F was used to label the Trp residue within the chromophore of CFP, so that the chromophore could be observed by distinct \textsuperscript{19}F spectra.

Instead of using \textsuperscript{19}F, other labeling methods such as \textsuperscript{1}H-\textsuperscript{13}C-\textsuperscript{15}N can also be used to isotopically label special residues to reveal the chromophore structure of GFP variants, which was reported more recently.

The diffusion constants D of a molecule can be calculated using the correlation time (\(\tau_c\)) obtained from the relaxation results by using the Debye equation:

\[
\tau_c = \frac{4\pi \eta r_s^3}{(3kT)}
\]

And Stoke-Einstein equation:

\[
D = \frac{kT}{f}
\]

where D is the diffusion constant, k is the Boltzmann constant, T is the temperature, and f is the friction coefficient.

In the PFG method, the attenuation of the spin-echo signal resulting from the dephasing of the nuclear spins due to the combination of the translational motion of the spins and the imposition of spatially well-defined gradient pulses is used to measure motion. The translational
motion of well-packed spherical-like molecules in solution has a direct correlation with the hydrodynamic radius and a molecular size according to the equation:

\[ D = kT / (6\pi\eta r_s) \]

where T is the temperature, \( \eta \) is the solvent viscosity, and D is the diffusion constant. At a given temperature and viscosity, the diffusion constant decreases as the radius increases.

Figure 4.1 Scheme of the relaxation and pulse-field gradient methods for determining molecular dynamics. In this representation of the relaxation method, the probe molecule is a sphere with an effective hydrodynamic radius.
The relaxation of NMR and magnetic resonance imaging (MRI) describe the multiple processes by which the nuclear magnetization switches from a non-equilibrium state to an equilibrium distribution. It can be measured by both spectroscopic and imaging methods.

The longitudinal (or spin-lattice) relaxation time T1 is the decay constant for the recovery of the z component of the nuclear spin magnetization, $M_z$, towards its thermal equilibrium value based on the following equation,

$$M_z(t) = M_{z,eq} - [M_{z,eq} - M_z(0)]e^{-t/T_1}$$

If M has been tilted into the xy plane, then $M_z(0) = 0$ and the recovery is simply,

$$M_z(t) = M_{z,eq}(1 - e^{-t/T_1})$$

The T1 values are usually measured by an inversion recovery experiment. The initial magnetization is inverted such that $M_z(0) = -M_z$ using the following equation.

$$M_z(t) = M_{z,eq}(1 - 2e^{-t/T_1})$$

The transverse (or spin-spin) relaxation time T2 is the decay constant for the component of M perpendicular to $B_0$, designated $M_{xy}$, MT.

$$M_{xy}(t) = M_{xy}(0)e^{-t/T_2}$$

The rotational correlation time ($\tau_c$) represents the time of rotation by one radian featured by a particle in solution exhibiting Brownian rotation diffusion. It is also dependent on the molecular size of the particle. For a globular protein, $\tau_c$ can be estimated by Stoke’s law.

The hydrodynamic radius can be estimated from the molecular weight of the protein (M) with the equation below:
\[ r \approx 3 \sqrt{\frac{3M}{4\pi \rho N_a}} + r_w \]

where \( \rho \) is the average density of the protein (1.37 g/cm\(^3\)), \( N_a \) is the Avogadro’s number and \( r_w \) is the hydration radius (1.6 to 3.2 Å). (citation: Cavanagh J., Fairbrother W.J., Palmer, A.G, Rance M., Skelton N.J. (2007) Protein NMR Spectroscopy: Principles and Practice, p21, Elsevier) The \( \tau_c \) value (in ns) of a protein is heuristically estimated approximately 0.6 times its molecular weight in kDa. The molecular weight of single EGFP is 27 kDa, so the \( \tau_c \) of EGFP mutants is roughly estimated to be 16 ns.

For rigid proteins having slow molecular motion (\( \tau_c >> 0.5 \) ns) in a high magnetic field (≥500 MHz) and molecular weight up to 25 kDa, a correlation exists between the \( \tau_c \) value, \(^{15}\)N \( T_1 \) (longitudinal relaxation), and \(^{15}\)N \( T_2 \) (transverse relaxation) which is expressed in the following equation,

\[ \tau_c \approx \frac{1}{4\pi \nu_N} \sqrt{\frac{T_1}{T_2} - 7} \]

where \( \nu_N \) is the \(^{15}\)N resonance frequency (in Hz). This equation is derived from Eq. 8 of (citation: L E Kay, D A Torchia, A Bax Backbone dynamics of proteins as studied by 15N inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease. Biochemistry: 1989, 28(23);8972-9) by considering only J(0) and J(wN) spectral density terms and neglecting higher frequency terms.

The \(^{15}\)N \( T_1 \), and \( T_2 \) relaxation times for a given protein can be measured by each residue, or directly getting an average by using 1D \(^{15}\)N-edited relaxation experiments (N A Farrow, R Muhandiram, A U Singer, S M Pascal, C M Kay, G Gish, S E Shoelson, T Pawson, J D For-
man-Kay, L E Kay. Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by $^{15}$N NMR relaxation. Biochemistry: 1994, 33(19);5984-6003). The 1D $^{15}$N spectra of a given protein is collected at the downfield backbone amide $^1$H region (10.5 to 8.5 ppm) to eliminate the contribution of unfolded regions of the protein. For CatchER, $T_1$ and $T_2$ was calculated for $\tau_c$ only from the residues in the beta-sheet, excluding the residues in flexible loops without well-defined secondary structure. The accuracy of the measured $T_2$ for large proteins decreases.

In this chapter, we will report our progress in 1D, 2D and 3D structural determination and analysis of CatchER and identifying key residues involved in conformational changes of CatchER induced by Ca$^{2+}$ binding by using heteronuclear expressed protein. In addition, we have also successfully investigated the $T_1$, $T_2$, NOE and $T_C$ value of monomeric CatchER in comparison to reported relaxation values of dimeric GFP.

4.3 Methods

$^{2}$H-$^{13}$C-$^{15}$N triple labeled protein CatchER expression in 100 mL SV media. One single E. coli BL21(DE3) colony was picked up and placed into 10 mL LB media with 6 $\mu$L Kanamycin. The cell culture was incubated at 37°C in a shaker for 8 hours. From the cell culture, 0.5 mL was transferred to 10 mL SV medium (4 mL D$_2$O (99.9%), 6 mL ddH$_2$O, 0.05 g $^{13}$C labeled glucose, 0.005 g $^{15}$N labeled NH$_4$Cl, 0.0794 g K$_2$HPO$_4$, 0.044 g KH$_2$PO$_4$, 0.0005 g MgSO$_4$$\cdot$7H$_2$O, 0.00007 g (NH$_4$)$_2$Fe(SO$_4$)$_2$$\cdot$6H$_2$O). In a 15 mL falcon tube labeled A, 0.05 g of $^{13}$C Glucose and 0.005 g of $^{15}$N NH$_4$Cl was dissolved into 4 mL of D$_2$O (99.9%). The mixture was filtered with a 0.2 $\mu$m HT Tuffryn® membrane into a 50 mL falcon tube labeled B. In flask c, 0.794 g of K$_2$HPO$_4$ 0.794 g and 0.44 g of KH$_2$PO$_4$ was dissolved into 60 mL of ddH$_2$O. In flask D, 0.5 g of MgSO$_4$$\cdot$7H$_2$O and 0.07 g of (NH$_4$)$_2$Fe(SO$_4$)$_2$$\cdot$6H$_2$O 0.07 g was dissolved into 20 mL
ddH₂O. After the components in flask C and D were completely dissolved, 0.2 mL of the mixture was transferred from flask D to C. Flask C was autoclaved and allowed to cool to room temperature. After cooling, 6 mL of the mixture was transferred to tube B containing the triple labeled chemicals. The 10 mL pre-culture was transferred to 100 mL SV medium (75 mL D₂O (99.9%), 25 mL ddH₂O, 0.5 g ¹³C labeled glucose, 0.05 g ¹⁵N labeled NH₄Cl, 0.794 g K₂HPO₄, 0.44 g KH₂PO₄, 0.005 g MgSO₄·7H₂O, 0.0007 g (NH₄)₂Fe(SO₄)₂·6H₂O). In a 500 mL flask labeled E, 0.794 g of K₂HPO₄ and 0.44 g of KH₂PO₄ was dissolved into 25 mL of ddH₂O. From the mixture in flask E, 0.2 mL was transferred to flask D. Flask E was autoclaved and allowed to cool to room temperature. In beaker F, 0.5 g of ¹³C Glucose and 0.05 g of ¹⁵NH₄Cl was dissolved in 25 mL of D₂O (99.9%) and filtered, as previously stated, into flask E. After adding 60 µl of Kanamycin to flask E, it was stored for protein expression on the second day. Tube B was centrifuged at 2700 rpm for 10 minutes decanting the supernatant out. Five milliliters of SV media from flask E was poured into tube B and shaken by hand to re-suspend the cell pellet. The re-suspended cell culture was poured into flask E and incubated in the 37°C shaker until the O.D. reached 0.6 to 0.7. Then, 20 µl of IPTG was added to flask E decreasing the temperature to 25°C for overnight expression.

Diffusion NMR experiments with PG_SLED pulse sequence. The CatchER protein concentration was 0.3 mM in 10 mM Tris pH 7.4. A standard sample of lysozyme was also prepared in the same conditions. A spectral width of 6600 Hz was used for the 500 MHz NMR spectrometer with the probe temperature at 25°C. The diffusion constants were calculated with the modified Stokes-Einstein equation as shown with two terms: with both monomer and dimer existing in solution and the original equation with one term for homogenous diffusion. In the equation, A is the integrated area of desired resonances at each array spectrum after sub-
straction of baseline; and $A_0$ is the integrated area of the desired resonances when the PFG strength is zero. The internal reference was lysozyme, and the unknown diffusion constant of CatchER was converted by the equation with that of lysozyme. In the equation, $C$ and $C_0$ are the combined constants of the molecule of interest and internal standard, respectively. The data were processed by NMR spectra using Felix98. The $A$ values were measured by integrating NMR signals of all the resonances in the identical regions at each 1D spectrum with the subtraction of the baseline. The diffusion constant $D$ was calculated by fitting $A$ as a function of gradient strength using equation. The baseline was corrected using a zero-order polynomial.

The terms of the equation are as follows:

$$A = A_0 \exp[-(\gamma \delta G)^2(\Delta - \delta / 3)D]$$

$$A = A_0 \exp(-CG^2)$$

$$D = D_0 C / C_0$$

$$A = A_{01} \exp(-C_1 G^2) + A_{02} \exp(-C_2 G^2)$$

$A_{01} = 0.75$

$A_{02} = 0.25$

$C_1 = 0.0014$

$C_2 = 0.015$

$r_{bys} \times C_{bys} = r_1 \times C_1$

$r_{bys} = 20.1$

$C_{bys} = 0.0024$

$r_1 = 34.5$

All NMR experiments were performed at 37°C using a Varian 800 or 600 MHz spectrometer. Typically, NMR samples contained 0.3 mM $^{15}$N- or $^{13}$C, $^{15}$N-labeled protein in 10 mM
Tris, 10 mM KCl, 10% D₂O, pH 7.4. Data were processed using NMRpipe (125) and analyzed with the program Sparky. For backbone assignment of ¹H, ¹³C, and ¹⁵N resonances, a HNCA spectrum was collected on a Varian Inova 800 MHz spectrometer, and a CBCA(CO)NH spectrum was collected on a Varian Inova 600 MHz spectrometer, both equipped with a cryogenic probe. For Ca²⁺ titration, {¹H, ¹⁵N} HSQC spectra were collected, and chemical shift perturbations were calculated using the equation $D_{\text{av}} = \{0.5[Dd(¹H)^2] + (0.2 Dd(¹⁵N))^2]\}^{1/2}$, where $\Delta \delta$ is the change in chemical shift between the apo and Ca²⁺-loaded form. Rotational correlation time ($\tau_c$) was measured using a shared, constant-time, cross-correlated relaxation (SCT-CCR) pulse sequence developed by Dr. Prestegard’s laboratory (126). In this measurement, a series of highly sensitive HSQC spectra were collected at relaxational acquisition times from 0 to ~100 ms. Residue-specific $\tau_c$ values were then extracted from the exponential decay rates(127). T₁ and T₂ were collected on a Varian Inova 600 MHz spectrometer. Integrations of peak collected at 0, 30, 60, 100, 240, 480, 720, 1000, and 1500 ms (T₁) and 10, 30, 50, 70, 90, 110, 130, and 150 ms (T₂) were fitted with $I = I_0 \exp(-t/T_{1/2})$, where $I_0$ is the intensity at zero decay, and t, the relaxation decay. $\tau_c$ values were calculated following the equation below:

$$\tau_c = (2\omega_N)^{-1} \cdot \sqrt{(6T_1/T_2 - 7)}$$

$$\omega_N = 2\pi \cdot f_N$$

4.4 Results

4.4.1 1D NMR Ca²⁺ titration of CatchER

Prior to the 2D HSQC calcium titration, a 1D NMR calcium titration started from 0.1 mM EGTA, 100 mM KCl up to 4.0 mM Ca²⁺ to roughly detect chemical shifts. Besides the
sidechains being dispersed around 0 to 6 ppm, the major chemical shifts of the NH groups were approximately 6.6 to 7.8 ppm which was later proved to be from sidechain NH groups. The region of 8 to 11 ppm did not exhibit obvious shifts due to the huge number of peaks overlapping together.

Figure 4.2 Stacked 1D NMR spectra of Ca\textsuperscript{2+} titration of CatchER, from 6.3 to 8.7 ppm of proton dimension. 100 µM CatchER was dissolved in 10 mM Tris, pH 7.4 with 10% D\textsubscript{2}O (vol/vol). The spectra was recorded in a 500 MHz NMR spectroscopy, in the presence of 0.1 mM EGTA, 0.1mM EGTA+100 mM KCl, 0.1 mM EGTA+100 mM KCl+1.0 mM Ca\textsuperscript{2+} (final concentration), 0.1 mM EGTA+100 mM KCl+2.0 mM Ca\textsuperscript{2+}, 0.1 mM EGTA+100 mM KCl+4.0 mM Ca\textsuperscript{2+}. 

![NMR spectra](image-url)
NMR 1D spectra show that in the presence of 100 mM KCl, Ca\(^{2+}\) can still trigger a conformational change in CatchER. The intensity of one peak around 6.4 ppm proton dimension increased after adding Ca\(^{2+}\) but was unchanged between apo protein and in the presence of 100 mM KCl (far right red arrow). Moreover, a similar intensity was observed for another peak around 7.0 ppm (the second left red arrow). By comparing peak intensities, several regions showed that the peak intensities were decreased, such as the multiple peaks around 7.7 ppm (far left arrow) and 6.8 ppm (the third right red arrow). This 1D NMR experiment demonstrated that CatchER has been well folded, and Ca\(^{2+}\) can trigger its conformational change in the presence of 100 mM KCl which correlates to the Ca\(^{2+}\) induced fluorescence intensity change detected by fluorometric analysis and provides the explanation of the structural basis of this phenomenon.

The full 1D NMR spectra of CatchER was also analyzed, and no obvious global conformational change was observed in comparison of the 0.1 mM EGTA and 1 mM Ca\(^{2+}\) spectra (Figure 4.1). The finger print regions of methyl groups below 0.0 ppm to 2.0 ppm can be well overlapped, and the aromatic ring region beyond 10.0 ppm is similar in both spectra, suggesting that Ca\(^{2+}\) cannot trigger a major global conformation change of CatchER. Comparing the specific chemical shift changes observed between 6-8 ppm, we can conclude that submillimolar to millimolar Ca\(^{2+}\) interacts with CatchER at a particular location due to the local conformational changes measured by 1D NMR, but no significant global conformational changes were observed. We assume that these local conformational changes provide the structural basis of the fluorescence enhancement during the fluorescence titration.
Figure 4.2 Stacked 1D NMR spectra of Ca$^{2+}$ titration of CatchER, from -2.0 to 12.0 ppm of proton dimension. 100 µM CatchER was dissolved in 10 mM Tris, pH 7.4 with 10% D$_2$O (vol/vol). The spectra was recorded in a 500 MHz NMR spectroscopy, in the presence of 0.1 mM EGTA, 0.1 mM EGTA+ 100 mM KCl+1.0 mM Ca$^{2+}$, respectively.
4.4.2 Diffusion NMR by pulse-field-gradient (PFG)

Figure 4.3 Stacked plot of 1D NMR spectra of CatchER from -1.0 ppm to 10.0 ppm in proton dimension with the increased pulse field gradient (PFG) strength (arrow).
NMR spectroscopy experiments were conducted to demonstrate that the fluorescence intensity changes come from calcium binding to our biosensor. Because EGFP variants have a strong tendency to dimerize which might influence the quality of HSQC spectra, we first applied pulsed-field-gradient NMR spectroscopy to explore the size of our designed sensor. The diffusion experiment was conducted at 25°C using a 600 MHz NMR spectrometer with 0.3 mM protein sample in 10 mM Tris buffer, pH 7.4. The diffusion experiment was carried at 25 °C.

Figure 4.4 The NMR intensity of CatchER decreased according to the increment of PFG strength (empty circle). The solid line is fitted with the equation X. The protein concentration was 0.3 mM, dissolved in 10 mM Tris, pH 7.4. The diffusion experiment was carried at 25 °C.

NMR spectroscopy experiments were conducted to demonstrate that the fluorescence intensity changes come from calcium binding to our biosensor. Because EGFP variants have a strong tendency to dimerize which might influence the quality of HSQC spectra, we first applied pulsed-field-gradient NMR spectroscopy to explore the size of our designed sensor. The diffusion experiment was conducted at 25°C using a 600 MHz NMR spectrometer with 0.3 mM protein sample in 10 mM Tris buffer, pH 7.4. The diffusion constants were calculated with the modified Stokes-Einstein equation with two terms. From the curve fitting data, there were two components of 75% (0.75) and 25% (0.25) inside the solution with different combination constants. Lysozyme was used as the external standard to calculate the size of our designed sen-
The calculation results indicated that the size of our designed sensor was about 34 Å, which is about one third larger than the published monomer data, so that our designed sensor did form a dimer at 25 °C with the concentration of the sample being 0.3 mM.

### 4.4.3 Temperature-dependent peak dispersion

The reality of calcium binding to the sensor can be further proved by calcium titration by NMR after the construction of the relationship between fluorescence intensity and calcium concentration. The condition of sample preparation is very important to the quality of the NMR spectra as the protein is composed of beta-sheets which give it a greater tendency to aggregate. Temperature is another factor that will affect the spectrum by impacting peak dispersion, so the spectral quality of CatchER was tested in different temperatures using a 500 MHz NMR spectrometer.
The peak number increases from 128 to 194 as the temperature increased from 20°C to 37°C. Because EGFP is composed of 238 amino acids, the optimal temperature for the experiment operation is above 37°C.
4.4.4 Protein concentration dependent peak dispersion.

[Graph showing protein concentration dependency with [Protein] = 0.6 mM]
The influence of protein concentration contributed to the dimerization was also examined by testing different concentrations of protein. The spectrum of 0.3 mM protein was more dispersed than that of 0.6 mM protein revealing that at higher protein concentrations, the sample would be non-covalently dimerized due to hydrophobic interactions. The tumbling rate of the protein is changed when the dimmer forms. However, the mutation F223E of

Figure 4.6 Protein concentration dependent peak dispersion of CatchER detected by 800 MHz NMR (UGA, CCRC) HSQC spectroscopy. Protein was dissolved in 10 mM Tris, pH 7.4, 10% D2O (vol/vol). The spectra were recorded at the protein concentration of 0.6 mM (A) and 0.3 mM (B), respectively, corresponding to increased number of single H-NH peaks from 167 and 194, respectively.

[Protein]= 0.3 mM
CatchER locates is one of three bulky hydrophobic residues F223, L221 and A206 which form the major hydrophobic interaction surface between two GFP monomers reported by Philips’ group (Yang, F., Nat. Biotech., 1996). We assume that by introducing the hydrophilic residues in this location, the calcium binding will possibly prevent the dimer formation. The protein concentration was determined by absorption intensity at 280 nm with an extinction coefficient of 21890 M⁻¹cm⁻¹.

4.4.5 HSQC Ca²⁺ titration of CatchER

Salt effects were examined to verify whether CatchER can nonspecifically bind to monovalent cations. One hundred micromolar EGTA was added into the sample as the starting point and then titrated with 10 mM KCl to monitor the chemical shifts. The two spectra overlapped. Based on these results, a high concentration of salt could not cause a conformational change in the protein; therefore, there is no nonspecific binding.
Figure 4.7 The overlay of the HSQC spectra of CatchER in the presence of 0.1 mM EGTA (green) and 0.1 mM EGTA +10 mM KCl. The protein at 0.3 mM was dissolved in 10 mM Tris, pH 7.4, with 10% D$_2$O (vol/vol) and the spectra were recorded with 600 MHz NMR (GSU).
The calcium binding perturbations to the individual residues was examined by HSQC calcium titration. The protein sample was 0.3 mM in 10 mM Tris buffer, pH 7.4 and 10% D$_2$O. The operation temperature was 40°C for the 600 MHz NMR spectrometer. One hundred micromolar EGTA and 10 mM KCl was regarded as the starting point. Ascacidium concentration

Figure 4.8 The overlay of the Ca$^{2+}$ titration HSQC spectra of CatchER in the presence of 0.1 mM EGTA +10 mM KCl (green) as starting point. Ca$^{2+}$ was sequentially added using 0.5 mM (red), 1 mM (blue), 2 mM (yellow), 4 mM (purple), and 6 mM Ca$^{2+}$ (orange). The protein at 0.3 mM was dissolved in 10 mM Tris, pH 7.4, with 10% D2O (vol/vol) and the spectra were recorded with 600 MHz NMR (GSU).
increased, several residues exhibited chemical shift changes which could be fitted by 1:1 ratio binding.

The calcium binding affinity calculated from NMR HSQC spectra is similar to the value obtained from the fluorescence titration. Residue 143Y and 153T exhibited $K_d$ values for calcium of 0.35 mM and 0.44 mM, respectively, which is comparable with the fluorescence titration overall $K_d$ of 0.30 mM. The binding affinity of the individual direct binding ligands could not be calculated due to lack of residue assignments. Although residues 143Y and 153T were somewhat far from the designed calcium binding site, as both have bulky sidechains and are located in the flexible loop region, they were hypothesized to be folded into the calcium binding site contributing coordinating oxygen ligands from the hydroxyl sidechain groups.

The chemical shift perturbation (CSP) by the calcium binding was summarized above with three catalogs: proton CSP, nitrogen CSP and HN-N overall CSP. The HN-N overall CSP was calculated with the equation mentioned above. From these plots, it is obviously concluded that there are two condensed regions exhibiting the biggest CSP among all the residues (except the residues without assignment). One of them was from residues L42 to F46 which neighbors beta sheet 11 containing two designed binding ligands (F223E and T225E). We assumed that there were firm mainchain hydrogen bonds between these two beta sheets (beta sheet 3 & 11) which would maintain the restricted secondary structure within this region. Although the CSP of beta sheet 11 was unknown, according to the big CSP of the neighboring beta sheet residues L42 to F46, it is sagacious to predict that our designed binding ligands in beta sheet 11 also experienced big chemical shift changes after binding to calcium. Another region is the residues close to ligand S147E. From these three plots, it is obvious that residues Y143 and T153 exhibited the largest increase in CSP among all of the residues. The regions
that exhibited the largest CSP during the calcium titration were restricted to the designed calcium binding site. All of the calculated $K_d$s of individual residues revealed the same value (several hundred µM) providing the evidence to exclude the cooperativity of a dual calcium binding site. We can conclude that calcium can specifically bind to the designed region of our sensor.

![Figure 4.9 Representative chemical shift of cross-peak Y143 at [Ca$^{2+}$] = 0, 0.5, 1, 2, 4, and 6 mM. Overlaid 2D [$^1$H-$^{15}$N] HSQC spectra of 0.3 mM CatchER in response to Ca$^{2+}$.](image)

The influence of calcium binding to the chromophore conformational change was further explored by carefully analyzing the chemical shift change of the residues close to the chromophore caused by calcium titration. First, we proposed that the mutation of the polar residues to the negative charge residues around this region would disrupt the mainchain hydrogen bond between these three beta sheets by the charge repulsion force, which would sequentially cause the distances between these beta sheets to increase. The neighboring residues (T203...
and E222) which were involved in the complex hydrogen bond network with the chromophore were also pulled away from the original location as binding ligands. These residues shared the same restricted beta sheet backbone. We assumed that the hydrogen bonds between the chromophore and its neighboring residues restricted in this region were partially unformed due to the charge repulsion force caused by the rational mutagenesis of the binding ligands, causing the fluorescence intensity to decrease. After calcium binds to this region, the fluorescence intensity would increase as the two positive charges brought by calcium ions would neutralize the regionally condensed five negative charges and rebuild the hydrogen-bond network of the chromophore and its neighboring residues. Fortunately, the fluorescence titration in vitro data supported this hypothesis, as the 510 nm emission intensity excited by either 398 nm or 490 nm wavelengths increased after calcium titration.

![Figure 4.10 Q69 chemical shift perturbation induced by Ca^{2+} titration. A minor peak was separated from the original single peak after adding 2 mM Ca^{2+}, and the ratio of integration of peak b to peak a increased from 0 to 2.27 as Ca^{2+} concentration increased from 1 mM to 6 mM.](image)

Residue Q69 which is deeply buried inside the beta can structure and only one residue from the chromophore (T65-G67) and also supposed to have hydrogen bonds connection to it,
revealed dual peaks after adding calcium. When the concentration of calcium in the buffer increased from 1 mM to 2 mM, the HSQC spectrum of Q69 was turned from a single peak to split double peaks, which indicated that residue Q69 transferred from one form to another form. The integrations of the dual peaks were calculated and it was shown that the new form of Q69 arose as the calcium concentration increased. If we further calculate the chemical shift difference between these two peaks with the unit of Hz, we are able to obtain the vibration rate between these two forms. From this analysis, we can conclude that the chemical shift changes of Q69 which has a close relationship with the chromophore provides circumstantial evidence that calcium influences the conformational change of the chromophore.

Figure 4.11 Scheme of hydrogen bond interaction between E222 and L42. (A) The NMR HSQC spectra of the chemical shift change of L42 induced by Ca$^{2+}$ (B) the $K_d$ calculation. (C) The structural model shows the proposed main-chain hydrogen bonds between E222 and L42 located in two anti-parallel beta sheets based on x-ray crystal structure of EGFP (pdb code: 1EMA).
E222 is another significant residue involved in the hydrogen bond network with the chromophore, but there is no published assignment data of this residue. Fortunately, its neighboring residue L42 was assigned, which was connected by the mainchain hydrogen bond from the analysis of x-ray crystal structure. We supposed that there was crosstalk between the mainchain chemical shift change of E222 and L42 for the existence of the hydrogen bonds between the amide and carboxyl groups as the figure revealed. Although E222 chemical shift is negligible, the chemical shift change of L42 can be the indicator. With the purpose of more scientific analysis, and the hypothesis that L42 chemical shift change directly came from the calcium perturbation of E222, the nonspecific binding effect of L42 should be excluded in advance. The sidechain of L42 projected inside of the beta can structure and itself is a non-polar residue, so that it is stable and inert to the metal concentration. The interesting results were that L42 had a chemical shift change that could be fitted by a 1:1 ratio binding equation, and the $K_d$ was determined to be around 0.7 mM which was in the same range as the $K_d$s of Y143 and T153. It revealed that all of these individual residues which displayed obvious chemical shift changes could be involved in the same calcium binding event with similar binding free energy change. For E222, it should have experienced conformational change caused by calcium concentration increase.
V150 is another very important residue which has hydrophobic interaction with the chromophore. Although it could not be assigned nor its other of hydrogen bond pair residue, its next sequential residue Y151 underwent a significant chemical shift change when the calcium concentration increased along with Y151’s mainchain hydrogen bond pair residue N164. We hypothesized that calcium concentration changes also influenced the conformational change of V150, according to its sequential neighboring residue chemical shift change.
Figure 4.13 Scheme of two pairs of hydrogen bonds between I167 and A179, V163 and Q183. The overlay NMR HSQC spectra of subtle chemical shift change of A179. (A) and A163 (C) induced by Ca$^{2+}$. The structural model showed the proposed mainchain hydrogen bonds between I167 and A179 (B), V163 and A179 (D) located in two anti-parallel beta sheets based on x-ray crystal structure of EGFP (pdb code: 1EMA).

Another two residues I167 and Q183 were also explored indirectly with the hydrogen bond pair theory to monitor the chemical shift change. From the overlap spectra of residue A179 and A163, which form the hydrogen bonds respectively with the two residues mentioned above, they revealed minimal chemical shift change even at high concentration of calcium. We supposed that I167 and Q183 also only underwent minimal conformational change with high concentration of calcium.
Figure 4.14 Combined chemical shift changes in combining a backbone amide proton and nitrogen between the Ca$^{2+}$-saturated and Ca$^{2+}$-free form. Ca$^{2+}$ influences the residues interacting with the chromophore or close to the designed Ca$^{2+}$ binding site. In addition, Y182, highly accessible to solvents, and G228 in the flexible C-terminal also exhibited more than a 0.2-ppm change in chemical shift. The secondary structure of CatchER, according to EGFP, was labeled on the top. All data were recorded at 37°C using a 600 MHz NMR spectrometer with 300 µM 15N-labeled samples in 10 mM Tris, 10 mM KCl, pH 7.4.
V61, Q94, R96 and F165 which were involved in the hydrogen bond network with the chromophore based on the X-ray crystal structure analysis of GFP and on the opposite side of designed calcium binding site were stable during calcium titration according to the direct HSQC spectra analysis. Only subtle chemical shift changes were detected during the Ca\(^{2+}\) titration, suggesting they were not involved in the calcium binding event.
Figure 4.16 Summary of the mainchain H-NH chemical shift perturbation triggered by Ca$^{2+}$ of the CatchER. These residues are spatially closest to the chromophore among all the residues of GFP based on the X-ray crystal structure analysis. (A) Structure scheme based on EGFP (pdb code: 1EMA) of the Ca$^{2+}$ induced mainchain H-NH chemical shift change of CatchER. The residues are classified with significant change (blue), proposed significant change (purple blue), minimal change (purple), proposed minimal change (pink), and unknown (Orange). (B) The table summarized the distance between the chromophore and the residues shown on the left with the same color.

Here is the summary of chemical shift perturbation of all the residues assumed to have hydrogen bonds with chromophore. The residues with blue and purple-blue color exhibited real or proposed significant chemical shift change respectively, based on the direct or indirect HSQC spectra change analysis. While for the residues with purple and pink color underwent truly or assumed minimal chemical shift change with the same analysis approach. Only two
residues T62 and V68 were unknown for the conformational change caused by adding calcium, which were deeply buried inside the hydrophobic core of the beta can structure and not solvent accessible. From the figure above, it is obvious that all the residues involved in the hydrogen bond network with the chromophore experienced significant chemical shift changes located close to our designed calcium binding site while the residues on the opposite side almost had no chemical shift change during calcium titration. It is reasonable to conclude that the designed calcium binding ligands function as the bridge to link the environmental calcium concentration to the fluorescence intensity quantitative change by the regional conformational changes both of the ligands and their neighboring residues involved in the hydrogen bonds, as the quantity of chemical shift changes of the residues are closely related to their distance to the designed calcium binding site.
4.4.6 HSQC Mn\textsuperscript{2+} titration of CatchER

Figure 4.17 The overlay HSQC spectra of 20 µM Mn\textsuperscript{2+} peak broaden effect of CatchER. 300 µM CatchER was dissolved in 10 mM Tris, pH 7.4, with 10% D\textsubscript{2}O (vol/vol) in the presence of 1 mM Ca\textsuperscript{2+} as the start point (red), and 20 µM Mn\textsuperscript{2+} added (green).
The paramagnetic metal Mn$^{2+}$ was also applied to explore the specific residues involved in calcium binding. But after adding 20 µM Mn$^{2+}$, more than 30 residues disappeared due to the paramagnetic metal broadening effects. The ideal case is that the broadened peaks are the same as those that exhibited chemical shifts during the Ca$^{2+}$ titration, suggesting that Mn$^{2+}$ specifically replaces Ca$^{2+}$ and binds the same location of the protein with the same binding ligands. However, the optimized experimental conditions with appropriate concentration of Ca$^{2+}$/Mn$^{2+}$ added in is highly related to the binding affinity ratio between these two metals to the protein for the metal competition or replacement. It is interesting that almost every glycine was negligible as these residues should not be able to bind to calcium. The broadened peaks

Figure 4.18 Structure scheme based on EGFP (pdb code: 1EMA) of the Mn$^{2+}$ broaden effect results. The residues are classified to be designed Ca$^{2+}$ binding site (red), Mn$^{2+}$ broadened peaks (orange), chromophore (blue), the rest residues (green).
are highlighted with orange color in the Fig. 3.X, and most of them are locate in the neighboring beta-sheets of the designed Ca\(^{2+}\) binding site.

4.4.7 Bacterial expression of $^2$H-$^{13}$C-$^{15}$N isotopic triple labeled and $^{13}$C-$^{15}$N double labeled CatchER and purification of double labeled sample by his-tag.

![Image of SDS-PAGE gel](image)

Figure 4.19 SDS-PAGE gel showing the molecular weight of bacterial expressing isotopic triple labeled (D-$^{13}$C-$^{15}$N) CatchER in comparison to non-labeled CatchER. Bacterial cell pellets collected after IPTG induce for 4 hrs (Line 1), overnight (Line 2), supernatant of overnight (Line 3), purified non-labeled CatchER (Line 4), marker (Line 5).

Heteronuclear $^{13}$C-$^{15}$N double labeled CatchER was expressed in Escherichia coli BL21(DE3) with the recombined pET28(a) vector containing a six-His tag. The cell were incubated at 37 °C with heteronuclear $^{13}$C-$^{15}$N double labeled SV media for the cell replication until the optical density 600 nm reaches 0.6. IPTG is introduced into the cell culture for protein expression and decrease the temperature to be 25°C for overnight expression, as the high temperature would prevent the correct cyclization of chromophore. The collected protein was purified with nickel-chelating column connected to a fast performance liquid chromatography (FPLC) system. The yield of the purified CatchER is 25 mg out of 1 L heteronuclear $^{13}$C-$^{15}$N double labeled SV medium. Small-scale expression of triple labeled CatchER was also con-
ducted with the established methods above. Line 1 and Line 2 of Fig. 5 revealed the protein expression level in the cell pellets was increased after overnight incubation, while almost no protein secreted to the supernatant. Compared with the non-labeled CatchER, the molecular weight of triple labeled samples was slightly bigger, as the bands of Line 1 and 2 were closer to the bottom of the SDS page than that of Line 4.

4.4.8 3D NMR HNCA and CBCACONH for mainchain assignment of CatchER.

The CatchER backbone HN-N was assigned by 3D HNCA, according to CA chemical shifts, and then CBCACONH data were collected to verify the correctness of the previous assignment. At the same time, CB chemical shifts were assigned. Compared with Khan’s published data, the biggest chemical shift perturbations came from the loop region (N23, E90, G160 and G228) because a different buffer system was applied. For CA chemical shifts, which were closely related to the secondary structure prediction, there were only three condensed regions exhibiting big chemical shift changes. One was the chromophore region, as V68 CA is about 5 ppm bigger than Khan’s wt GFP. Another two were close to the designed calcium binding site regions, which indicated that the mutations cause the regional secondary structure change.
Figure 4.20 Selected strips of NMR HNCA-HSQC assignment. The G138 carbon alpha was identified based on the featured chemical shift of glycine alpha around 40 $^{13}$C ppm in HNCA strip, and the corresponding mainchain H-NH peak of G138 was assigned with the cross spontaneously.
Figure 4.21 Selected CatchER 3D HNCA spectra from I14 to E17, with sequential and intraresidual Cα–Cα connections indicated by red lines.
In order to explore whether the mutagenesis would influence the secondary structure of EGFP, the secondary structure prediction was conducted by the protocol provided in Wishart’s published paper. CA was used as the index of secondary structure prediction. From the plotted results, beta sheet 1-9 could be well predicted, while the last two were not so accurately predicted as the lack of assignment. It concluded that our designed calcium sensor maintained the overall beta can structure even after five residues mutagenesis.

Figure 4.22 Difference in Cα chemical shift between published and our data. Most laboratoryeled residues exhibiting more than a 1.5 p.p.m. chemical shift difference were sequentially close to the chromophore or the designed Ca^{2+} binding site (blue). 1-5 represent E147, D202, E204, E223, and E225, respectively. Unassigned CatchER residues are gray in the structure. All the data were recorded at 37°C using an 800 MHz NMR spectrometer with a cryogenic probe and a 300 µM ^{13}C-^{15}N double-laboratoryeled sample in 10 mM Tris, pH 7.4. (D-G) CatchER 2D (^{1}H-^{15}N) HSQC spectrum recorded at 0 mM Ca^{2+} (black) and 6 mM Ca^{2+} (red). A subtle chemical shift change was observed for Q94 at 6 mM Ca^{2+} but no change for R96, F165, or V61. (H) Sidechains of R96, Q94, F165, and V61 (red) protruding toward the chromophore (green sticks) on the opposite side of the designed Ca^{2+} binding site (blue). All data were recorded at 37°C using a 600 MHz NMR spectrometer with a 300 µM ^{15}N laboratoryeled sample in 10 mM Tris and 10 mM KCl, pH 7.4.
4.4.9 $T_1, T_2$ and NOE of CatchER

Figure 4.23 Representative fitting of peaks integrations collected at 0, 30, 60, 100, 240, 480, 720, 1000, and 1500 ms $T_1$ delays.

Figure 4.24 Overlay of $T_1$ delay spectra from selected region: 0 ms (black), 1500 ms (red).
The relaxation of our protein was also explored by measure the $T_1$, $T_2$ and heteronuclear NOE. For $T_1$, $T_2$ data, the designed protein behaved like a small protein compared with Seifert’s published wt-GFP data, as the average of $T_1$s of catchER is about half while $T_2$ is doubled compared with Seifert’s published wt-GFP data. It is plausible to explain that catchER is a monomer while Seifert’s wt-GFP is a dimer as they used the deuterated sample. These results will be further examined. The heteronuclear NOE spectra indicated that several residues were flexible. 232G and 231H were two of the flexible residues located in the C terminal. 25H and 151Y were placed in the loop region.

4.4.10 Tau C calculation

![Figure 4.25](image)

Figure 4.25 The monomerization of CatchER is supported by measured rotational correlation time $\tau_C$ with high-field nuclear magnetic resonance spectroscopy. $\tau_C$ directly determined by the SCT-CCR experiment performed on an 800 MHz NMR spectrometer (gray square) or calculated using Equations (16) and (17) with relaxation times $T_1$, $T_2$ determined on a 600 MHz NMR spectrometer (black circle) (see Methods). The secondary structures of corresponding residues are marked above.
The rotational correlation time ($\tau_C$) of CatchER was calculated by $T_1-T_2$ with 600 MHz NMR spectrometer with the equation provided within Taq A. Holak's GFP dynamics paper (Biochemistry, 2003 Mar 11; 42(9):2500-12.) $\tau_C$ was measured using an 800 MHz NMR spectrometer (UGA) with SCT-CCR pulse sequence. The average of $\tau_C$ calculated from $T_1/T_2$ is 10.38 ns and $\tau_C$ directly measured in 800 MHz is 12.39 ns by calculating 60 amino acids in beta-sheet. Holak's $T_1$ and $T_2$ data were collected with very high protein concentration of 0.75~0.9 mM (20-25mg/ml) and calculated an apparent mass for GFPuv of 45 ± 10 kDa under the conditions used in NMR experiments which is significantly larger than the value of 27 kDa for the monomeric GFPuv but still smaller than the value of 54 kDa expected for a dimer, suggesting that GFPuv in solution exists as a mixture of monomers and dimers. All our samples were only 0.3 mM which explained the difference between our data and published results. If we increase the protein concentration, we may get similar values.

4.5 Discussion

The initial purpose of using dynamic NMR to investigate the structural properties of GFP after tens of X-ray crystal structures of GFP variants have been reported was that the proposed three steps of p-hydroxybenzylideneimidazolidinone chromophore forms based on X-ray crystal structure could not explain the photoisomerization, photoconversion and on-off blinks of GFP. Moreover, more and more evidence suggested that chromophore should exhibit internal vibration instead of stable state which was in conflict to the well-defined chromophore structure with low B factors in most X-ray crystal structures. However, the chromophore is deeply buried within the beta-barrel structure not accessible to the bulk solvent, preventing investigation with regular $^1$H-$^13$C-$^15$N isotopic labeled samples by NMR. The $^{19}$F NMR method can specially labeled Trp by replacing the $^1$H by $^{19}$F in the proton-nitrogen bonds within the aromatic rings, and
observe the labeled residues by distinct chemical shift changes of $^{19}$F providing a direct measurement of the dynamics of chromophore containing Trp. Cyan fluorescent protein (CFP), with only one residue difference (Y66W mutant of GFP) in the chromophore, exhibits more than 40 nm blue shifted excitation and emission spectra compared to GFP making it a good model for $^{19}$F NMR study. Other than W66 only possessed by CFP, W57 close to the chromophore exists in both CFP and GFP and was also labeled. Different from single $^{19}$F peak of W57 in GFP, both W57 and W66 in CFP were observed with two split peaks, suggesting a slow exchange process of the chromophore. Besides the protein existing in the monomer and dimer form and cis trans conformation of the chromophore, the highest possibility of the slow exchange process was due to neighboring residue H148 which exhibited two states with further verification needed by X-ray crystallography. This work mainly focused on discussing special phenomena of split peaks of $^{19}$F labeled W66 in CFP observed in the NMR study, while there is limited discussion of the contribution of other residues close to the chromophore. Moreover, for a monomeric protein of the size (around 245 amino acids) of GFP, due to the Stokes-Einstein relation, a total correlation time around 14 ns (310K) is expected, as the maltose-binding protein (MBP, 370 amino acids) $\tau_C$ is 17ns (310 K). However, the NMR spectra of CFP were collected at 303 K, with $\tau_C$ determined to be 22 ns. Instead of existing as monomers, most of the protein stayed in the dimer form, indicating the non-optimal conditions of the NMR sample. The authors proposed that dynamic NMR would help to elucidate the vibration of chromophore by directly observing isotopic labeled chromophore structure. Several following studies of the GFP structures of NMR reported later, and the R1, R2 values of GFPuv were measured to be 0.5 s$^{-1}$ and 27 s$^{-1}$, respectively, at 310 K (37 °C) with 20-25 mg/ml protein (750-930 μM) using a 600 MHz NMR spectrometer.
Rotational correlation time ($\tau_c$) is related to temperature, molecular size, and the viscosity of the solution. The measurement is based on the intensity modulation as a function of relaxation delay. The relaxation mechanism of interest is effective, and the other mechanisms are often eliminated by internal pulse sequences or cancel out by combination of two sets of data (Liu, Y et. al.).

The significance of NMR structural determination of GFP and its variants are multi-layered: 1) It allows investigation of the backbone dynamics by relaxation, correlation time, and HD exchange experiment in a large time scale from nanosecond to months. 2) It allows for further exploration of the denatured states (strong acid, mild acid, urea, guanidinium chloride, guanidinium thiocyanate) of GFP which had not been crystallized, providing experimental results for calculating the folding energy barrier between denature and folding states. 3) It helped to understand the dark form of photoconvertable fluorescent protein Dropona which would lose its dark form at extremely low temperature (80 K) common to X-ray crystal structures, as the sample preparation and experiment conduction of NMR were performed at room temperature.

1D proton NMR spectra showed robust peaks in low field from 9.0 ppm to 7.0 ppm representing the backbone mainchain amine of CatchER, suggesting the protein was well folded. There were obvious chemical shift changes within both the low field and high field of the stacked 1D NMR spectra in the absence and presence of Ca$^{2+}$, indicating Ca$^{2+}$ could trigger conformational changes of CatchER which are the unique properties of CatchER as other GFP variants were not sensitive to Ca$^{2+}$.

Moreover, we further verified that the oligomerization of CatchER was both temperature and concentration dependent. The molecular weight of CatchER was estimated to be 33 kDa, containing 27 kDa of the 238-residue GFP scaffold and more than 30-residue his-tag. A 66
kDa dimer form of CatchER was out of range for the molecular weight and could not be measured by HSQC 600 MHz NMR due to the inaccurate transverse relaxation ($T_2$) value of molecules larger than 25 kDa. The monomer CatchER would show better NMR spectra than the dimer. There were three experimental evidences to support the first claim. One, the number of mainchain amine peaks of HSQC spectra continued increasing when the temperature rose from 20°C to 37°C and maintained 197 distinct peaks from 37°C to 43°C, suggesting the oligomer state of the protein changed as a function of temperature. Second, the effective hydrophobic radius of CatchER (33 kDa) at 300 µM from the PFG diffusion results at 25°C were fitted to be 34.5 Å from more than 70% of the mixture compared to 20.1 Å for lysozyme (14.7 kDa), suggesting that CatchER was mainly a dimer at this temperature. However, with the same protein concentration, the $\tau_c$ of the backbone amines was measured to be 12 ns at 37°C, similar to the heuristical estimation of 14 ns (0.6 times its molecular weight) for the monomer being smaller than 22 ns of GFPuv measured at 25°C reported in the literature.

Besides the temperature, the protein concentration was also influential to the oligomerization of CatchER. More amine peaks were shown in the 800 MHz NMR HSQC spectra at 300 µM than that of 600 µM at fixed 37°C possibly due to the shorter $T_2$ values caused by dimerization with higher protein concentration. Though L223 and L225 were mutated to hydrophilic residues in CatchER and the original X-ray crystal structures of GFP showed the dimer form of the protein was mainly caused by the hydrophobic interaction of bulky residues A206, L223 and L225 on the surface, CatchER is a beta-berral protein that is prone to aggregation at high protein concentration in contrast to alpha helix protein. Moreover, we excluded the possibility of the conformational change as a function of protein concentration since no apparent chemical shift changes were observed between these two spectra. Most of the peaks at high
protein concentration were only broadened. The diffractional centrifigation results reported from another group indicated that GFP would form form dimers at concentrations higher than 100 µM, suggesting the oligomerization of GFP variants was concentration dependent.

75% of CatchER have been assigned for the backbone $^{1}\text{H}^{13}\text{C}^{15}\text{N}$, CB

The secondary structure of CatchER was maintained based on the carbon alpha chemical shifts.

CatchER exhibited metal selectivity between Ca$^{2+}$ and K$^+$. CatchER also exhibited Ca$^{2+}$ induced chemical shift changes in the presence of 10 mM K$^+$. Calcium binding to catchER was monitored by NMR metal titration. Several residues close to the calcium binding pocket exhibited chemical shift changes which could be fitted by the 1:1 ratio binding equation with all of the fitted $K_d$s in the same range.

CatchER Q69 and E222 exhibited large calcium-induced chemical shift changes. These residues are close to the calcium binding pocket and involved in hydrogen bonding with the chromophore. In contrast, R96, Q94, F165 and V61 which are on the opposite side of calcium binding pocket was not influenced by calcium binding according to NMR HSQC calcium titration spectra.

Calcium perturbation results: 15 residues with sidechain distance to the chromophore within 5 Å in the crystal structure. And the chemical shift changes of these residues can be classified to five groups. Major chemical shift change (from direct observation or indirect observation). Minimal chemical shift change. Mainchain hydrogen bond interaction.
The average $T_1$, $T_2$ of GFPuv were measured to be 2000 ms and 37 ms, respectively, ($R_1=0.5 \text{ s}^{-1}$ and $R_2=27 \text{ s}^{-1}$) with 20-25 mg/ml protein (750-930 µM) at 310 K (37 °C) with 600 MHz NMR. However, our CatchER $T_1$, $T_2$ values were 800 ms and 80 ms, respectively, with 300 µM protein at the same temperature (37 °C) with the same magnetic field strength (600 MHz). The paradox can be explained by the partial dimmerization of GFPuv (238 amino acids) at high protein concentration, as their rotational correlation time ($\tau_c$) was measure to be 22 ns, much larger than estimated 14 ns of a monomer form GFP (0.6 times of molecular weight in

![Figure 4.26 The relations of longitudinal relaxation ($T_1$), transverse relaxation ($T_2$), rotational correlation time ($\tau_c$) and molecular weight. The estimated $T_1$, $T_2$ values of CatchER or GFP mutants were at the cross of the black dash line according to $\tau_c$ value.](image)
kDa), even larger than a experimental 17 ns of the maltose-binding protein (MBP), with 370 amino acids, whereas the $\tau_c$ of CatchER was measured 12 ns using a shared, constant-time, cross-correlated relaxation (SCT-CCR) pulse sequence developed by Dr. Prestegard’s lab based on the well-defined secondary structure of backbone amine excluding the flexible loop regions. From Figure 4.X, the correlation time of GFP measured by both two groups are within $10^{-8}$ s scale, and $T_1$ value increases while $T_2$ value decreases in the function of molecular weight, especially at high magnetic field (black dash line), suggesting the dispency of the relaxation time constants of same scaffold protein measured by two groups is mainly due to the dimmerization of protein at high concentration. Our group successfully investigated the relaxation time constants of the monomer form of CatchER.

Mn$^{2+}$ broaden effect results. Ideal results, Mn$^{2+}$ has the similar coordination geometry to Ca$^{2+}$, which should bind the same location as Ca$^{2+}$. If the particular residues are perturbed by Ca$^{2+}$, the same residues should be broadened by Mn$^{2+}$. (Yang, W. et. al. 2005, JACS) Most Gly were broaden, similar to Seifert’s report about the pH sensitive residues of GFP. (several Gly residues disappear at low pH).

The significance of each individual NMR experiment. 1) Temperature dependent dimerization of GFP variants. (Monomer 300 uM at 37 C, Tau C condition with calculated value 12 ns. Mixture of monomer and dimer at 25 C, results of diffusion NMR and HSQC.) 2) Concentration dependent dimerization. (600 uM and 300 uM peak numbers.) 3) HSQC titration results correlate to the fluorescence titration results. $K_d$ 4) split peak spatial close to the chromophore. 5). Secondary structure prediction based on the carbon alpha chemical shift change indicate the beta-barrel structure of CatchER. So that the major assignments of the CatchER is correct.
6) $T_1, T_2$ calculation and relaxation. 7) Mn$^{2+}$ broaden effects, most Gly broaden, corresponding to the pH sensitive residues of Gly assigned by Seifert.

### 4.6 References


CHAPTER 5: PINPOINTING A CLAW-SHAPED METAL BINDING SITE IN CATCHER BY X-RAY CRYSTAL STRUCTURES.

5.1 Introduction

X-ray crystal structure of enhanced green fluorescent protein (EGFP, S65T mutation of GFP) was initially solved and published in 1996, reporting a 1.9 Å resolution structure of the monomer folded protein consisting of an 11-stranded beta barrel with a coaxial helix forming the chromophore. The p-hydroxybenzylideneimidazolidinone chromophore structure was solved and observed to be completely buried inside the beta barrel structure protected from the bulk solvent being formed by a spontaneous cyclization and oxidation of sequence -Ser65(or Thr65)-Tyr66-Gly67-. The proposed hydrogen bonds between chromophore and sidechains of spatial neighboring residues surrounding the chromophore further stabilize the chromophore structure and generate the environment that facilitates fluorescence emission during excitation. Moreover, the site-directed mutagenesis of Thr203 to Tyr or His will produce red-shifted variants. A contemporary and independent green fluorescent protein (GFP, S65) X-ray crystallography paper was published with the similar beta-barrel structure; however, the dimmer protein was formed by hydrophobic interaction with bulky sidechains of residues A206, F225 and L223. A direct and systematic comparison of X-ray crystal structures of GFP and EGFP was reported in 1997. The conformations of T203, H148 and E222 close to the chromophore were identified to play important roles in the wide spectrum of GFP variants, and a more dynamic hydrogen bond network close to chromophore was established by analyzing the duality of sidechain conformations for these residues due to high B factors.

In early X-ray crystal structure analysis of GFP, it was proposed that the fluorescent dynamics of GFP was due to an intramolecular Forster-cycle. However, it cannot explain the
photoconversion nor the complex on-off blinking and switching behavior. The crystal structure provides the chemical evidence that the twisting of double bonds within the chromophore is crucial for the photoisomerization; however, it was not able to determine the conformational changes quantitatively by molecular simulations.

$\text{Ca}^{2+}$ acts as a ubiquitous signaling molecule in the regulation of numerous biological functions including heart beat, muscle contraction, cell development and proliferation(12, 128). $\text{Ca}^{2+}$ signals exhibit different amplitude and durations as the ions flow between subcellular compartments. It functions as first messenger in the central nervous system and works as an extracellular ion source for postsynaptic ligand gated channels(129). The endoplasmic reticulum (ER) functions as an intracellular $\text{Ca}^{2+}$ store and the release of ER $\text{Ca}^{2+}$ triggers a series of biological processes via the binding of $\text{Ca}^{2+}$ with intracellular $\text{Ca}^{2+}$-sensing proteins like calmodulin (CaM) and troponin C (TnC)(130). Hence, determination of free $[\text{Ca}^{2+}]_{\text{ER}}$ has been of extensive interest, which has stimulated the development and improvement of tractable biological $\text{Ca}^{2+}$ indicators.

Many efforts have been devoted to green fluorescent protein (GFP)-based $\text{Ca}^{2+}$ fluorescent indicators, such as cameleons and pericams developed by Miyawaki, Perschini and Nagai, based on either fluorescence resonance energy transfer (FRET) between two different GFP variants or the ionization state change of chromophore with more sensitivity to pH of circularly permutated GFP(10, 131-133). However, one common property of these sensors is that they involve the insertion of naturally occurring $\text{Ca}^{2+}$-sensing proteins like CaM, which are limited to a perturbation of intrinsic calcium signaling and suffer from difficulty in qualification(3, 134, 135). Although many improvements for these types of $\text{Ca}^{2+}$ sensors have been made to reduce the limitations(1, 90, 136), there is still a strong need to design $\text{Ca}^{2+}$ sensors without
using natural calcium signaling proteins. We have reported a new strategy for creating Ca\(^{2+}\) indicators by engineering an exogenous Ca\(^{2+}\)-binding motif (EF-hand loop III of CaM) into the fluorescence sensitive location of GFP\((71)\). These sensors exhibit an affinity for binding Ca\(^{2+}\), producing large ratiometric fluorescence and absorbance changes in response to Ca\(^{2+}\) at specific subcellular locations especially in high Ca\(^{2+}\) environment without perturbing the cellular environment Ca\(^{2+}\) signaling. Thus, to a large degree, this design overcomes the disadvantage of using the natural Ca\(^{2+}\) binding protein.

An alternative approach to rationally designed Ca\(^{2+}\) sensors has been developed by introducing an endogenous Ca\(^{2+}\) binding site via site-directed mutagenesis of selective residues in the fluorescent sensitive location of enhanced GFP (EGFP) Tang et al. Residues Ser147, Ser202, Gln204, Phe223, and Thr225 of wild type EGFP (wtEFGP) were selected as the components of the designed Ca\(^{2+}\) binding site. A series of Ca\(^{2+}\) sensors were designed with different mutations (based on these five residues), with fast response to Ca\(^{2+}\) and optical property changes. These genetically encoded Ca\(^{2+}\) indicators allow real-time measurement and exhibit single-wavelength fluorescence enhancement and K\(_d\) values from 0.1 mM to 1 mM upon binding to Ca\(^{2+}\) (Tang, in press). In particular, the single EGFP-based Ca\(^{2+}\) biosensor termed CatchER has been created by the introduction of S147E, S202D, Q204E, F223E and T225E, as a designed Ca\(^{2+}\) binding site (Figure 1). CatchER provides multiple advantages in reliably monitoring Ca\(^{2+}\) signaling in high [Ca\(^{2+}\)] environments: (1) it exhibits high signal to noise ratio and metal selectivity for fluorescent change in response to Ca\(^{2+}\) both in vitro and in vivo; (2) CatchER binds Ca\(^{2+}\) in a simple 1:1 binding mode quantized by NMR metal titration and thus avoids the cooperativity of CaM or TnC based calcium sensors; (3) CatchER shows fast kinetic response to Ca\(^{2+}\) changes within milliseconds and can successfully detect multiple calcium
spikes during muscle contraction and relaxation; (4) the $K_d$ of CatchER (approximately 1 mM) indicates an intrinsic weak binding affinity in SR (sarcoplasmic reticulum)/ER in situ, which allows the accurate calibration of SR Ca$^{2+}$ signaling; (5) no invasive methods are required for injecting CatchER in living organelles compared with current natural Ca-binding protein based dyes.

In this report, we investigate the Ca$^{2+}$ binding site and the structural basis for the fluorescent change based on crystallographic analysis of CatchER. Crystal structures of CatchER in the absence of Ca$^{2+}$ [CatchER(apo)], in the presence of Ca$^{2+}$ (CatchER-Ca$^{2+}$), and soaked with Gd$^{3+}$ (CatchER-Gd$^{3+}$) were obtained. In addition to the spectroscopic study of CatchER with Gd$^{3+}$, the heavy metal Gd$^{3+}$ was also chosen to guide the position of the metal ion binding site, since Ca$^{2+}$ as a lighter metal with fewer electrons may be less visible in the crystal structure. X-ray crystal structures of CatchER and its complexes can assist the development of protein-ligand interaction based biosensors for the detection of various physiological molecules.

5.2 Experimental Section

Expression, Purification and Crystallization. BL21(DE3) cells transformed with pET28a vector containing the protein DNA were pre-cultured in 10 ml of LB media containing 6 µl of 50 mg/ml kanamycin (30 mg/ml) and were cultured overnight at 37°C. The pre-culture was then transferred to 1 L of LB media containing 30 mg/ml of kanamycin and were cultured at 220 rpm at 37°C until the O.D. reached ~0.6 followed by the induction of protein expression with the addition of 200 µl of 1 M IPTG (0.2 mM) and a reduction in temperature to 25°C. Cells were collected by centrifugation at 7,000 rpm for 30 min at 4°C. Cell pellets were re-dissolved in extraction buffer (20 mM Tris pH 8.0, 100 mM NaCl, 0.1% Triton X-100) and sonicated. Cell lysate mixture was centrifuged for 30 minutes at 17,000 rpm and 4°C. Supernatant was fil-
tered using a 0.45 µm Whatman filter and purified by FPLC using a 5 ml Hitrap chelating column (Amersham Biosciences, Sweden) loaded with Ni\(^{2+}\). High purity fractions were then concentrated to 1-2 mL and purified further using size exclusion chromatography with a Superdex 75 100 mL column (Pharmacia Biotech) at a flow rate of 1 ml/min with 10 mM N-[2-hydroxyethyl]piperazine-N’-2-ethanesulfonic acid (HEPES) buffer pH 7.4 to ensure high purity for crystallization.

*Crystals of Ca\(^{2+}\) free and Ca\(^{2+}\) loaded CatchER* were obtained via the hanging drop method of vapor diffusion using 2 µl protein:2 µl reservoir solutions at room temperature using 24-well VDX plates (Hampton Research, Aliso Viejo, CA). Ca\(^{2+}\) free crystals (0.9 mM protein, 5 µM EGTA) grew in mother liquors containing 53 mM HEPES pH 7.0, 1 mM β-mercaptoethanol, 50 mM (NH\(_4\))\(_2\)SO\(_4\), and 16% PEG 4000 or 51 mM HEPES pH 7.0, 1 mM β-mercaptoethanol, 50 mM NaOAc, and 17% PEG 4000. The Ca\(^{2+}\) loaded CatchER complex was created by adding 50 mM CaCl\(_2\) into a 0.9 mM protein solution (final concentration = 0.45 mM). Crystals grew in mother liquors containing 51-53 mM HEPES pH 6.9-7.4, 1 mM β-mercaptoethanol, 50 mM NaOAc, and 16-17% PEG 3350. Crystals of CatchER-Gd\(^{3+}\) were obtained via the soaking technique. Crystals of apo CatchER were soaked in a solution of mother liquor with the final concentrations ranging from 1 mM to 4.5 mM of GdCl\(_3\) for 1 to 2 days. The crystals were mounted in liquid nitrogen with the cryoprotectant of 20-30% (vol/vol) glycerol. X-ray diffraction data for the crystals were collected on the SER-CAT beamline of the Advanced Photon Source, Argonne National Laboratory, Argonne, IL.

*X-ray diffraction.* X-ray diffraction data were processed with HKL2000(137), and the structures were solved by molecular replacement using MOLREP CPP4i suite of programs(138, 139) with the wtEGFP structure [Protein Data Bank (PDB) entry 2OKW](140).
as the starting model. The structures were refined with SHELX(141, 142) and refitted using Coot 0.5.2(143). Alternate conformations were modeled for CatchER residues where observed. The solvent was modeled with a few hundred water molecules, metal ions and solvent molecules according to the observed electron density maps. Anisotropic B factors were applied for all the structures. Hydrogen atom positions were calculated in the last stage of refinement, using all data after other parameters, including disorder, had been modeled. In CatchER(apo) structure, residue Arg73 and Glu225 are refined with the same free variable number in SHELX due to their close distance of two alternative conformations. The mutant crystal structures were compared with each other and with the wild type GFP (PDB entry 1EMA) by superimposing their Cα atoms using SUPERPOSE in CPP4i suite of programs(139, 144). Structural figures were made using PyMol(145).

5.3 Results & Discussion

5.3.1 Crystallographic analysis of CatchER structures

Crystal structures of CatchER of apo form, Ca²⁺ loaded form and Gd³⁺ loaded form were determined to identify the Ca²⁺ binding site in the designed mutant of EGPF. The crystallographic data collection and refinement statistics are shown in Table 1. The crystal structures of CatchER(apo), CatchER-Ca²⁺, CatchER-Gd³⁺ were refined to R factors of 0.17, 0.15 and 0.17 at resolutions of 1.40, 1.10 and 1.66 Å, respectively. These three structures are in different space groups: CatchER(apo) structure in C2, CatchER-Ca²⁺ in P2₁2₁2₁ and CatchER-Gd³⁺ in C222₁. In CatchER(apo), two non-crystallographic molecules with residues labeled 1-229 and 1'-229' were solved in the asymmetric unit, while in CatchER-Ca²⁺ and CatchER-Gd³⁺, only a single molecule of residues 1-229 was observed.
Because of the high resolution of the diffraction data, the solvent was modeled with about 300 water molecules for CatchER-Ca\textsuperscript{2+}, over 150 for CatchER-Gd\textsuperscript{3+} structures, and around 370 waters for CatchER(apo) with two protein molecules. The non-water solvent molecules were: two Ca\textsuperscript{2+} ions both with 0.50 occupancy in CatchER-Ca\textsuperscript{2+}; two partial Gd\textsuperscript{3+} ions with the relative occupancy of 0.70/0.30 and one half occupancy glycerol molecule in CatchER-Gd\textsuperscript{3+}. These solvent molecules were identified by the shape and peak height in the electron density maps, B-factors and potential interactions with other molecules.

Alternative conformations were modeled for a total of 36 (21 for monomer A and 19 for monomer B), 18, and 9 residues in CatchER(apo), CatchER-Ca\textsuperscript{2+} and CatchER-Gd\textsuperscript{3+} structures, respectively. More alternative conformations were refined in CatchER(apo), and CatchER-Ca\textsuperscript{2+} structures per molecule of protein than seen in CatchER-Gd\textsuperscript{3+} due to the higher resolution X-ray data. Residues with alternative conformations, such as Leu15, Leu18, Ser30, Gln80, Lys131, Glu132, Arg168, Glu172 and Thr186, are mostly located on the β-strands and loops with side chains at the protein surface. At the loop 155-159 region, although the electron density map is less well defined than in the interior of the protein, two alternate positions were observed for main chain carbonyl group of Asp155 in CatchER-Ca\textsuperscript{2+} and Asn159 in CatchER(apo). In CatchER(apo) structure, almost identical residues showed alternate conformations in both non-crystallography symmetric monomers, except for Lys26 and Lys124. In all three structures, Glu222 consistently shows two alternate conformations with clear electron density. Among the five designed mutations located on three neighboring β-strands, Glu223 and Glu225 have two alternate conformations in CatchER(apo) and CatchER-Ca\textsuperscript{2+}, while there were two conformations of Glu147 in CatchER-Ca\textsuperscript{2+}. Due to the surface location of these five mutated residues and the potential for radiation damage to the carboxylate side chains,
Glu204, Glu223 and Glu225 showed poor electron density and negative difference density in different CatchER complexes.

### 5.3.2 Identification of metal ions in CatchER

Crystals of CatchER-Ca$^{2+}$ were grown in high concentrations of Ca$^{2+}$, however, the solvent peaks near the mutated residues and elsewhere were indistinguishable from those of water in the electron density maps (Figure 5.3). Therefore, the presence of Ca$^{2+}$ was deduced from the interactions with nearby residues and waters and calculations from the bond valence method as described by Harding and Trzesowska et al (146, 147). The two Ca$^{2+}$ ions were refined at 0.5 occupancy (Table 5.1) in the crystal structure of CatchER-Ca$^{2+}$. We cannot rule out the possibility of Ca$^{2+}$ ions in other regions of the protein structure. Consequently, the structure of CatchER-Gd$^{3+}$ was obtained from apo crystal soaked in high concentrations of the metal ion. Two Gd$^{3+}$ ions were identified near the mutated residues. The higher 0.7 occupancy Gd$^{3+}$ was identified unambiguously by a large peak represented in $F_o-F_c$ omit map contoured at 4.0$\sigma$ (Figure 5.3) and the lower 0.3 occupancy Gd$^{3+}$ was deduced based on the B-factor and the peak in the electron density map and potential interactions with neighboring atoms in comparison with the other possible ions like chloride.

Compared to the wtEGFP (PDB code: 1EMA), CatchER bears five mutated residues S147E, S202D, Q204E, F223E, and T225E. They are located on three neighboring β-strands, pointing out of the protein β-barrel (Figure 5.1), and the selected mutations form a pentacarboxylate ionic environment. In CatchER(apo), one of the carboxyl oxygen of Glu147 forms two hydrogen bonds with the carboxylate of Asp202 based on the distance measured, and this indicates that the Glu147 is protonated (Figure 5.3A). Otherwise, the two negative charged residues will show repulsion effects. Ca$^{2+}$ has 18 electrons orbiting the nucleus, while the Gd$^{3+}$...
has 61 orbital electrons. Therefore, it is difficult to locate the Ca$^{2+}$ compared with Gd$^{3+}$, which has 9 folds increased diffraction over Ca$^{2+}$, especially when the Ca$^{2+}$ is partially bound with protein. Thus, the first Ca$^{2+}$ with 0.5 ratio occupancy in CatchER-Ca$^{2+}$ was placed between Glu147 and Asp202, with hydrogen-bonding distances of 2.1 Å to Glu147, 2.7 Å and 3.0 Å to Asp202, 1.8 Å, 2.5 Å and 3.0 Å with three water molecules (Figure 5.3B). This location was deduced from the bond valence calculation(146, 147) for calcium, short interatomic distances and coordination with two residues of the designed binding site and surrounding solvent molecules. However, this deduction cannot exclude the possibility of a Na$^+$ and a water molecule which are from the protein buffer or crystallization condition. The major 0.7 occupancy Gd$^{3+}$ ion was identified unambiguously from the very high peak in the electron density indicative of a heavy metal ion. This major peak in CatchER-Gd$^{3+}$ is located also between Glu147 and Asp202, forming three hydrogen bonds with these two residues at 2.2 Å, 2.6 Å and 2.7 Å distance and one with a nearby water molecule at 2.1 Å (Figure 5.4C). The second Gd$^{3+}$ with 0.3 occupancy was deduced not only from the short interaction distance with Glu204 at 2.4 Å, and two water molecules at 2.4 Å and 3.1 Å, but the positive peak observed when a water molecule or a partial occupancy Na$^+$ ion was refined at this site. The two Gd$^{3+}$ ions are bound with CatchER by the three residues of the designed Ca$^{2+}$ binding site. Location of the other Ca$^{2+}$ is proposed from the major Gd$^{3+}$ position when superimpose CatchER-Ca$^{2+}$ and CatchER-Gd$^{3+}$ structures, and a previously refined water molecule was changed to the second partial occupancy Ca$^{2+}$.

Superposition of CatchER-Ca$^{2+}$ and CatchER-Gd$^{3+}$ (Figure 5.3D) has shown that both Ca$^{2+}$ and Gd$^{3+}$ are bound within the designed claw-shaped Ca$^{2+}$ binding site. However, Glu223 and Glu225 are not involved in direct interactions with the metal ions. However, the side chains
of these two residues were not well defined in the electron density maps, possibly due to radiation damage. Also, introduction of F223E and T225E increases the extent of the potential metal binding site. Therefore, it is unclear whether these two mutations are essential for the metal binding properties of CatchER.

5.3.3 Structural changes around the chromophore

The chromophore interactions were compared for the CatchER(apo) structure and wtEGFP (PDB entry 1EMA), which was determined in space group of $P_{2_1}2_12_1$ at 1.9 Å resolution\(^{(109)}\). The $p$-hydroxylbenzylideneimidazolidinone chromophore (CRO66) is clearly visible in the electron density for all structures, as shown in Figure 2(a). It is buried centrally in the protein molecule and well protected from solvent\(^{(109)}\). The chromophore can exist in neutral and anionic forms, which produces the two absorbances at 395 nm and 475 nm with a ratio of 3.0 in vitro (Tang, et al.), respectively\(^{(148)}\) (Figure 5.3A). The spectroscopic characterization of CatchER shows two absorption maxima with a major peak at 398 nm and a smaller peak at 490 nm, and thus, resembles wtGFP with two similar excitation wavelengths (Figure 5.3B).

Like the intricate network of polar interactions with the chromophore in wtEGFP, the hydrogen bond interactions are conserved in the vicinity of the carbonyl group of the imidazolidinone ring of chromophore\(^{(109, 149)}\). However, two major changes are seen in the CatchER structures compared with wtEGFP around the tyrosyl group of Tyr-CRO66 and the side chain of Thr-CRO66 associated with Thr302 and Glu222 (Figure 5.3C,D). The side chain of Thr203 is rotated away from Tyr-CRO66 and the main chain moves towards the chromophore. This results in the elimination of direct hydrogen bond interactions between hydroxyl of Thr203 and tyrosyl of CRO66 but allows a couple of hydrogen bonds between the main chain carbonyl group and tyrosyl of Tyr-CRO through a water molecule (Wat2031). The omit map of Asp202-Ala206 to-
gether with CRO66 is shown in Figure 5.2B. In addition, Glu222 has two conformations of the side chain in CatchER with 0.56/0.44 relative occupancy. In one conformation of Glu222, the side chain is considered to be deprotonated and forms a hydrogen-bonding network through Ser205 and Wat2031 to Tyr-CRO66, which is similar to the hydrogen bond interactions in wtGFP. The other conformation of Glu222 has no interactions with CRO66 but only participates in the hydrogen bond network with surrounding residues. The intricate hydrogen bond network around the chromophores of CatchER, wtGFP and wtEGFP is shown in Figure 5.3C, 5.3D and 5.3E(149).

Previous research has reported the profound but opposite effects of residues Thr203 and Glu222 via point mutagenesis and analysis of crystal structures(149-152). T203I in wtGFP retains the 399 nm peak but lacks the 475 nm peak, whereas E222G retains the 480 nm peak but lacks the 395 nm peak(151, 152). Crystal structures of wtGFP and wtEGFP revealed that the side chain of Thr203 can stabilize a negative charge on the chromophore through a direct hydrogen bond with Tyr-CRO66, but the carboxylate of Glu222 can maintain the neutral form of chromophore through the electrostatic repulsion and hydrogen-bonding network via water and Ser205(149). In the CatchER structure, the orientation of Thr203 and proposed protonated form of the chromophore are consistent with its spectroscopic properties. The hydrogen-bonding network via water and Ser205 is achieved by one conformation of Glu222 in deprotonated or negatively charged state. However, even though the other conformation of Glu222 does not contact Thr-CRO66 as in wtEGFP(109), this secondary conformation with almost half occupancy is proposed to be protonated and can no longer maintain the neutral form of chromophore. Therefore, we suggest that the chromophore of CatchER has a mix of neutral and negative charged states, as observed in wtGFP, which has two conformations of Thr203(149).
Overall, the crystal structure of CatchER(apo) has reaffirmed support for the proposed excited-state photon transfer mechanism for the photoisomerization of wtGFP, which is based on the structural data and spectroscopic work(9, 149).

5.3.4 Relationship between mutations and chromophore fluorescence excitation

CatchER was selected from a series of designed Ca\(^{2+}\) sensors by introduction of different mutations to form the Ca\(^{2+}\) binding site, among which D8, D9, D10, D12 are the CatchER variants with selective mutations of the five residues (Tang, in press). They all show an increase at peak 398 nm and a decrease at peak 490 nm to different extents. D8 has only two mutations of S202D and F223E, while the other variants have additional mutations, which indicate that S202D and/or F223E may play important roles in the absorbance change for D8-D10, CatchER and D12. Based on the analysis of structural changes around the chromophore, Thr203 and Glu222 are two key residues that account for the altered chemical environment of chromophore. Therefore, the mutations S202D and F223E, which are located next to Thr203 and Glu222, respectively, might lead to conformational change of these two residues and even the observed main chain shift. Introduction of S202D results in the movement of main chain of residues 202 to 206 closer to the chromophore by 0.7-0.9 Å and the rotation of Thr203, leading to protonation of the chromophore (Figure 5.5A). However, no direct change was observed for F223E next to Glu222, and F223E did not participate in metal binding in CatchER-Ca\(^{2+}\) and CatchER-Gd\(^{3+}\) crystal structures (Figure 5.5B). Mutation S147E appears mainly to function as the anion for interacting with metal cations, while S202D is also involved in metal coordination based on our crystal structures.

Ca\(^{2+}\)-free CatchER exhibited a major absorption peak at 395 nm and a minor peak at 490 nm, which is similar to the wtGFP, with a ratio of 395 nm to 490 nm 3.0 measured in vitro.
(Tang, et. al.). From the crystal structures of Ca2+ free and loaded form CatchER (Fig. 2.), the sidechain of 222 rotated to change the distance of hydrogen bonds between carboxyl group of sidechain Glu222 and Ser205 and hydroxyl group of chromophore. The proposed hydrogen networks surrounding the chromophore are based on the previous reported crystal structure of wtGFP (pdb code: 1EMB) and EGFP (pdb code: 1EMA). The previous reported wtGFP from A jellyfish has two absorption peaks at 390 nm (major) and 490 nm (minor), suggesting a mixture of two forms of chromophore co-existed in one fluorescent protein (Heim, 1996). Though from the DNA sequence alignment, the site directed mutagenesis S65T is the cause of the major difference, however, the chromophore of wtGFP and EGFP can be overlapped well from the crystal structure, but the sidechain surrounding the chromophore exhibited different conformation, especially for Thr203 and Glu222 (Baird, 1997). In wtGFP, the mainchain of Thr203 distantly interacts with the chromophore through a water molecule (Reminton, 1996 and Tsien, 1998), while for EGFP, the polar sidechain hydroxyl oxygen interact oxygen atom of chromophore directly, forming a short hydrogen bond 2.5 Å, and stabilize the chromphore at anionic form, causing a major absorption peak at 490 nm. This stabilization is further enhanced by the special orientation of sidechain carboxyl group of E222 (Fig. 1), the only negative charged residue protrude toward the chromophore, forming a restricted hydrogen network among E222, V61 and T65 conjugated within the chromophore. However, the oxygen of Tyr66 of wtGFP only directly interacts with H2O without forming hydrogen bonds with polar residues, maintained neutral form, contributing to the major absorption peak at 395 nm. The two oxygen atoms of the carboxyl group of E222 are equally partially charged forming hydrogen bonds with Ser205 and chromophore. A hydrogen bridge between the hydroxyl group of T65 and Y66 is formed via E222, S205 and a water molecule, ensuring an efficient electron transfer between
polar residues within the chromophore. An interesting observation of carboxyl group of E222 rotating between Ca2+ free and loaded form CatchER from the crystal structure, altering the distance of the hydrogen bonds between the carboxyl group of E222 to S205 and chromophore. Up to now, the rotation of Glu222 triggered by analyte binding has not been reported, especially correlated with the optical properties change. It is possible that the E222 sidechain rotation in response to Ca2+ may contribute to the fast kinetics of CatchER as a single residue rotation is faster than long-range protein-protein interaction in FRET pair based sensor. However, this sidechain rotation of Glu222 was not observed in comparison of Gd3+_free and Gd3+_soaking CatchER structure (Fig. 4), it is plausible that the residues buried inside the beta-barrel structure of GFP did not exhibit further conformational change after crystallization, even during the metal soaking, though fluorescent intensity of CatchER was dramatically enhanced after adding Gd3+ during in vitro titration. (Ying, please help to check whether this part is correct.) The other key residue Thr203 of CatchER maintained one hydrogen bond between the mainchain oxygen and water close to chromophore in all of metal-free, Ca2+_loaded and Gd3+_loaded form CatchER, similar to the wtGFP, suggesting that the fluorescent intensity of Ca2+_loaded CatchER only recover 50% of EGFP is possibly due to the fixe hydrogen bond network close to the phenol group of Tyr66 of chromophore maintained similar to wtGFP.

5.4 Acknowledgements

The author sincerely thanks Ying Zhang, Florence Reddish, You Zhuo and Dr. Irene Weber’s contribution to this chapter, also thank Dr. Johnson Agniswamy for valuable discussions. The author is grateful for the assistance of the staff at the SER-CAT beamline at the Advanced Photon Source, Argonne National Laboratory. Use of the Advanced Photon Source was sup-
5.5 References

Table 5.1 Major parameters of CatchER crystal structures.

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<th>Complex Name</th>
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### Average B-factors (Å²)

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<td>0.7/0.3</td>
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**Figure 5.1** Structure of EGFP CatchER with the locations of mutated residues (red sticks) Glu147, Asp202, Glu204, Glu223 and Glu225.
Figure 5.2  (a) $F_o-F_c$ omit map showing the chromophore from CatchER-Ca$^{2+}$ contoured at 4.0σ; (b) $F_o-F_c$ omit maps showing residues from D202 to A206 from CatchER(apo) contoured at 3.0σ with chromophore; (c) $F_o-F_c$ omit maps showing the two conformations of Glu222 from CatchER(apo) contoured at 3.0σ.
Figure 5.3 (a)-(c) The hydrogen bonding interactions and selected $F_o-F_c$ omit maps contoured at 4.0$\sigma$ in (a) CatchER(apo), (b) CatchER-Ca$^{2+}$, (c) CatchER_Gd$^{3+}$. Ca$^{2+}$, Gd$^{3+}$ and water molecules are represented as sphere in magenta, cyan and red, respectively. The distance is indicated in lengths Å. The numbers (0.4/0.4, 0.7/0.3) besides the ions are the percentage occupancy of the alternate conformations of the corresponding ions. (d) Superposition of CatchER-Ca$^{2+}$ and CatchER-Gd$^{3+}$; the proteins are shown as cartoon in grey and the chromophore is represented as green sticks. The five mutational residues and ions are shown in sticks and as spheres in magenta for CatchER-Ca$^{2+}$ and in cyan for CatchER-Gd$^{3+}$. The cyan arrow points the overlapped ions Gd$^{3+}$ with Ca$^{2+}$. 
Figure 5.4 Structural scheme of protonated chromophore of CatchER. (a) Protonation states of chromophore of GFP and their responsible excitation wavelength; (b) Absorbance intensity ratio at 395 nm and 488 nm for CatchER and wtEGFP.
Figure 5.5 Schematic diagram of the hydrogen-bonding interactions between chromophore and surrounding residues and water molecules. Hydrogen bonds are shown as dashed lines. The hydrogen bond distance range is set to 2.6-3.2 Å.
Figure 5.6 E222 and T203 are Key Residues to Determine the Optical Properties of wtGFP and EGFP. Proposed hydrogen networks between the chromophore and its neighboring residues. The key residues T203 and E222 are highlighted in box and the absorption and emission spectra (Tsien, 1998) of wtGFP (pdb code: 1EMB) (A) and EGFP (pdb code: 1EMA) (B).
Figure 5.7 Rotation of Glu222 in Comparison to wtGFP and EGFP (S65T). (A) The overlap of the chemical structure of chromophore and Glu222 from wtGFP (orange) and EGFP (black). (B) The absorption spectra of wtGFP and EGFP correlated to their chromophore structure. (C) The distance of hydrogen bonds between the sidechain carboxyl oxygen of Glu222 to hydroxyl group of Ser205 and Tyr66 of wtGFP (black) and EGFP (red).
Figure 5.8 (A) Rotation of Glu222 in Comparison of Ca\textsuperscript{2+}_Free (green) and Ca\textsuperscript{2+}_loaded CatchER (blue) with correlated absorption spectra. (B) The distance of hydrogen bonds formed between carboxyl oxygen and Ser205 and chromophore are measured by Pymol.
CHAPTER 6: MAGNESIUM-BINDING SITES OF PROTEINS

6.1 Biological functions of Mg$^{2+}$

Mg$^{2+}$ is one of the most abundant physiological divalent metals. Similar to total tens of millimolar intracellular Ca$^{2+}$ concentration, magnesium has a total concentration around 25 and 100 mM in prokaryote and eukaryotic cells, respectively. The free intracellular Mg$^{2+}$ concentration is reported around 0.3-0.6 mM with a negligible concentration gradient between plasma membranes, tightly controlled by large amount of Mg$^{2+}$ chelators, such as ATP and parvalbumin. In contrast, the free Ca$^{2+}$ concentration inside different sub-cellular organelles varies from sub-micromolar in nucleus and cytosol to sub-millimolar in endoplasmic reticulum and Golgi. Such significant difference in homeostasis between Mg$^{2+}$ and Ca$^{2+}$ is likely a result of large differences in metal coordination chemistry, binding affinity, selectivity, and chemical properties of intracellular chelate molecules. The Mg$^{2+}$ homeostasis between intracellular and extracellular prokaryotic cells is regulated by several plasma membrane anchored Mg$^{2+}$ transporters of which the high-resolution X-ray crystal structures have been solved recently. The Mg$^{2+}$ transporters have also been found in squid giant axons and barnacle muscle cells in a Na$^{+}$- or ATP-dependent way.

6.2 Metal coordination chemistry

Mg$^{2+}$ has a radius of 0.6 Å that is significantly smaller than that of Ca$^{2+}$ (0.95 Å). The resulting great charge/radius ratio of Mg$^{2+}$ to Ca$^{2+}$ dictates the main differences in coordination properties between Mg$^{2+}$ and Ca$^{2+}$. Different from the pentagonal bipyramidal geometry of binding site of Ca$^{2+}$ surrounded by seven oxygen atoms with average Ca$^{2+}$-O distance about 2.4 Å, up to six oxygen atoms octahedrally coordinate Mg$^{2+}$ with average Mg$^{2+}$-O distance 2.05 Å (Figure 6.1A, B).
Usually at the physiological pH range (around 7-8), Mg\(^{2+}\) is hydroxylated with six H\(_2\)O molecules with a large hydration energy to form a complex with big radius around 5 Å. In addition to protein ligands, Mg\(^{2+}\) also strongly interacts with phosphate ligands from nucleotides such as ATP and DNA due to its high charge/radius ratio. Coordination number and spatial distribution of water molecules surrounding the Mg\(^{2+}\) influence its binding thermodynamics with the protein. Mg\(^{2+}\) and its associated water or phosphate complex binds protein with 3-5 coordinating oxygen atoms from protein, and the sidechain carboxylate oxygen plays a major role in the protein binding ligands. The classification of several representative Mg\(^{2+}\) binding protein sites based on the number of sidechain carboxylate are listed in Table 6.1 Mg\(^{2+}\) often regulates numerous enzymes through water-mediated interaction. Further, the stable octahedron of Mg\(^{2+}\) and higher surface charge also lead to 10\(^4\) times slower exchange rate between the Mg\(^{2+}\) and water than that of Ca\(^{2+}\), which determes that Mg\(^{2+}\) cannot function as a fast trigger metal inside of cells.

In addition to maintaining the three-dimensional conformation of DNA and RNA and interacting with phospholipids to influence the membrane fluidity and permeability, Mg\(^{2+}\) binds diversified classes of proteins such as DNA/RNA polymerases, reverse transcriptases, telomerasers regulate numerous important cellular processes, and a wide-range of enzymes involved in the metabolism of phosphorylation, or the glycolytic and tricarboxylic acid pathways. Many crystal structures of enzymes interacting with Mg\(^{2+}\) have been solved, though debates existing about the possibility of the electron density to be interpreted is in fact not Mg\(^{2+}\) but rather some other metal, or even a water molecule due to the same number of electrons contained by both Mg\(^{2+}\) and water. The types of Mg\(^{2+}\) binding proteins can be classified into several categories based on the arrangement of ligand residues, binding coordination properties, metal binding
specificity, and ligand types such as with cofactors (ATP and other nucleotide triphosphates) in a wide range of enzymes.

6.3 Metal selectivity and affinities of metalloproteins

The selectively binding Mg$^{2+}$ rather than other physiological metals of metalloproteins is achieved by several factors. For the monovalent metals, such as Na$^+$ and K$^+$, the high charge to radius ratio of Mg$^{2+}$ assists the strong electrostatic attraction of binding sites consisting of several negatively charged ligands Asp/Glu, even though these monovalent metals are present at tens to one-hundred millimolar concentration inside the cells.

In contrast to hard metal Mg$^{2+}$ prone to binding oxygen mainly from the sidechain carboxylates of Asp and Glu, the physiological divalent metals such as Zn$^{2+}$ and Cu$^{2+}$ are soft metals prefer to bind nitrogen from the sidechain His, preventing them from rivaling the major Mg$^{2+}$ binding sites, though Zn$^{2+}$ was found to bind different sites from Ca$^{2+}$ in Ca$^{2+}$ binding protein such as calmodulin. Moreover, the approximately $10^6$ lower concentration of free Zn$^{2+}$ and Cu$^{2+}$ than that of Mg$^{2+}$ at $10^{-4}$ M further reduces their occupancy of abundant Mg$^{2+}$ binding sites. The several-magnitude concentration difference of free metals also assists the selectivity of metalloproteins between Mg$^{2+}$ and Ca$^{2+}$, as the latter is at $10^{-6}$ - $10^{-7}$ M. In addition, different from the monodentate ligand interaction between carboxylate oxygen and Mg$^{2+}$, Glu bidentately binds Ca$^{2+}$, enhancing the metal selectivity of EF-hand to Ca$^{2+}$ due to its common appearance at position 12 of the binding loop. Moreover, the calmodulin biological functions are carried out by its interaction with numerous target peptides after a dramatically global conformational change triggered by Ca$^{2+}$ binding in general, however, only minor perturbation of the binding loop was observed during Mg$^{2+}$ interaction, preventing subsequent interaction between Mg$^{2+}$-loaded calmodulin and target peptides.
The distinct ligand chemistry of special metals also dictates metal binding affinity, as the dissociation constants of Mg\(^{2+}\) to numerous intracellular molecules are not lower than \(10^{-4}\) M, in contrast to \(10^{-7}\) M of Ca\(^{2+}\).

6.4 Major types of Mg\(^{2+}\) binding protein sites

The major types of Mg\(^{2+}\) binding protein sites can be classified as: 1) the conserved continuous non-EF-hand peptide containing multiple acidic residues, 2) EF-hand binding motif and Ca\(^{2+}\)/Mg\(^{2+}\) selectivity, 3) the discontinuous binding site formed by sequentially distant residues, 4) protein motif binding multiple Mg\(^{2+}\) ions, 5) Mg\(^{2+}\) binding site assembled by different subunits/ several monomers, 6) Hybrid of phosphate and protein ligand binding site.

6.4.1 The conserved continuous non-EF-hand peptide containing multiple acidic residues.

Different from Ca\(^{2+}\) binding proteins, in which the continuous binding motifs can be identified by primary sequence due to well-established theories and methods(42), it is difficult to define general Mg\(^{2+}\) binding motifs, and only a few short continuous Mg\(^{2+}\) binding sequences with high sequence homology have been discovered among the Mg\(^{2+}\) regulated enzymes, such as - NADFDGD-, and –GDD- mainly identified in different RNA polymerases, -D-NSLYP- and –K-NS(L/V)YG-, found in DNA polymerase, and –YXDD or -LXDD- motifs in reverse transcriptase and telomerase, respectively (153). The acidic residues in bold among these sequences directly binds Mg\(^{2+}\), similar to high preference of D/E as Ca\(^{2+}\) binding ligands. The relatively shorter sequence with fewer binding ligands of Mg\(^{2+}\) binding site in compare to Ca\(^{2+}\) binding site is plausibly due to constant hydrolysis of Mg\(^{2+}\) and fewer coordination number of Mg\(^{2+}\) binding site. A conserved Mg\(^{2+}\) binding sequence \(^{281}\)EFMP\(^{289}\)ELKWS plays an important role in metal selectivity of CorA channel(154). The mutation of E281 and K287 to other residues does not affect the metal selectivity, so the electrostatics of these two residues do not
play significant roles in the biological function of the CorA channel. However, the mutation of E285 will substantially impair the function of the CorA channel, though the variant with Ala or Lys in this position maintained highest activity, suggesting the minimal contribution of electrostatic of this residue. Though these results are distinct from the traditional theory of the strong electrostatic interaction between divalent metal ion and acidic residues, it is hypothesized that this conserved loop mainly interacts with the hydrolyzed Mg$^{2+}$ ion but not with the dehydrated Mg$^{2+}$ ion.

**6.4.2 EF-hand binding motif and Ca$^{2+}$/Mg$^{2+}$ selectivity.**

Endogenous Ca$^{2+}$ binding proteins are mainly EF-hand proteins, classified to Ca$^{2+}$ sensors, like calmodulin, transferring the chemical signals (intracellular Ca$^{2+}$ concentration change) to diversified biological responses (interacting with numerous targeting peptides through Ca$^{2+}$ binding induced conformational change), and Ca$^{2+}$ buffers, like parvalbumin, with strong binding affinity to control the free Ca$^{2+}$ concentration, without experiencing global conformational change.

Due to similarity of coordination chemistry of magnesium and calcium and high physiological Mg$^{2+}$ concentration, Mg$^{2+}$ is often observed to bind endogenous calcium binding proteins. Thus, calcium binding motifs such as EF-hand binding motifs are often classified as calcium specific, magnesium specific or mixed type.

Mg$^{2+}$ competes for Ca$^{2+}$ binding sites at some level, especially in the intracellular environment where both of them are at millimolar total concentration. EF-hand is a Ca$^{2+}$ binding motif with helix-loop-helix structure originally reported in the X-ray structure of parvalbumin which is intracellularly abundant. Other Ca$^{2+}$ binding proteins such as calmodulin, troponin C and the regulatory domain of scallop myosin are reported to have this motif in high-resolution
structure and further defines the geometry of the EF-hand. The canonic EF-hand is featured by a continuous 12 amino acid loop containing multiple D/E occupied at position 1, 3, 5, 9 and 12 as Ca$^{2+}$ binding ligands flanked with two helices. Special computational algorithms have been designed to predict the EF-hand based Ca$^{2+}$ binding sites, based on the structural study of this motif. The alternation of amino acid sequences enables EF-hands bind Ca$^{2+}$ with K$_d$ from $10^{-4}$ to $10^{-9}$ M (34) even they have a similar secondary structure. A recent study demonstrated that a non-Ca$^{2+}$ binding protein fused this isolated 12 amino acid loop originated from the third EF-hand of calmodulin can bind Ca$^{2+}$ with K$_d$ around $10^{-5}$-$10^{-4}$ M even without two helices, though these helices can influence the Ca$^{2+}$ binding capacity and maintain the secondary structure of EF-hand. This short loop wraps around the Ca$^{2+}$ or Mg$^{2+}$ ion to form a binding cavity in pentagonal bipyramid and octahedral coordination, respectively, with different binding affinities. One of the key factors to determine the coordination is the amino acid at position 12, as Glu with long sidechain favorably forms bidentate with Ca$^{2+}$ whereas Asp interact Mg$^{2+}$ monodentately. In general, EF-hand motif exhibits high Ca$^{2+}$/Mg$^{2+}$ selectivity featured with glutamate occupied this position. Moreover, the Ca$^{2+}$/Mg$^{2+}$ metal selectivity and binding affinity of this motif can determine the biological functions of some EF-hand proteins, such as calmodulin in cytosol and parvalbumin in muscle.

Calmodulin has four canonical EF-hand motifs coupled (two pairs) distributed in N and C domains, respectively, with hydrogen bonds formed between the loops and positive cooperativity within each pair. The calmodulin interaction with Mg$^{2+}$/Ca$^{2+}$ was explored by nuclear magnetic resonance (NMR) heteronuclear single quantum coherence (HSQC) titration, and the results suggest(67): 1) Ca$^{2+}$ triggered global conformational change, Mg$^{2+}$ binding only influences the local loop. 2) Mg$^{2+}$ traveled to the same location as Ca$^{2+}$, but possibly binding differ-
ent ligands. 3) Mg\(^{2+}\) has a preference of binding EF-hand loop I and IV. Only high concentration of Mg\(^{2+}\) at a ratio of 1:10 (protein: Mg\(^{2+}\)) can trigger significant conformational change in loops II and III. 4) Mg\(^{2+}\) binding will influence calmodulin binding affinity to its target peptide. 5) The presence of Mg\(^{2+}\) will not influence the binding of Ca\(^{2+}\), as Ca\(^{2+}\) can still induce conformational change in the presence of Mg\(^{2+}\). 6) The presence of Mg\(^{2+}\) will occupy more than 90% of loop I of calmodulin and slow down the k\(_{off}\) value of Ca\(^{2+}\). The metal selectivity between Ca\(^{2+}\)/Mg\(^{2+}\) are determined by the metal binding preference of specific loops, and the biological function of calmodulin is specially carried by interacting Ca\(^{2+}\) as only Ca\(^{2+}\) loaded calmodulin experiencing significant conformational change to interact numerous targeting peptides.

Parvalbumin is a 12 kd endogenous calcium buffer protein with two canonical EF-hands, however, no cooperativity among the binding sites was demonstrated though hydrogen bonds were reported to exist between the two antiparallel binding loops. Different from calmodulin, the metal selectivity of parvalbumin between Ca\(^{2+}\)/Mg\(^{2+}\) is achieved by a different binding coordination. The Ca\(^{2+}\) ion has a completed binding of 5 ligands of the binding loop and bidentately binding to glutamate at position 12, however, Mg\(^{2+}\) only binds monodentately to the glutamate. The metal binding specificity of Ca\(^{2+}\) and Mg\(^{2+}\) is determined by glutamate or aspartate in position 12 of the loop, respectively. The D/E replacement at position 12 determining the metal selectivity has been supported by computational simulation, based on E101D mutant\((155)\). The in-depth study of coordination difference between Mg\(^{2+}\)/Ca\(^{2+}\) binding is due to high-resolution X-ray crystal structures of metal loaded parvalbumin that have been solved. Moreover, parvalbumin does not interact with any peptide, so neither Ca\(^{2+}\) nor Mg\(^{2+}\) binding can trigger significant conformational change (Figure 6.1C,D).
Calbindin D9k has one pseudo EF-hand. In contrast to parvalbumin and calmodulin featured with Glu in position 12 of the binding loop, Asp in this position decreases the metal selectivity between Ca\(^{2+}\) and Mg\(^{2+}\), as the sidechain of Asp is too short to coordinate Ca\(^{2+}\) with bidentate, leaving an unsealed binding cavity in comparison to Ca\(^{2+}\) loaded EF-hand. (Figure 6.2A) Similar to calbindin D9k, the position 12 of RLC loop of scallop myosin is also Asp, providing a more preferential site for Mg\(^{2+}\).

For the other endogenous EF-hand proteins, Mg\(^{2+}\) binding also helps to stabilize the conformation of protein from the molten globule apo state in the absence of Ca\(^{2+}\). Calcium-and integrin-binding (CIB) protein, calcium binding protein 1 (CaBP1) and guanulyl cyclase activating protein 1 (GCAP1) are still able to respond to sub-micromolar Ca\(^{2+}\) in the presence of millimolar Mg\(^{2+}\).

### 6.4.3 The discontinuous binding site formed by sequentially distant residues.

A highly conserved Mg\(^{2+}\) binding motifs containing only three discontinuous residues (DED) has been discovered in the RNase H family \(156\), supported by the Mg\(^{2+}\) loaded X-ray crystal structures of three RNase H from E. coli (1rdd), human immunodeficiency virus type 1 (HIV-1) (1o1w), and maloney murine leukemia virus (MMLV) (2hb5). The secondary structure of each individual residue is beta-sheet (first D), alpha-helix (E), and a connection between alpha-helix and beta-sheet (second D). A distinct equilibrium of bidentate and monodentate interaction between the second D and Mg\(^{2+}\) was discovered in the MMLV RNase H, whereas the other sidechain carboxylates of DED motif from E. coli, HIV-1 and the first D and E from that of MMLV monodentately bound Mg\(^{2+}\) (Figure 6.2B).
6.4.4 Protein motif binding multiple Mg\(^{2+}\) ions.

Two distinct protein motifs binding multiple Mg\(^{2+}\) ions were discovered in a recent report of high-resolution structure of bacterial Mg\(^{2+}\) transporter MgtE(157). A discontinuous sequence containing three glutamates (E216, 255 and 258) and two aspartates (D259 and 418) with their sidechain carboxylates coordinating two Mg\(^{2+}\) ions formed the first metal-protein binding site. The second site was composed by sidechain carboxylates of five aspartates (D91, 95, 226, 247 and 250), one glutamate (E59) and mainchain hydroxyl from glycine 136 and alanine 223, binding four Mg\(^{2+}\) ions (Figure 6.2C). Though some binding ligands were shared by more than one Mg\(^{2+}\) ion, the octahedral coordination geometry of Mg\(^{2+}\) preserved, supported by the other octahedron metal Co\(^{2+}\) and Ni\(^{2+}\) binding these two same sites of MgtE in new crystal structures. Such multiple divalent metals clustered within a high negatively charge-dense cavity is very similar to calcium binding proteins such as C2 domain of protein kinase c-beta and cadherins with more than two Ca\(^{2+}\) ions bind to the protein.

6.4.5 Mg\(^{2+}\) binding site assembled by different subunits.

The integrins, a family of cell adhesion molecules non-covalently interacting diversified ligands to integrate the extracellular and intracellular informations, play important roles in immunology. In vertebrates, the integrins are heterodimers containing 1 alpha and 1 beta subunit, and a total of 24 different alpha beta pairs are formed by 18 alpha and 8 beta subunits. The Mg\(^{2+}\)-binding sites in integrins have been reported in crystal structures of several subfamilies and are crucial to influence integrins binding their ligands. One universal magnesium binding site called metal ion dependent adhesion site (MIDAS) was initially discovered on the surface of alpha subunit (alphaM I domain) of integrin CR3, and the high-resolution crystal structure showed that MIDAS was formed by a continuous binding loop featured by DXSXSD.
and two Asp from the other part of the protein. A single Mg$^{2+}$ octahedrally coordinated with three sidechain hydroxyl oxygen atoms of S142, S144 and T209, two water oxygen atoms and one carboxylate oxygen atom of E314 of a neighboring A domain with short bond distance around 2.0 Å. After the first shell was fully occupied by six ligands, D140 and D242 did not directly coordinate Mg$^{2+}$ but formed hydrogen bonds with the binding ligands and maintained in the second shell. One carboxylate oxygen atom from both D140 and D242 bound to the same water molecule and the other one bound to the sidechain hydroxyl oxygen atom, respectively (D140 to S142 and D242 to S144). However, this open-form alphaM I domain MIDAS can switch to close-form via replacing E314 by D242 as Mg$^{2+}$ binding ligand, maintaining the same number of negatively charged ligand between these two forms. The site-directed mutagenesis of the second shell binding ligand D140A and D242A not only abolished the affinity of the recombined protein to divalent metal, but also reduced its binding to ligand iC3b and NIF, and a similar affinity decrement was observed in the presence of divalent metal chelators, EDTA without introducing mutations, suggesting the crucial role of Mg$^{2+}$ in the protein-protein interaction for achieving the biological functions of integrins.

Mg$^{2+}$ binding site also influences the binding between integrin and ligands from other subfamilies. Instead of forming Mg$^{2+}$ binding sites within integrin, the Mg$^{2+}$ coordination ligands can come both from the integrin and its ligands. High-resolution crystal structures showed that Mg$^{2+}$ bound to MIDAS on the surface of αL I domain, and the carboxylate group of E37 from the ligand ICAM-3 also directly coordinated Mg$^{2+}$ (PDB codes: 1T0P) (Figure 6.2D). A similar Mg$^{2+}$ binding site of lymphocyte function-associated antigen-1 (LFA-1) was identified, and the site-directed mutagenesis of this identified Mg$^{2+}$ binding site decreased the binding affinity between LFA-1 and its ligand ICAM-1, suggesting that the Mg$^{2+}$ ions rather than particular resi-
dues play an important role of Integrin and ligand binding. Moreover, E34 of ICAM-1 directly coordinated Mg$^{2+}$, as an analogy to E37 of ICAM-1 in binding $\alpha L$ I domain.

Moreover, the crystal structure of maltoporin complex showed an assembled Mg$^{2+}$ binding site formed within a small triangle space among three parallel beta-can maltoporin monomer with three D78 as binding ligands provided by each monomer (Figure 6.2E).

### 6.4.6 Hybrid phosphate and protein ligand binding site.

Magnesium binding sites of protein also widely contain the binding ligands from the phosphates and nucleotides functioned as cofactors. One example is the DNA mismatch repair protein (MutS) which plays an important role in recognizing and repairing mismatched DNA base pairs and inserts or deletes short DNA sequences. Magnesium influences DNA mismatch repair protein (MutS) molecular switch, as it can accelerate the ATP binding process and enhance the binding affinity, influencing the switching of DNA mismatch repair. These conclusions are supported by the mutation experiments that the impaired Mg$^{2+}$ binding site will prevent the fast switching and DNA mismatch repair(158).

Magnesium binding ligands of MutS are a hybrid of phosphate from ATP or ADP and the sidechain hydroxyl group of S621, and four water molecules directly coordinate with Mg$^{2+}$ in the first binding shells. The carboxylate oxygen atoms of E694 and D693 form hydrogen bonds with water molecule and S621 in the second shell to stabilize the binding site (Figure 6.2F). The non-Mg$^{2+}$-binding crystal structure of site-directed mutagenesis of D693N suggests the significance of this stabilization to the Mg$^{2+}$ binding.

### 6.5 Computational algorithm to predict and design Mg$^{2+}$/Ca$^{2+}$ binding sites

Based on rapid understanding 3D structures of Mg$^{2+}$ binding motifs and large databank of structures available, the prediction of Mg$^{2+}$ binding domains has evolved from amino acid
sequence alignment to 3D structure homology based computational algorithms(153), which overcomes the limited Mg$^{2+}$ binding sequence patterns available, difficulties of identifying discontinuous Mg$^{2+}$ binding motifs, and they are efficient to screening large-scale databank. The optimized prediction parameters rely on the common features of existing Mg$^{2+}$ binding cavities, and the concept of describe Mg$^{2+}$-binding ligands by the first and second shells is also used in computational algorithms to predict Ca$^{2+}$-binding sites independently(101).

6.6 Acknowledgements

The authors are grateful for Natalie White's helpful editing and Yunmei Lu's insight suggestions.

6.7 References

6.8 Figures and legends

Figure 6.1 Comparison of coordination difference between Ca$^{2+}$ binding site and Mg$^{2+}$ binding site. (A) Octahedral coordination of Mg$^{2+}$ binding site. Mg$^{2+}$ (black circle) locates in the center, surrounded by 1 H$_2$O (empty circle), 4 protein ligands (black circle) and 1 Asp or Glu as an anchor (gray circle) in which one sidechain carboxylate oxygen atom monodentately interact Mg$^{2+}$. (B) Pentagonal bipyramidal coordination of Ca$^{2+}$ binding site. Ca$^{2+}$ (black circle) locates in the center, surrounded by 1 H$_2$O, 4 protein ligands (black circle) and 1 Glu as an anchor (gray circle) in which two sidechain carboxylate oxygen atoms bidentately interact Mg$^{2+}$. (C) Mg$^{2+}$ loaded EF-hand II of parvalbumin (PDB code: 4PAL). The Glu (cyan stick) in position 12 of the binding loop monodentately (black line) interacts Mg$^{2+}$ (blue circle). (D) Ca$^{2+}$ loaded EF-hand II of parvalbumin (PDB code: 4CPV). The Glu (cyan stick) in position 12 of the binding loop bidentately (black line) interacts Ca$^{2+}$ (orange circle).
Figure 6.2 Major classes of Mg<sup>2+</sup> binding sites. (A) Mg<sup>2+</sup> loaded pseudo EF-hand of calbindin D9k (PDB code: 3ICB). The E65 in position 12 of the binding loop is too far to interact Mg<sup>2+</sup>. (B) Discontinuous Mg<sup>2+</sup> binding site of RNase H from maloney murine leukemia virus (MMLV) featured with DED residues (PDB code: 2hb5). (C) Multiple Mg<sup>2+</sup> ions binding site in Mg<sup>2+</sup> transporter MgtE (PDB code: 2ZY9). Four Mg<sup>2+</sup> ions clustered within one domain, and three Mg<sup>2+</sup> ions partially share the same binding ligands. (D) Mg<sup>2+</sup> binding site formed by metal ion dependent adhesion site (MIDAS) of integrin αL I domain and E37 of its ligand ICAM-3 (PDB code: 1T0P). (E) Mg<sup>2+</sup> binding site is assembled by three maltoporin monomers (red, green and yellow), and each monomer provides D78 (blue stick) as a binding ligand. (PDB code: 1MPM). (F) Hybrid phosphate and protein binding site from DNA mismatch repair protein (MutS) (PDB code: 1W7A). The binding residues (blue stick) from protein are S621, D693 and E694, and the β-, γ-triphosphate (orange stick) of ATP bind Mg<sup>2+</sup>.
Table 6.1 Classification of representative Mg$^{2+}$ binding proteins based on the number of sidechain carboxylate.

<table>
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<td>D53</td>
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CHAPTER 7: DESIGNING A NOVEL CLASS OF THROMBIN SENSORS WITH FAST RATIO-METRIC RESPONSE.

7.1 Abstract

Thrombin is involved in numerous cellular signaling pathways and plays a crucial role in blood coagulation, which converts fibrinogen to fibrin clot and support platelet aggregation. It is also vital for trafficking of inflammatory cells into sites of injury and tissue repair by enhancing expression of endothelial cell. The abnormal activity of thrombin will result thrombosis, one of the most common factors to cause thromboembolism, myocardial infarction and stroke.

Currently, there is a strong need to develop protease sensors with strong protein substrate specificity and quantitatively measurement of the enzymatic action in cell/vivo in real time. Here we report our achievement in designing a novel class of single EGFP-based thrombin sensors. These designed protease sensors exhibit rapid kinetic responses and large ratiometric fluorescence change.

7.2 Biological functions of thrombin and detection methods.

Thrombin has been researched in various aspects since it was discovered in the 19th century. Thrombin is involved in many different processes, for example, cell signaling and memory, but the most important function of it is in the blood coagulation. A very important concept, thrombosis is the most common reason to cause the death no matter via venous thromboembolism, myocardial infarction or stroke, which all involve the inappropriate activity of thrombin.

Thrombin biological function can be divided to be two major parts: the first one is related with the coagulation cascade, as thrombin convert fibrinogen to fibrin clot and support platelet aggregation. The second role is related with cellular effects, as thrombin is vital for traf-
ficking of inflammatory cells into sites of injury. It also plays a major role in tissue repair by enhancing expression of endothelial cell.

Thrombin inhibitors are useful in medical applications, as the inappropriate amount of thrombin expression will cause venous thrombosis, inflammatory and fibrotic disorders, neuronal disease and cancer. Anti-thrombin therapies are now widely used in clinics with two major commercial inhibitors: heparin and warfarin.

Thrombin has a small A-chain and a catalytic B-chain. The catalytic tetrad, which involves of His 57, Asp 102, Ser 195 and Ser 214 is present at the bottom of a canyon-like cleft shaping the catalytic pocket. Thrombin’s catalytic activity contains recognition domains, which bind to the substrates, and insertion loops, which regulate the stability of the enzyme(94).

Thrombin is a highly specific cleavage enzyme and from this table, it is found that the cleavage site is Arg-Gly or Arg-Ser preference. The thrombin optimum recognition sites of the substrate now have a universal standard after more than two decades research. In the optimized sequence of P4-P3-Pro-Arg-P1’-P2’, P3 and P4 should be hydrophobic amino acid and P1’ and P2’ are nonacidic amino acids according to the study of the natural substrates of thrombin(159). In 1990s, small peptides which are composed of P4 to P1 sequence and a leaving group which function as a cleavage indicator were composed for the study of optimizing the P4 to P1 sequence(95). Thrombin and interaction models are well studied for the P1’ to P3’ sequence optimizing(160). Another method to optimizing P1’ to P3’ is grafting thrombin recognition sites into a protein as a frame and then fixing P4 to P1 sequence but mutate P1’ to P3’ amino acid randomly to construct the P1’ to P3’ library and then select the most optimized sequence according to the steady-state kinetic parameters(161).
In this project, the major purpose is to construct a sensitive thrombin sensor both in vitro and in vivo which has advantages compared to the existed thrombin sensors. Currently, the major thrombin sensors can be divided to be three classes, first, electrode signal changed sensors, for example: DNA aptamer with nano-particles(162); the second one, small peptide with leaving group(163); the third one, the fluorescent resonance energy transfer pairs (FRET)(164). But all of them have limitation, for the nano-particles, it cannot be applied into the living cell image although it is very sensitive to detect thrombin. The P1' to P3' sequence cannot be optimized for the small peptide with leaving group as all of these sites are occupied by leaving group. For the FRET pairs, the photobleaching problem is very common during the experiment as the structure of the whole protein is well exposed to the solution(165). Another problem which can not be ignored is that the proton transfer can happen between different molecules if the distance between the donor and acceptor is appropriate so that the signal change does not specially come from the proton transfer inside one molecule but also from the molecule and molecule interaction(165). In this project, studies of the steady state kinetic parameters were carried out to promote the detection of thrombin activity in living cells with real time.
Figure 7.1 The major biological functions of thrombin and related diseases.
7.3 Structural analysis of complementary interaction between the active site of thrombin and cleavage site of substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>P4</th>
<th>P3</th>
<th>P2</th>
<th>P1</th>
<th>P1'</th>
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Figure 7.2 The statistic analysis of the cleavage sequence from P4 to P3’ of natural thrombin protein substrates, which interacts S4 to S3’ sequence of thrombin active site, respectively, shown as a lock and key scheme on the top. The thrombin substrates P2 and P1 site are highly conserved for Pro and Arg, respectively. The P3 and P1’ sequence (in red rectangle) are highly variable.

7.4 Our strategy to design single GFP-based ratiometric protease sensors.

Thrombin sensor designed by our laboratory is one variant of enhanced green fluorescent protein which is called EGFP N188R. The majority of the structure is the same as wide type EGFP while E hand and F hand come from calmodulin is grafted into it. Between the E
hand and F hand, there is one linkage which involves the cleavage site of thrombin. The expression process of thrombin sensor is similar to the one of the other variants of EGFP.

Figure 7.3 Scheme of grafting approach to engineering single EGFP-based thrombin sensor by genetically inserting a short sequence with a desired thrombin cleavage sequence into the fluorescent sensitive location of EGFP.
7.5 **Key factors to determine the substrate specificity.**

Thrombin is a trypsin like enzyme and it comes from the chymotrypsin family of serine protease; similar to trypsin, it has a positive charged amino acid at the P1 position of the cleavage bond.

Thrombin exhibits high recognition specificity of substrate. Arg-Gly or Arg-Ser is optimized recognition sequence in P1-P1’. Hydrophobic amino acid in P3-P4 and non-acidic amino acids in P2’-P3’ are favorable according to the static study of the natural substrates of thrombin.
This figure shows the stereo pairs of surface of thrombin. Colors are according to electrostatic potential, A, (red for negative and blue for positive), or hydrophobicity (green), B. The active site is filled in this figure by the reactive center loop of AT from P4 to P2' (yellow rods). Substrate recognition within the active site depends on the preference of interactions between

Figure 7.5 Electronic potential of thrombin surface and sequential interaction between thrombin active site and substrate P2' to P4.
the P1 residue and the S1 pocket, and the aryl-binding pocket is between hydrophobic residues N-terminal to P1 (often P2 proline and an aromatic residue at P4) in the hydrophobic groove. The active site cleft of thrombin is unusually deep because of the presence of the 60- and c-insertion loops above and below the active site. Two basic exosites on the surface of thrombin have been identified as critical for substrate and cofactor recognition: the so-called anion-binding exosites I and II (ABEI and ABElI). Although the figure shows a wide open thrombin active site, thrombin can exist in a less active, closed conformation without cofactor, substrate or Na+, which co-ordinates near the site indicated.

7.6 Methods

*Bacterial expression of protein.* The thrombin sensor DNA sequence is transferred into the E. coli cell BL21 (DE3). LB is the cell growth media. 0.03uM Kanamycin is used to select the clone of interest. When the O.D 600 reaches to be 0.6, IPTG is added into the media to induce the T7 promoter. From the cell growth curve above, it shows that the final O.D 600 value will reaches 2.200.

*Kinetic study of Thrombin Sensor with Michaelis Menten equation.* In order to compare the specificity of the thrombin sensor designed in our laboratory and the commercial ones, it is necessary to know the steady state kinetic parameters of the sensor. Two experiments have been done for the kinetic study. Both of them react in the high salt Tris buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl$_2$; pH=8.0). The absorbance change of the substrate is used to monitor the initial rate of the steady state kinetics.

The Michaelis Menten equations are based on the mathematical steady state kinetic interaction model between a single substrate and enzyme.
E represents the enzyme; S represents the substrate. ES is the complex of enzyme and substrate as an intermediate. P is the final product. $k_f$ is the forward reaction rate constant, $k_r$ is the reverse reaction rate constant. $k_{cat}$ is the turnover number, the maximal number of substrate molecules converted to product per enzyme molecule per second. The enzyme and substrate interaction step is reversible.

$$v_0 = \frac{V_{\text{max}} [S]}{K_m + [S]}$$

$$\frac{1}{v_0} = \frac{K_m + [S]}{V_{\text{max}} [S]}$$

$$\frac{1}{v_0} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \frac{1}{[S]}$$

$v_0$ represents the initial rate, $V_{\text{max}}$ is the maximal rate constant. $K_m$ is the substrate concentration at which the reaction rate is at half-maximum, and also indicates the affinity between substrate and enzyme. $[S]$ is the substrate concentration. The condition for this equation is that the substrate concentration is much higher than that of enzyme.

In order to get the Lineweaver-Burk (LB) plot, firstly the steady state kinetic parameters should be obtained. Y intercept is the value of $1/V_{\text{max}}$, X intercept is the value of $-1/K_m$, slope is the value of $K_m/V_{\text{max}}$. $[S]$ is the concentration of the substrate while slope is the ratio of the absorbance and the time. When the substrate is completely cleaved, absorbance of the product is measured to calculate the ratio of the absorbance and the concentration which is approximately to be a constant 0.0374 $\mu$M$^{-1}$. Then the $V_0$ is obtained from the slope value divided by the constant 0.0374 $\mu$M$^{-1}$. 
7.7 Results

7.7.1 Expression and purification of our designed thrombin sensors.

In order to monitor the quantity of the product produce after substrate catalyzed by the enzyme, the relationship between the concentration of product and the intensity of the absorbance change is constructed. The absorbance at 490 nm is measured after thrombin sensors of different concentrations cleaved by thrombin for overnight on purpose to calculate the extinction coefficient constants. The constants are different according to the influence of different inserted sequence to the EGFP and the data are shown in table below.

Table 7.1 The extinction coefficient constants of absorbance at 490 nm of designed thrombin sensors.

<table>
<thead>
<tr>
<th>Thrombin sensor</th>
<th>Extinction coefficient constants (ε) (µM⁻¹ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin sensor I</td>
<td>0.0356 ± 0.0056</td>
</tr>
<tr>
<td>Thrombin sensor II</td>
<td>0.0307 ± 0.0018</td>
</tr>
<tr>
<td>Thrombin sensor III</td>
<td>0.0203 ± 0.0023</td>
</tr>
</tbody>
</table>

7.7.2 Comparison of dynamic range

The dynamic range indicates quantity of signal change before and after enzyme catalysis so that the absorbance at 490 nm and 398 nm is measured and the ratio is calculated as referred previously. From the table showed below, thrombin sensor I has the largest dynamic range and sensor II and III have similar and comparatively smaller dynamic range which according to the different cleavage site design.
Table 7.2 The dynamic range in term of absorption ratio 490nm/398 nm of designed thrombin sensors after and before cleaved by thrombin.

<table>
<thead>
<tr>
<th>Thrombin sensor</th>
<th>Dynamic range (Dr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin sensor I</td>
<td>2.716 ± 0.055</td>
</tr>
<tr>
<td>Thrombin sensor II</td>
<td>2.050 ± 0.271</td>
</tr>
<tr>
<td>Thrombin sensor III</td>
<td>1.815 ± 0.086</td>
</tr>
</tbody>
</table>

7.7.3 Thrombin cleavage monitored by SDS PAGE.

At first, in order to testify that the sensor can be cleaved by thrombin, a very low concentration of enzyme cleavage experiment is applied to prove that. The final thrombin concentration is 0.48X10⁻³ µU (1:50), 0.24X10⁻³ µU (1:100), 0.048X10⁻³ µU (1:500) and the substrate concentration is 20 µM. The reaction system is in the high salt tris buffer. (Tris 50 mM, NaCl 150mM, CaCl₂ 2.5mM; pH=8.0).

SDS-PAGE and UV are applied to monitor the cleavage process and the SDS-PAGE shows that the substrate is obviously cleaved although in such a low concentration of enzyme while the UV figure does not show an apparently change.
• Marker
• N188R Before cleavage
• N188R 1:50 cleavage 20min
• N188R 1:50 cleavage 45min
• N188R 1:50 cleavage frozen 5d
• N188R 1:100 cleavage 20min
• N188R 1:100 cleavage 45min
• N188R 1:100 cleavage frozen 5d
• N188R 1:500 cleavage 20min
• N188R 1:500 cleavage 45min
The figure above shows the absorbance does not change obviously although the substrate is cleaved. It is attributed to the high concentration of substrate which overcomes the absorbance change caused by the new products.

Figure 7.5 The molecular size of cleaved thrombin sensors investigated by SDS-PAGE. (A) Thrombin sensor 1 cleaved by thrombin at the concentration ratio of 1:50, 1:100, 1:500 (thrombin: sensor). (B) Thrombin sensor 1 incubated with thrombin for 15, 30, 60, 120, 240 minutes and overnight. (C) Thrombin sensor 2 incubated with high does thrombin for 5, 10, 20 and 60 minutes.
In order to see the absorbance change, another experiment with high concentration of enzyme is applied to demonstrate it.

The enzyme concentration is increased to be 10U/ml in the reaction system while the substrate concentration is fixed to be 20.84 µM. The reaction buffer is the same while the temperature increased to be 37 °C. Samples are collected during the experiment at specific time points.

The figure above shows the 280 nm absorbance change. Although it is supposed that the 280 nm abs will not change quite a lot, from this figure it is found that the overall trend is the abs increase after cleavage.
The figure above shows the absorbance change after thrombin is added. From this figure, it is found that the absorbance change obviously at this concentration of enzyme. Although the last data is not very accurate as it shifts, the overall trend is visible. The absorbance at 490 nm increases while the absorbance at 398 nm decreases.

These two SDS-PAGEs are applied to monitor whether the overnight cleavage is completely. After cleavage, the initial substrate is separated to be two parts and in the SDS-PAGE gel, compared with the sample without cleavage, there are two new bands with small molecular weight generate. From these two figures, the 10 U enzyme cleavage is not complete.

7.7.4 The steady state kinetic study of thrombin sensor with michaelis menton equations.

Table 7.3 The detected initial velocity ($V_0$) and ratio of initial velocity to enzyme concentration ($V_0/E$) in response to 25 unit of thrombin at different substrate concentrations of designed single EGFP-based thrombin sensors 1.

<table>
<thead>
<tr>
<th>[S] (µM)</th>
<th>$V_0$</th>
<th>$V_0$ (µM/s)</th>
<th>$V_0/E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.741</td>
<td>1.4954</td>
<td>0.0004</td>
<td>0.0063</td>
</tr>
<tr>
<td>5.2992</td>
<td>2.8544</td>
<td>0.000763</td>
<td>0.012025</td>
</tr>
<tr>
<td>8.4057</td>
<td>3.9074</td>
<td>0.001045</td>
<td>0.016461</td>
</tr>
<tr>
<td>10.461</td>
<td>4.5918</td>
<td>0.001228</td>
<td>0.019344</td>
</tr>
<tr>
<td>16.309</td>
<td>6.1443</td>
<td>0.001643</td>
<td>0.025884</td>
</tr>
<tr>
<td>21.791</td>
<td>6.9235</td>
<td>0.001851</td>
<td>0.029167</td>
</tr>
<tr>
<td>35.678</td>
<td>8.6935</td>
<td>0.002325</td>
<td>0.036623</td>
</tr>
</tbody>
</table>
The steady state kinetic parameters can be easily derived from the LB double reciprocal plot, so $1/V_0$ and $1/[S]$ are calculated from the original data.

As $1/V_0=1/V_{\text{max}} + K_m/V_{\text{max}}*1/[S]$ is the LB equation, $y = 1767 + 43808x$ which is derived from the LB plot can be fitted to be $1/V_{\text{max}} = 1767$ and $K_m/V_{\text{max}} = 43808$, so $K_m = 24.79 \mu M$.

From the derivation above, it is found that the $K_m$ is around 20 $\mu M$ while that experiment focused on the low concentration of the substrate so that the points around 20 $\mu M$ are not enough for the accuracy. Another problem is that the noisy of the initial rate plot is very huge. In order to reduce the noisy, longer time of reaction values should be recorded and the concentration of the enzyme should be increase as the vibration of the solution will cause huge errors to the final results. According to the drawback factors referred above, another experiment is designed in order to make remedy of that.

In this experiment, 25 Units of thrombin is added into each reaction system which is 2.5 times of the previous experiment. Seven different concentrations of the enzyme are applied for the curve fitting. The calculation process and the equation is the same as above.

The kinetic parameters $k_{\text{cat}}$, $K_m$ and $k_{\text{cat}}/K_m$ are calculated by measuring enzymatic reaction initial rate according to the intensity of absorbance at 490 nm change because of the huge dynamic range change at this absorbance wavelength. Three sensors are composed systematically in order to discover optimized linker location and cleavage sequence. Sensor I is designed as wild type EGFP inserted with calmodulin EF-hand III as a cleavage linker from No. 172 amino acid and the thrombin cleavage site is designed inside the loop as indicated in table I. Sensor II and sensor III also have EF-hand linker at the same insert location of EGFP as sensor I but the cleavage sequence is designed into the F hand. From the data showed in the table below, the specificity of enzyme to substrate which is represented by the parameter
of $k_{cat}/K_m$ is increased by about 30 fold and the $k_{cat}$ increases by 4 fold and the $K_m$ decrease about 5 to 10 fold after the cleavage site and sequence change according to the comparison of sensor I and sensor II and III.

Figure 7.7 Steady-state kinetic study of designed thrombin indicator 5 by fitting the initial rates of absorption change with Michaelis Menten equation.
Table 7.4 Summary of the steady state kinetic parameters of designed single EGFP-based thrombin sensors 1-5 in compared to natural thrombin substrate fibrinogen A and small synthesized peptide-based thrombin sensor EGR-pNA.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Sequences</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor 1</td>
<td>KDGR</td>
<td>GY</td>
<td>0.057 ± 0.01</td>
<td>20.92 ± 0.96</td>
</tr>
<tr>
<td>Sensor 2</td>
<td>FNPR</td>
<td>GF</td>
<td>0.27 ± 0.02</td>
<td>4.22 ± 0.69</td>
</tr>
<tr>
<td>Sensor 3</td>
<td>FNPR</td>
<td>SF</td>
<td>0.23 ± 0.01</td>
<td>2.86 ± 0.28</td>
</tr>
<tr>
<td>Sensor 4</td>
<td>FTPR</td>
<td>GF</td>
<td>0.67 ± 0.06</td>
<td>11.4 ± 1.8</td>
</tr>
<tr>
<td>Sensor 5</td>
<td>FTPR</td>
<td>SF</td>
<td>0.93 ± 0.14</td>
<td>12.8 ± 3.5</td>
</tr>
<tr>
<td>Fibrinogen A*</td>
<td>GGVR</td>
<td>GP</td>
<td>75 ± 5 75 ± 5</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>EGR-pNA*</td>
<td>EGR</td>
<td>pNA</td>
<td>0.15 ± 0.02</td>
<td>2540 ± 270</td>
</tr>
</tbody>
</table>
7.8 Discussion

**Site 172 of EGFP as the linker insertion location.** EGFP-based thrombin sensors were designed by fusing one flexible linker containing enzymatic cleavage site into the EGFP as the scaffold. The efficiency of the sensitivity was monitored both by ratiometric fluorescence and absorbance intensity change at specific wavelengths. The previous work of creating EGFP-based calcium sensors and trypsin sensors in our laboratory provided the valuable information about the fluorescence-sensitive site either for metal ions binding or enzymatic cleavage. Finally, we focused on EGFP site 172 as the linker insertion location which has both the optimized dynamic range before and after sensors cleaved by thrombin and the fluorescence
quantum of the intact protein compared with other different sites such as 144 and 157. The rationale for the scarce qualified insertion sites in EGFP is that it should satisfy two limitations: one is that both of the specificity of wavelength and the quantum of fluorescence should survive after the linker inserted into the wild type EGFP; and the other one is that the fluorescence change should be linearly related with the activity or the concentration of the enzyme at least at a restricted range such as 0-150 nM which covers all of the thrombin concentrations in physiological condition. Due to the long sequence (more than 30 amino acids) and bulk conformation (variants of whole or partial EF-hand III of calmodulin) of the inserted linker which may impair the hydrophobic beta-sheet can around the chromophore to quench the fluorescence as a result, our discovered qualified sites were less than the reported ones (166) and only site 172 was committed conductive to render EGFP sensitive to proteolysis with our standards.

The rationale of EF-hand as the linker. The coinage of posing the EF-hand III of human calmodulin as the protease cleavage linker was derived from the EGFP-based calcium sensors generated in our laboratory. Compared to most of the reported protease sensors’ cleavage linkers which were only the loops with amorphous conformation involving the enzyme recognition sites, our EF-hand linker, with its figurate helix-turn-helix motif, was supposed to take advantage to protrude the protease recognition site alienated from the rigid beta sheets out of the bulk EGFP scaffold much more, which was conductive to avoid the stereo hindrance happening during the protease active sites binding to the recognition sites. The loop, however, between the two helixes and functioned as the calcium binding pocket initially, was not the optimal candidate mutated to be protease recognition sites according to the more than 20 fold lower enzymatic specificity indicated by the \( \frac{k_{\text{cat}}}{K_m} \) of sensor I compared to the other sensors.
in the table mentioned previously. The conceivable reason is that the mutant P1 site Arg which was initially Asn and with other four ligands from this loop were coordinated to calcium in a pentagonal bipyramidal configuration was possibly to protuberate to the core of the loop where loaded the calcium which came from the systematic buffer with the concentration of 2.5 mM rather than project outside which may be more available for the serine protease recognition. The significance of the specificity of P1 site for thrombin has been well proved both with steady state kinetic study of $k_{cat}/K_m$ and X-ray crystal structures. And Dr. Huntington with the fame of sublime contribution on thrombin structure and function indicated that the antithrombin, combined with heparin characterizing high efficiency of inhibition by blocking the active site of thrombin, initialized a global conformational change involving exposing the hinge region of the reactive center loop (RCL), which release the P1 Arg to extend to the S1 pocket of thrombin. In contrast of the flexibility of RCL, the rigid of the loop between two alpha helixes of EF-hand, especially when it binds to calcium in the form of a pentagonal bipyramidal configuration is remarkable. Surprisingly, both of the reaction rate $k_{cat}$ and the specificity $k_{cat}/K_m$ of sensor II to V were dramatically increased more than 20 fold when the cleavage site was moved to the F helix which was supposed to be much more rigid and figurate than the loop. The P1 site Arg in new location is possible more available to S1 pocket of thrombin as the side chains of alpha helix putatively project out of the helix which provides a solid backbone scaffold. Different from the randomly oscillation of the sidechain in loop, the chance of P1 site Arg in alpha helix with a solider configuration to protuberate into the narrow cleft of the recognition site and finally bound to the S1 pocket of thrombin is more generous. On the other side, P1 site Arg is least likely to be benefited from the solid scaffold in sensor II to V as the alpha helix of F-hand may not be completely formed since we mutated P4 to P2' sites totally six amino acids which were
originally to form F-hand for optimizing the specificity of protease recognition. Except that both of P4 and P2’ site Phe have a high preference to form alpha helix, residues in P3 to P1’ are all rare to appear in alpha helix. Although the authentic conformation of the recognition sequence and the contribution of that to the specificity of enzymatic recognition are concealed to us, what we explore with the aim of discovering the insertion linker to avoid the stereo hindrance of the bulk EGFP is clearly known to be EF-hand.

**Optimizing the recognition sequence.** The non-optimized P3, P4 and P2’ sequence in sensor I compared with the other mutants may also contribute to the low specificity in some degree.

The rational of utility of EGFP as the scaffold of the sensors is that EGFP has both of strong fluorescence and absorbance signals which can be sensitively detected with different methods. And it can also be well expressed in both of bacteria and mammalian cells with strong fluorescence which can be applied in both of in vitro and in living cells. Another advantage of EGFP are that it has been widely applied in body as indicators which demonstrates the EGFP-based sensors can also be further applied into the human body for detection.

In our thrombin sensor design, it is aimed to discover the optimal enzymatic cleavage linker and particular cleavage sequence without destroy the strong fluorescence of EGFP. Two different cleavage locations of the linker are designed and for each cleavage location, the P4 to P2’ sequence are mutated independently for comparison. The cleavage sequences mimic the natural substrates which are highly specifically recognized by thrombin. From the statistic results, majority of natural substrates of thrombin prefer Arg at P1 site, Pro at P2 site and hydrophobic residue at P4 and P2’ sites while P1 site perfer two dominant residues Ser and Gly and for P3, there is no statistic results for special residue preference. The evidence of structure
study of thrombin can support this conclusion. As we know, the S pockets in the enzyme can one to one residue recognize the P site of substrate, so the sequence of S pockets of the enzyme is critical information for optimizing the cleavage sequence of substrates. S1 pocket of thrombin is Asp, which is an acidic amino acid so that it prefers basic residue to contact itself. This presume well bolster the statistic result that thrombin has highly preference of Arg which is the most basic amino acid at P1 site. For P2 and P4 pockets, it indicates that they are apolar binding pockets which prefer non-polar amino acids which also well support the statistic results. For S1 pocket, it is Lys which has a long side chain and prefers small amino acid so that small residues Ser and Gly are the preference at P1’ site. For our design, P1 and P2 sites are fixed to be Arg and Pro respectively. P4 and P2’ are both designed with Phe. P1 is designed with Ser and Gly separately to compare which residue is better while the steady state kinetic parameters after calculation of these two sensors seems to be at the same level. For comparison of cleavage site location in the linker, two series of thrombin sensors are designed. One kind is inserting the cleavage site inside the loop of EF-hand and another one is inserting it inside the F-hand. For the results, all the steady state kinetic parameters of the two sensors with different P1 site seems to be at the same level which indicate Ser and Gly have the similar contribution to the thrombin recognition at P1 site of this substrate. For the different cleavage site location comparison, the \( \frac{k_{cat}}{K_m} \) dramatically increases about 30 to 40 fold after removing the location from loop to F-hand. As we know, the three-dimension structure of F-hand is alpha helix while the loop does not have fixed three-dimension structure without binding to calcium. Compared these two structure, it is hypothesized that when the cleavage site insert into F-hand, the alpha helix will help for the exposure of cleavage site into the thrombin recognition sites while for the loop the structural contribution is not strong.
7.9 Conclusions

High sensitive thrombin sensors were successfully developed through grafting approach in our laboratory. The kinetic parameters of thrombin sensors were systematically investigated. The specificity of recognition region of sensors for thrombin was optimized as following:

1. P4 to P1 of thrombin sensors are prefer to Phe-Asn-Pro-Arg.
2. Ser or Gly in P1' position have similar capability for thrombin recognition.
3. Cleavage linker at location 1 is possibly due to less exposure and less accessibility for thrombin.

7.10 References


50. Lisa Jones, W. Y., Anna L. Wilkins, Alice Kearney, Anton P. van der Merwe, and Jenny J. Yang. (2005) Rational Design of a novel calcium binding site adjacent to the ligand binding site on CD2 increases its adhesion function Submitted to PNAS.


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CHAPTER 8: CONCLUSIONS AND MAJOR FINDINGS

8.1 Conclusions

We have successfully created genetically encoded Ca\(^{2+}\) reporters and thrombin reporters based on scaffold protein GFP with rational design approaches. We have further demonstrated the application of designed Ca\(^{2+}\) reporter (CatchER) in studying real-time endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) Ca\(^{2+}\) signaling with fluorescent microscope. CatchER has been successfully expressed in FDB of mice skeletal muscles to measure fast SR Ca\(^{2+}\) release and recover during short-pulse voltage stimulation. CatchER has been structurally analyzed and determined with high-field nuclear magnetic resonance spectroscopy (NMR) and X-ray crystallography to understand novel fluorescent enhancement of GFP triggered by Ca\(^{2+}\) binding, and key residues close to the chromophore were identified to play an important role in the hydrogen network for the fluorescent change. The major classes of Mg\(^{2+}\) binding proteins were reviewed and classified and the structural basis of the metal selectivity between Mg\(^{2+}\) and Ca\(^{2+}\) of the metalloproteins are summarized, which will help to identify and differentiate Ca\(^{2+}\) and Mg\(^{2+}\) binding proteins and understand the biological function difference of Ca\(^{2+}\) and Mg\(^{2+}\) carried by the metalloproteins. GFP-based thrombin sensors have also been created by inserting a thrombin cleavage peptide into the fluorescent sensitive location of GFP with systematically improved kinetics \(k_{cat}/K_m\) up to \(10^4\) magnitudes higher than that of small peptide based absorption indicator EGR-pNA.
8.2 Major findings in rational design of Ca\textsuperscript{2+} binding sites on the surface of beta sheet protein.

Our lab uses a special design for Ca\textsuperscript{2+} binding protein via introducing four to five negative charged residues as the binding ligands in a square or pentagonal arrangement mainly on the surface of beta-sheet protein cell adhesion protein CD2. The alternation of the sidechain size such as Asp to Glu or from non-charged to charged residues can change the dissociation constants in tens of micromolar to low millimolar, with the assistance of computational algorithms—MetalFinder. This generates a new theory by changing the geometric arrangement of binding ligands on the surface of beta-sheet protein to tune the Ca\textsuperscript{2+} binding affinity, which is very different from the classic EF-hand based Ca\textsuperscript{2+} binding sites. It is important to find the biological application of this technique, and the widely used green fluorescent protein with its colorful analogies playing a tremendously significant role in cellular biology is a very promising candidate. I identified the fluorescence sensitive locations to introduce Ca\textsuperscript{2+} binding ligands via site-directed mutagenesis with high solvent accessibility near several key residues involved in hydrogen networks with the chromophore. This approach is different from the previous design of Ca\textsuperscript{2+} binding CD2 variants by emphasizing the optimized putative pentagonal bipyramidal geometry of Ca\textsuperscript{2+} coordination and compromising the location selection by computational algorithm, but proposing that conformational change of the designed ligands induced by Ca\textsuperscript{2+} binding will be transferred to those key residues to alter the hydrogen networks and triggering fluorescent change with a fast kinetics correlated to the real-time conformational change. Several variants with different number of negatively charged residues dispersed in three anti-parallel beta-sheets of EGFP in the vertex of a pentagon have been generated, using semi-rational design with the assistance of previous computational prediction results. The single-
wavelength fluorescent enhancement in response to Ca\(^{2+}\) of these variants was gradually improved according to the biochemical study of the bacterially expressed and purified protein, and the most optimized variant is called “CatchER”.

**8.3 Major Findings in ER/SR Ca\(^{2+}\) signaling of non-excitable and excitable cells measured by CatchER.**

Spectroscopic, kinetic, and structural studies show that CatchER has several unique merits, including a new kindle mechanism, tunable and optimal metal binding affinity without complicated cooperative binding activity, unprecedented off-rate, and a simple calibration equation. Its designed calcium binding domain negligibly perturbs natural SR Ca\(^{2+}\) signaling, and its intrinsic low Ca\(^{2+}\) binding affinity eliminates the possibility of Ca\(^{2+}\) buffering capacity. CatchER, the smallest genetically encoded Ca\(^{2+}\) biosensor, allows us to examine SR Ca\(^{2+}\) in various cell types and to define the aging-related coupling of sarcolemmal excitation and SR Ca\(^{2+}\) release without a decrease in Ca\(^{2+}\) concentration in myofibers.

A resting SR Ca\(^{2+}\) concentration of 512 and 573 µM was measured by CatchER in myofibers from young and old mice, respectively (122). Some studies have reported an SR Ca\(^{2+}\) concentration of ~1mM in frog muscle fibers (167, 168), while a 3-4-fold lower value (~308µM) was noted using the Ca\(^{2+}\) biosensor D1ER (40). We found higher basal SR Ca\(^{2+}\) concentration than that reported in mouse tibialis anterior muscle (40). Moreover, a significant residual SR Ca\(^{2+}\) after prolonged myofiber depolarization, and a larger SR Ca\(^{2+}\) depletion in myofibers from young compared to old mice. By using a more physiological repetitive stimulation, a train of pulses was sustained until CatchER’s signal reached a plateau, a more considerable residual Ca\(^{2+}\) was recorded in the SR from old mice. Residual SR Ca\(^{2+}\) was quantified based on converting CatchER’s signal into Ca\(^{2+}\) concentration according to published methods (108, 169).
These experiments support the conclusion that CatchER is suitable for measuring Ca$^{2+}$ in a high concentration cellular environment; that residual SR Ca$^{2+}$ concentration is significant beyond physiological demands; and that it is higher in myofibers from old compared to young adult mice.

8.4 Major Findings in structural determination of CatchER by NMR.

CatchER can be bacterially expressed and purified heteronuclear isotopic single- ($^{15}$N) double- ($^{13}$C-$^{15}$N) and triple-($^{2}$H-$^{13}$C-$^{15}$N) labeled CatchER for different pulse-sequence NMR experiments. With more than 70% mainchain assignment of residues by 3D NMR and Ca$^{2+}$ titration by 2D NMR, I have identified several key residues involved in the conformational changes in response to Ca$^{2+}$, both close to the chromophore and designed Ca$^{2+}$ binding site, and the quantified chemical shift changes are highly correlated to the dissociation constant measured by fluorescent response. These structural determination results supports the original hypothesis that chemical interaction on the surface of EGFP can influence the hydrogen networks of the hydrophobic chromophore via transferring conformational change to these designed ligands’ neighboring residues interacting with chromophore. Moreover, the calculated correlation time ($\tau_c$) of CatchER suggests that it maintained as a monomer at 37 °C even at 300 $\mu$M, suggesting that CatchER preserves its monomerization in live mammalian cell or animal imaging.

8.5 Major findings in structural determination of CatchER by X-ray crystallography.

The high-resolution X-ray crystal structure is indispensible to elucidate the fluorescence mechanisms of CatchER by direct seeing the chromophore and sidechain conformation. Crystal structures of Ca$^{2+}$-free, Ca$^{2+}$-loaded and Gd$^{3+}$ soaking form CatchER were grown with maximal 1.1 Å resolution. The crystal structures have been analyzed. I have identified
sidechain carboxyl groups of E222 rotating between Ca\(^{2+}\) free and Ca\(^{2+}\) loaded form CatchER crystal structures, altering the distance of the hydrogen bonds between the carboxyl group of E222 to S205 and chromophore, which is similar to E222 sidechain conformation of wtGFP and EGFP, respectively. However, T203 of CatchER maintained one hydrogen bond between the mainchain oxygen and water close to chromophore in all of Ca\(^{2+}\)-free, Ca\(^{2+}\)-loaded and Gd\(^{3+}\)-loaded form CatchER, similar to the wtGFP, suggesting that the fluorescent intensity of Ca\(^{2+}\)-loaded CatchER only recover 50\% of EGFP is possibly due to the fixed hydrogen bond network close to the phenol group of Y66 of chromophore maintained similar to wtGFP.

8.6 Major findings in analyzing Ca\(^{2+}\) and Mg\(^{2+}\) binding sites of protein.

There are major biological differences between Ca\(^{2+}\) and Mg\(^{2+}\). Ca\(^{2+}\) is a fast triggering metal, at low free concentration within cytosol (10\(^{-7}\) M) regulating numerous biological functions via interaction of multiple Ca\(^{2+}\) binding proteins, fluxing between membranes of various subcellular organelles through numerous calcium channels and receptors, and active transport via calcium pumps to generate several magnitudes concentration gradient. However, Mg\(^{2+}\) has slow exchange rate with H\(_2\)O, 10\(^4\) times slower than Ca\(^{2+}\), due to stable octahedral coordination with its ligands, high free concentration within cytosol (10\(^{-4}\) M), highly hydrolyzed forming non-covalent bonds with H\(_2\)O, mainly interacting with phosphates and nucleotides. To date, there is no convincing evidence of significant concentration gradients between different subcellular organelles. I reviewed and classified the major Mg\(^{2+}\) binding sites of proteins based on structural differences, and identified several key factors to determine Mg\(^{2+}\)/Ca\(^{2+}\) selectivity with binding constants difference up to 10\(^4\) in several types of metalloproteins, and mainly focused on EF-hand proteins, which will be very helpful for further design and optimize Ca\(^{2+}\) indicators with high metal selectivity.
8.7 Significance and impact of design calcium sensor studies.

The significance and high impact of our proposed study is multi-faceted. First, our study develops a novel methodology for designing Ca\(^{2+}\) biosensor by creating a Ca\(^{2+}\) binding site on GFP with site-direct mutagenesis, which not only overcomes the limitations of current Ca\(^{2+}\) sensors, but also can be utilized in various other fluorescent proteins with different optical properties for the further application in tissue and animal imaging. Second, our study explores a new approach to accurately measure the real-time Ca\(^{2+}\) concentration in ER, which enhances our understanding of Ca\(^{2+}\) signaling in ER, correlated to its biological function. To date, there are few successful protein-based ER Ca\(^{2+}\) sensors that are published, as most of the Ca\(^{2+}\) binding motifs originate from natural Ca\(^{2+}\) binding proteins. Third, our study allows us to enhance sensors with different signal peptides and multiple-magnitude binding affinities, which can help in detecting Ca\(^{2+}\) signaling response to different agonists in various subcellular organelles of diverse cell types. Fourth, our study provides a novel approach to detect calcium signaling in sarcoplasmic reticulum (SR) of muscle cell where the total calcium concentration is around several mM, far beyond the K\(_d\)s of most of published calcium sensors but suiting the most sensitive calcium concentration detection range of our designed sensors. The adenovirus infection system will be applied to our designed calcium sensors to maximize the DNA transfection rate to primary mammalian cells which is more efficient than loading the organic dye into the SR of mammalian cells. Fifth, our study establishes the methodology of GFP structural and dynamic investigation by NMR experiments and provides the protocol of hetero triple (D-\(^{13}\)C-\(^{15}\)N) or double (\(^{13}\)C-\(^{15}\)N) labeling GFP variants with high expression rate. Sixth, our proposed study of the relationship between the fluorescence change and hydrogen bonds around chromophore, based on NMR experiments, will deepen our understanding of the mechanism
of particular molecules influencing the environment and conformational change of the chromophore. This will provide solid theoretical evidence for the development of GFP-based biosensors detecting diverse molecules. Seventh, since the ER/SR Ca\(^{2+}\) signaling is broadly related to numerous cardiac diseases, our study will also provide insight into molecular basis of heart failure associated with ER/SR Ca\(^{2+}\) metabolism, and consequentially generate the novel therapeutic approaches to treat the abnormal Ca\(^{2+}\) handling.

CatchER is a genetically encoded calcium indicator designed in our laboratory which can specifically target ER and maintain inside. ER calcium measurement can be achieved in the intact cells instead of permeabilizing the cell applied with calcium dyes (Fluo-5N, Fura-2), which extends the ER calcium explore in a wider field as several experiments can only be done with the intact cells, including EC-coupling, and study the receptors in the plasma membrane and also make it possible of multiparameter fluorescence imaging measurement in the same cells.

The unrevealed mechanism of fluorescence alternation of CatchER triggered by calcium hampered further rational improvement of optical properties of this series. We can still hypothesize this based on the existing calcium indicator with disclosed mechanism. Our design mimics single-wavelength calcium dyes featured with 1:1 binding mode and the fluorescence enhancement are triggered by calcium itself rather than the other genetically encoded calcium indicators with four calcium binding domain. The fluorescence changes are induced by the protein-protein interaction. The early calcium-sensitive indicators, nonfluorescent metallochromic dyes, murexide, arsenazo III, antipyrylazo III exhibited absorbance change in response to calcium (Tsien, Monitoring cell calcium, 1999). The mechanism is calcium replacing H\(^{+}\) to trigger absorbance change, which render them pH-sensitive, with low selectivity against Mg\(^{2+}\) and
high dissociation constant around 1 mM. All these three features occurred to D11, indicating the feasible of this mechanism and preliminary stage of our current design. The future advance of our work should benefit from the knowledge accumulation both from calcium dyes and fluorescent proteins and their frontier research. The high convenience and flexibility of intentionally adjusting small molecules in comparison to scaffold protein as even protein folding prediction with high accuracy is still under-explored allows fast development of rational design of small molecules.

### Table 8.1 Application of CatchER in studying diseases required by material agreement transfer (MTA).

| B: Bacillus subtilis, Bipolar disorder, Burn injury |
| C: Calcium homeostasis-related diseases, Cancer genetics, Cardiac arrhythmias, Cardiac hypertrophic signaling, Cardiac physiology, Cardiovascular diseases, Cell apoptosis, cell necrosis, Cellular stress, Central core disease (CCD), Cerebrovascular disorders, Chronic heart failure, Chronic inflammatory disease, Chronic lymphocytic leukemia, Chronic obstructive airway disease, Chronic obstructive pulmonary disease, Congenital heart disease (CHD), Congenital heart defects |
| D: Diabetes, Diabetes Mellitus, Disease-causing mutation |
| E: Endocrinology, Epilepsy, ER stress |
| F: Fatigue |
| H: Heart diseases, Heart failure, Hereditary disease, HIV, Hormonal disorders, Human disease, Human, Huntington’s disease, Hypertension, Hypertrophy (Hyp), Hypokalemic periodic paralysis, Hypoplastic left heart syndrome |
| I: Immature heart, Immune system diseases, Immunity deficiency, Inflammatory disease, Intravenous anesthetics, Irritants |
8.8 Major findings in designing a novel class of thrombin biosensors with fast ratiometric responses.

Thrombin is involved in numerous cellular signaling pathways and plays a crucial role in blood coagulation, which converts fibrinogen to fibrin clot and support platelet aggregation. It is also vital for trafficking of inflammatory cells into sites of injury and tissue repair by enhancing expression of endothelial cell. The abnormal activity of thrombin will result thrombosis, one of the most common factors to cause thromboembolism, myocardial infarction and stroke. Currently, there is a strong need to develop protease sensors with strong protein substrate specificity and quantitatively measure of the enzymatic action in cells in real time. I designed a novel class of single EGFP-based thrombin sensors by inserting a thirty-amino acid short peptide with a thrombin cleavage site into the fluorescent sensitive location of EGFP. These designed protease sensors exhibited rapid kinetic response and large ratiometric fluorescent/absorption
change, with optimized steady state kinetic parameters measured by Michaelis Menten equations, $k_{\text{cat}}/K_m$ up to $10^4$ magnitudes higher than that of small peptide based absorption indicator EGR-pNA. The substrate specificity is also high, as the measured $K_m$ value is in below 10 $\mu$M, in the same magnitude as that of natural thrombin substrate Fibrinogen A, but much lower than that of EGR-pNA.
Appendix

Constructing calsequestrin tethered CatchER.

>CSQ pRSETb 04282010 T7-terminal sequence (Original file)
>CSQ-1-T7-Term_A01.ab1

NNNNNNNTCTCCGGGCTTTTGGTAGCAGCCGGGATCAAGCTCAGTTCCGAAATCCGTT-
TAACCGGGCCCTCCTAGACTAGTCGTCGTACATCGTCATCGTACATCGTACATCGTCCTG-
CATCGTCGTACATCCTCTGTGGATGATCTCGCCCTCAGCAAGGTCTCCAAAGTACCC-
TTAGGTCTCGGTCGCTACATC
AGGCATCAGATCGTCTTTCTGCAAAGGCGACAGTGGAGTTCCATCCAGGTCAGTCA-
CTCCAGGTCACATACTCAT
TCAGGGCTTTCTCAGGTTATCTCTGTGCTACTCACAGTCAACTCTCCACGACTCACAATCTCC-
CTCTCACTTGGGTGTTGCT
TGGGATGCTATAGGCTTCTTCCATGAAGGCGCTCGAGAAATCAATCTCATTCAACTTCA-
GAGTCAGCTCCTTCTTGCCTACT
TGCTGTCGAAGGTAGCGAAGAAAGGGATGAGGATGGAACCTCTCAGCTGCGTCC-
TCGAGGGTTCTGAATGCTCTGAG
TCTTTGCTTGAAGTAGCTTTGATTTCCATCTTCAATTTCTCAAATGCTGCT-
GAGCTCTGTCACCTATC
CAACCTCACAGGCTCTTCTAGACATCAAGCAGAAACTCCACCAGAGTGTGCAG-
GAAACTCCGCCGTCATATGCTAATGA
CTTCATCTCCTTTGAGACACATACAAAGCTGCTTTCTCAGTTCATGCTACTTCTGCTGCTG-
GCCACGGCTCCTATCTC
TCCACGGCAGGAGCCACACCTTGGCTTCTTACTANACTTGGGCTGAATGTGAAGAGTCAT-
CTCTTCCTCCCTCAATAG
TCTCTGC

CSQ-1-translation:
(start from skeletal CSQ site 76, Kye Won Park, 1998, Gene) QRFEMEELILELAAQX-
LEDKVGFGLVDSEKDAVAKKLGLTEEDSVYVFKGDIEYD
GEFSADTLVEFLLVLEDVPELIEGERELQAFENIEDEIKLIGYFKSKDSEHYKAYEDAA
EEFHPYIPFFATFSKVAKLTLNEIDFYEAFMEEPMTIDPKPNSEEEIVSFVEEHR
STLRKLKPESMYETWEDDLLGHTVAFAEEADPDYEFLETLKAVAQDNTENPDSLIIW
DPDDFPLLVPYWEKTFDIDLSAPQIGVGNVDADSIWMEMDEADLPSADELEDWLEDVL
EGEIINEDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDD

>CSQ pRSETb 04282010 T7-terminal sequence Reverse and complementary
GCAGAGAGAATTGAGATGGAGAGCTAATCCGGAGTTAGCAGCCCAAGTGTAGTGA-
GACAGGGGTGTTGGCTTGGCCTGTTGGAAGTCAGAGAACAGATGCAGTGGCAGC-
AAAAAAGGCTCAGACTAAGGAGACAGCCTTATGCTGTTTAAAGGAGATGAGTCAT-
GAATATGACGCGAGTTTTCTGCGACACACTCTGGTGAGTTTCTGCTTGATGTCCTCA-
GAAGACCCTGTAGAGTTGATTGAAGGTGAACGAGAGCTGCAGGCATTTGAGAATAT-
GAGATGAAATCAAACTCATTGGCTACTTCAAGAGCAAAGACTCAGAGCATTACAAAGCC-
TACGAGAGCGAGCGCTAAGAGTTTTCTGCTACCTTCGACAGCAAGTTGCAAGGCTATTTGA-
ATGAAGATTGATGTTTTCTACGAGGCTTT-CATGGAAAGCCCTATGACCATCCAGACAAGCCAGGGCCACAGTTGAAGAGATTGTGAGC-
TCGTTGGAGGAGGACACAGGAGATCAACCCTGAGAAACTGAAGCGCTAGATGTAG-
TACGAGACTTGGGAGGATGACCTGGATGGAATCCACACTGTCGCCTTTTGCAAGAGAGAAG-
CAGATCCTGATGGGCTATGAGTTCTTAGAGACTCTCAAGGCTTGAGCCAAAGACACACT-
GAGAAGCGCGACCTCAGTATCATCTGGATTGAGATGATGTTGATGACAGGTAACTCATTCTG-
CTGATGAGCTGGAGGAGGACTGGCTGGGAGGGCGAGATCAACACAGAGATGACGACGAC-
GAGAAGGGCCCGTTTAAACC

Linker sequence:

R S P R P R D N N R R R M D P
CGT TCT CCT CGT CCT CGT GAT AAT AAT CGT CGT CGT ATG GAC CCT

1) (Poly D) C-terminal sequence long  BamHI cleavage sequence G|GATCC

5'-TGATGACGATGATGACGACGAC

CGTTCTCCTCGTCCTCGTGATAAT

AATCGTCGTATGGACCCT

GGATCCTTTTTT -3'

a) Primer: C-terminal sequence  Reverse and complementary:

5'-AAAAAGGATACCAGGGTGTCCTACAGCAGACGATTATTATCATGACGAGGACGAGGA-
GAACGGTCGTCGTATCATCGTATCA -3'

Tm: 11x4+11x2=44+22=66 C

(Poly D) C-terminal sequence short no linker BamHI cleavage sequence

5'-

TGATGACGATGATGACGACGAGACGACCGATCCTTTTTTT -3'
b) Primer: C-terminal sequence Reverse and complementary:
5' - AAAAAAGGATCCGTCGTGCATCATCATGTCATCA -3'

2) (no D) C-terminal sequence BamHI cleavage sequence G|GATCC
5' - CTGGAGGCGGAGATCAACAGAGCGTTCTCTCGTCCTCGTGTA-TAATAATCGTCGTGCATATGGACCCCTGGATCCTTTTTTTT -3'

  c) Primer: C-terminal sequence Reverse and complementary:
  5' - AAAAAAGGATCCAGGTCCATACGACGACGATTA -3'
  Tm: 14x4+10x2 = 56+20 = 76 C

  (no D) C-terminal sequence BamHI cleavage sequence
  5' - GAGGGCGAGATCAACACAGAGGTCTTTTTTTTTTTT -3'
  Tm: 12x4 + 9x2 = 48 + 18 = 66 C

d) Primer: C-terminal sequence Reverse and complementary:
5' - AAAAAAGGATCCCTCTGTGTTGATCTCGCCCTC -3'

3) N-terminal sequence HindIII cleavage sequence A|AGCTT
5' - AAAAAA|AGCTTATGGGGGCCAGAGCAGTGTC -3'

e) Primer: AAAAAAAGGCTTATGGGGGCCAGAGCAGTGTC
Tm: 13x4 + 7x2 = 52 + 14 = 66 C

---

Primer e) and b) PCR/ HindIII and BamHI cleavage CSQ sequence inserted in D11cyto sequence

Proposed sequence:
TATTACGTGTTTCGTGGCTaGCGTtTaACTTAgCGTTATGGGGGCCAGAGCAGCAGTGCTCGAGCTCGCGGCTGGCAGACTGCTGTGTTTGTACTTGGTGCTAGGGAGCGCCCAGGTTAGG-GTCCAGGGGGAAGATGGGTGACTTTG CCTGAGACGGTGTGATGACAGGAGGTGGACCGTGTGAT-CATGTGAATGCAAGAACCTCAGAAGATGGAGGGGTCTTGGGCTGAGACAGATGGTGCTGAGGTGGAGGCAGAAGTCGAATCTGGTGGAGTTCGAAGGCCCTCAGCTACGACTGAGATGCAGAGGTGGACCGTGTTTCAGAGCAGAAAGGTGGTGGC
CGTGGACAGAAGATGGGTTGGACTTCGGTACAGGACGTGGTCCTAGAAGACCCTGTAGAGTTGATAG-GAGCTAATCCTGGAGTTAGCAGCCAGTGTTGGCCAGTAGTTTCTGCTTCGCTTCTGGGCTGAGACAGAAGATGGGTTGGACTTCAGAGAAGGATGCAGCTGTGGCCAAGAAACTAGGACTAACTGAAG
GACAGCGTTTATGTGTTCAAAGGAGATGAAGTCAATTGAATATGACGGCGAGTTTTCTG
CAGACACTCTGGTGAGTTTCTGCTTGATGTCCATAGACAGACCCTGTAGAGTGGTATT-
337

GAAGGTGAACGAGAGCTGCAGGCATTTGAGAATATTGAAGATGAAATCAAACTCATTGGCTACTTCAAGAGCAAAGACTCAGAGCATTACAAAGCCTACGAGGACGCAGCTGAAGAGTTCCATCCCTACATCCCTTTCTTCGCTACCTTCGACAGCAAGGTGGCAAAGAAGCTGACTCTGAAGTTGAATGAGATTGATTTCTACGAGGCCTTCATGGAAGAGCCTATGACCATCCCAGACAAGCCCAACAGTGAAGAGGAGATTGTGAGCTTCGTGGAGGAGCACAGGAGATCAACCCTGAGGAAACTGAAGCCTGAGAGTATGTACGAGACTTGGGAGGATGACCTGGATGGAATCCACACTGTCGCCTTTGCAGAGGAAGCAGATCCTGATGGCTATGAGTTCTTAGAGACTCTCAAGGCTGTGGCCCAAGACAACACTGAGAACCCCGACCTCAGTATCATCTGGATTGATCCTGATGACTTCCCGCTGCTGGTCCCGTACTGGGAGAAGACCTTTGACATTGACCTGTCAGCTCCACAAATAGGAGTTGTCAATGTTACAGACGCGGACAGCATATGGATGGAGATGGATAACGAGGAGGACCTGCCTTCTGCTGATGAGCTGGAGGACTGGCTGGAGGACGTGCTGGAGGGCGAGATCAACACAGAGGATGACGACGACGATGACGACGATGACGATGATGACGATGATGACGACGACGGATCCGGGCCCTCTAGAATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACGAGCACAACGTCTATATCACGGCCGACAAGCAGAAGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGGACACCGAATCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGGAGGTGGAGGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAGAATTCT(EcoR1)GCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGTT
TAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGT
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KNYKNVFKKYEVLALLYHEPPEDDKASQRQFEMEELILELAAQVLEDKGVGFGLVDSEKD
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FMEEPMTIPDKPNSEEEIVSFVEEHRRSTLRKLKPESMYETWEDDLDGIHTVAFAEEADP
DGYEFLETLKAVAQDNTENPDLSIIWIDPDDFPLLVPYWEKTFDIDLSAPQIGVVNVTDA
DSIWMEMDNEEDLPSADELEDWLEDVLEGEINTEDDDDDDDDDDDDDDDDDGSGPSRMVS
KGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVT
TLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNR
IELKGIDFKEDGNILGHKLEYNYNEHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHY


QQNTPIDGPVLLPDNYLDSEALSKDPNEKRDHMVLLEEVEAAGITLGMDLYK- 
EFX
XQISSTVAAARVRARLNPILSLCFL-PAICC

Add aa linker between CSQ and D11
5' - AATCGTCGTCGTATGGACCCTATG GTGAGCAAGGGCGAGGAG -3' (Start from D11)
Tm: 13x4+8x2=52+16=68 C

f) primer: 5' - AATCGTCGTCGTATGGACCCTATGGTGAGCAAGGGCGAGGAG -3'

(CSQ C-terminal Poly D)
5' - ATGATGACGATGATGACGACGACC CGTTCTCCTCGTCCTCGTGATAAT -3'
Tm: 11x4+12x2=44+24=68 C

Reverse and complementary

g) Primer: 5' - ATTATCACGGAGCAGGAGGAACGGTCGTCATCATCGTCATCAT -3'

(CSQ C-terminal no D)
5' - GAGGCGAGATCAACACAGAGCGTTCTCCTCGTCCTCGTGATAAT -3'
Tm: 12x4+9x2=48+18=66 C

Primer: (CSQ C-terminal no D) reverse and complementary

h) 5' - ATTATCACGGAGCAGGAGGAACGGCTCTGTGTTGATCTCGCCCTC -3'
Table. Ca\textsuperscript{2+} triggered mainchain amine \textsuperscript{1}H and \textsuperscript{15}N chemical shift changes of CatchER by 600 MHz NMR (GSU).

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<th>Residues</th>
<th>H apo</th>
<th>H 6Ca</th>
<th>Holo-Apo H</th>
<th>N apo</th>
<th>N 6Ca</th>
<th>Holo-Apo N</th>
<th>Holo-Apo</th>
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<td>7LH-N</td>
<td>7.604</td>
<td>7.576</td>
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<td>117.48</td>
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<td>113.75</td>
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<td>121.19</td>
<td>121.02</td>
<td>-0.17</td>
<td>0.081</td>
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<tr>
<td>12VH-N</td>
<td>9.152</td>
<td>9.177</td>
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<td>132.03</td>
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Commands in Sparky:

pa (select all the peaks)
oc (copy all the peaks)
op (paste all the peaks)

st (open the window to change the reference) and then change
pc (the peak will move to the center)

pb (to choose which file)

st (add reference)

ct (change the color)

xx (reverse the axis) choose the file

zo (zoom out)
zi (zoom in)

ct (negative level: 0; positive level: 19; lowest 5e+05)

F8 (select the peak)

F11+square with mouse (select the region)

at (assign it)

ol (overlap)

zi (zoom in) F11+select area

st (change the ppm as the reference)

tb (see the assignment of the peak)

To calculate the integration of the peaks:

Assign the peaks.
pa (select all the peaks)/ or choose the peaks need to be selected.

it (for the integration calculation, Keep: use data above lowest contour, group peaks in contour boundary)

pi (calculate the integration of all the peaks)

pb (show the window of the data) and then choose the file and update.

Commands of NMR HSQC:

```
  cd name.fid
  ls
  cd nmrpipe
  ls
  ./var2pipe_2d.com  (change to 2D data)
  ./ft2d.com  (process)
  csh
  nmrDraw

  file/ select  gnhsqc.ft2
  gedit ft2d.com

  (analysis data)
  pipe2ucsf -21 gnhsqc.ft2 ~/Sparky/Save/MAT/name.mat
```
Sparky (file-open-MAT)

xx (reverse the axis) choose the file
zo (zoom out)
zi (zoom in)

t (negative level: 0; positive level: 19; lowest 5e+05)
F8 (select the peak)
at (assign it)
ol (overlap)
zi (zoom in) F11+select area
st (change the ppm as the reference)
tb (see the assignment of the peak)

Commands for NMR data process HSQC

NMRpipe (hsqc)

Hing’s computer

Ctrl + R (open the window)

ls (list all the files)

cd data

cd file.fid (go to the file) change the parameters: gedit var2pipe-2d.com

ls

cd nmrpipe

ls
cp -r nmrpipe ../name

./var2pipe_2d.com (from varian to pipe) cd ../name

./ft2d.com (process data)
csh (c shell)
nmrDraw

(file, right click, select file)
click gnhsqc.ft2 (po -7.0)
click read/Draw 1phase. (mouse). (1D vertical. Horizontal)
put the cross out the window press the middle button and move the mouse on the
bench to change the negative of the peaks.

(file-- Quit the program)

pipe2ucsf -21 gnhsqc.ft2 ~/Sparky/Save/MAT/name.mat
sparky
click-file-open-MAT-choose the file
xx (reverse the axies)
zo (zoom out)
zi (small letter)
ct (negative level “0”, positive level “19”, lowest 1.13e+0.5) change the number to be
bigger 5e+5, dots change to be smaller. (usually, 5e+05 is OK)
select the peak (F8)
click F8-pc (selection will move to the center of the peak)
square select the peak. (center)

at (assignment)

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Click-apply (sparky manual from google)

Quit the program

gedit ft2d.com

po (the value -7.0)

click-save

./ft2d.com (select file-save)

st (add reference)

ct (change the color)

---

Tau c data process:

Here are the procedure on how to process the data.

You have split the fid from one to 9 fids.
1a, compile the script separate_2d.c (on the unix prompt type: gcc separate_2d.c)

1b, once you have compile it correctly, you will see a file call a.out

1c, you will just execute the script cut.sh (on the unix prompt type: cut.sh)

1d, if the script work correctly, you will see 8 additional fids

2. Now, process the data you will need to process all 9 fids (varian [return] read parameters)

2a, you can use the varian command in NMRPipe to read in the parameters, since there are 9 fids. So you will need 9 separate script but you can keep them in one file, I have attach the example as, var2pipe_2d_9.com (ft_2_8.com)

2b, once you have convert the fid to the NMRPipe formats, now you need to process the data, you will also need 9 separate script (or you can do them one by one) and you can keep them in one file, ft2d.com (pay attention to where you fid is, my fid is stored at one directory above.)

2c, note, the experiment is sensitive enhanced, the Rance-Kay command is placed inside the ft2d.com instead of var2pip2_2d_9.com. You can put it at either file, it will work the same. Make sure you check the phase of the spectrum, they all should have the same phase.

2d, once you have finish, you should be able to check the *.ft2 spectra in NMRDraw, convert them into Sparky format so you can process it.
3. Assign the spectra and calculate TauC.

3a, you should check every spectrum when you transfer the assignment over in Sparky, because the peak will move from TROSY position to the normal de-couple position in the 9 spectra increment (make sure you pick the head at assigned peak position only.)

3b, once you have assigned, type pa to select all of the peaks, then type rh to open the relaxation peak fitting window. Type set up to open another window, select the spectra and assign the time, it should be 0, 10, 20, 30, 40, 50, 60, 70 and 80 ms. Then type rh in one of the spectrum, it will calculate the value. Now you want to save this raw-data.list.

3c, you will use the “getcro.pl” script to convert the data to frequency, (in unix prompt type:./getcro.pl raw-data.list>data.cc)

3d, now to calculated the TauC, use the “calcTauC.pl”, (in the unix prompt type:./calcTauC.pl data.cc>data.TauC) this will give you the TauC. The first column should be residue number, the second column should be TauC, and the third column is error.

Diffusion data processing:
Process the data as 2D data

Data type: complex

Acquisition Mode: States or States TPPI

Correct 1st point: lpf

Solvent Suppression: cnv

Window Function: exponential

FT type: complex

Phasing Mode: Interactive

Baseline Correction: None

Extract Half Spectrum: None

Output Level: Verbose

CNV parameters

Convolution Function: Sinebell

Function Width: 10

Extrapolation: Linear

Exponential multiplication parameters

Method: parameter

Line Broadening (Hz): 2

Phase the spectrum, keep.
Ignore processing D2 by clicking cancel.

Under “Preference”, select “Reference” and change D1 axis type to “point”

Click the icon for realtime 1D slice.

Open Macro file: inter2.mac

Define the region you wish to look into (you should select peaks that are free from interference of the buffer signals, and choose larger signals)

Type “dr” in the command line to see the curve

Then “ld 1 40” to list your data (40 is the number of data in the array)

Prior to next step, make sure to zero it by input “ze” and “stb 1” sequentially.

Then select 10 points from the baseline on both sides of the peak and run the macro again.

Use the following equation to calculate the corrected integrated area:

\[ C' = C - \frac{(A/M + B/N)}{2} \times L \]

Where \( C' \) is the corrected integrated area

\( C \) is the measured integrated area

\( A \) is the measured area of the baseline on the left of the signal

\( M \) is the number of points under area A

\( B \) is the measured area of the baseline on the right of the signal
N is the number of points under area B

L is the number of points under area C

---

Date Transfer from 800 MHz CCRC (Frey computer)

Click ‘Start’ and choose ‘System Tools’ and click ‘Terminals’

(Transfer the data from 800 MHz NMR to weiyang’s account in UGA)

cd Ada (or any directory you want)

ssh jyang@128.192.9.242

password:

cd /data/backups/inova800/current

cd weiyang

cd vnmrsys

cd exp7 (any experiment you have done)

cd (go to the home directory)

cp –r /data/backups/inova800/current/weiyang/vnmrsys/exp7/acqfil gfp-7e15e-hsqc-800-062508.fid

(Transfer the data from weiyang’s account in UGA to Frey in GSU)
Open another terminal

cd Ada

`scp –r iyang@128.192.9.242:/home/iyang/gfp-7e15e-hnca-800-062508.fid .`

Password:

(Transfer the data from Frey to cherrio)

tar cvf hnca.tar gfp-7e15e-hnca-800-062508.fid

`ftp cherrio`

account: yang

password:

cd Ada/nmr/foreign

put hnca.tar

quit

(Please ftp transfer the data from cherrio to your computer to process the data)

CBCACONH

EGFP-7E15E 13C 15N double labeled protein

600 MHz cold probe Jeff
cexp700 (create experiment 700)

mp (400, 700) (copy exp400 to exp700)

su

Adjust the lock

Click ‘active lock’ to be off and adjust z0 (-4431)

Then click ‘active lock’ to be on

Click ‘Gradient Shim’—‘Acquire Trial Spectra’—‘Automake Shimmap’—‘Display Shimmap’—‘Gradient Autoshim on Z’—‘Quit’

ni=1

phase=1

ss=2

nt=8

pw=9

g a

array

pw

8.6 (start point)

0.2 (increment)

g a

pw=7

cexp(701)

jexp701
Click ‘Experiment’—‘Protein Triple-Resonance Experiment’—‘NH-Detection’—
‘CBCA(CO)NH’

Text output
dg

Open a terminal
Probe
cd probes
cd HCN

Click ‘probe’
dsp (check probe)
sw=8384
np=2048
ss=2
nt=16
ga
wf
aph(autophase)

presat='n'
watergate='y'
flag3919='y'
pw=1

d1=1.2

lp=0

rp=0

cexp(703) (set the exp to be HN(CO)CA)
jexp702 (set the exp to be water)
presat='n'

watergate='y'

jexp700 (gNhsqc)

sw=8384

np=2048

dg

JNH=98 (T2 is shorter for big protein)

nt=256

ss=128

jexp701

JNH=98

Jexp703

JNH=98

(exp700 1D gNhsqc)
Dynamic T1, T2, NOE (GFP)

Experiment operation:

600 MHz in GSU

(NOE on and off)

jexp6 (seq: gHsnc)

Click ‘main menu’ ⇒ ‘file’ ⇒ ‘data’ Choose the existed NOE file (e.g. 5g4_ca_NOE_on_1000807.fid) ⇒ ‘load’

temp=37
relaxT=4.000 (NOE on)

gain

(try to find the appropriated pw and gain value)

jexp7 (seq: gNhsqc)

Click ‘main menu’ ⇒ ‘file’ ⇒ ‘data’ Choose the existed NOE file (e.g.

5g4_ca_NOE_off_1000807.fid) ⇒ ‘load’

temp=37

relaxT=0.000 (NOE off)

gain

(try to find the appropriated pw and gain value)

When these experiments are finished, save the NOE data and go to set up T1

jexp6 (seq: gNhsqc)

Click ‘main menu’ ⇒ ‘file’ ⇒ ‘data’ Choose the existed T1 file ⇒ ‘load’

temp=37

adjust pw

T1= ‘y’

T2= ‘n’

relaxT=0

adjust gain

gain

jexp7
mp(6,7) (copy exp6 parameters to exp7)
relaxT=0.010
ga

jexp8
mp(6,8)
relaxT=0.060
ga

jexp9
mp(6,9)
relaxT=0.130
ga

jexp10
mp(6,10)
relaxT=0.230
ga

jexp11
mp(6,11)
relaxT=0.340
ga
When these experiments are finished, save the T1 data and go to set up T2

jexp6 (seq: gNhsqc)

Click ‘main menu’ ⇒ ‘file’ ⇒ ‘data’ Choose the existed T2 file ⇒ ‘load’
temp=37
adjust pw equal to the value from T1 experiment
T1= 'n'
T2= 'y'
relaxT=0.010
adjust gain
ga

jexp7
mp(6,7)
relaxT=0.030
ga

jexp8
mp(6,8)
relaxT=0.050
ga

jexp9
mp(6,9)
relaxT=0.070
ga
When the experiments are finished, save the T2 data
Dynamic experiment T1 setup:

dynamic 600 MHz (GSU)

Exp6 gNhsqc
Temp=37 oC
exp6 T1='y'
relaxT=0
exp6~16 parameter setup:
relaxT=0, 10, 60, 130, 230, 340, 480, 740, 1000, 1500
array start point: 19

jexp6 ga
jexp7 ga
jexp8 ga
...
jexp16 ga

Dynamic experiment T2 setup:

Jexp6
T2='y'
T1='n'
relaxT=10, 30, 50, 70, 90, 110, 130, 150
d1=2
pw=49
gain=38

Dynamic experiment NOE setup:
Load NOE exp6
5g4_ca_NOE_on_100807.fid
pw=4.9
temp=37.0
relaxT=4.000 s
jexp7
5g4_ca_NOE_off_100807.fid
ralaxT=0

TauC (tumbling time) setup
exp1
jexp2 array: CaM
temp=37 su
jexp1 da
phase? da
phase=0
ni=1
array Delta
array 8
sw1=2200

ejexp2
Shimming
svs('071408.shim') (save the shimming)
[Main menu]-[File]-[Data]-[load shims] su

ejexp1
pw=5.08
compH=0.99

Delta=0, 0.03
gain=28
array Delta 8 steps
0, 0.003
8
0
0.003
dg
phase=1,2
da
array='Delta, phase'
da
dg
time
ni=64
nt=80
ss=32 (31 hours)
nt=88
phase (180)
f full
pl pap page

Convert T1 and T2, to R1 and R2, respectively.
Six files
mfparam
S2
mfinput: don’t change anything
mfdata: R1, R2, NOE (put in data)
mfpdb: only keep ATOM

mfmodel

./run.mf

When it is finished, mfout will be generated.

Sum=(exp-pred)^2/uncert^2, Xsum=(01

model  1-2, 3-4, 5

select

 TauC

Split the fid from 1 to 8

./var2pipe_2d_8.com

./ft2d_8.com

nmrDraw

Sparky

[Convert]

process TauC data

Diffusion  CaM

500 MHz in GSU

jexp9 (Seq: diffusion_dbwg) (Only show specific range of peaks)

array
gzlvl10
40 (steps)
100 (start point)
400 (increment)

Open a terminal

cd vnmrsys/shapelib

more Pbox.inp

{g3 1250 1650}

(1250/500=2.5 ppm, width of the peaks)
(1650/500=3.3 ppm, distance from the water peak to the center of the peaks)
(The peaks range: (4.7+3.3) ± 2.5/2 ppm)

Choose ‘Text Editor’

Open ‘file’ and choose ‘Pbox.inp’

Adjust the peaks range

(Always use the following command in terminal after adjust the peak range)

inova500:yang2 19>Pbox -p 52 -l 9.6

600 MHz in GSU
jexp4 and load the power sequence: diffusion_wsledwg (apply water gate to suppress the water)

\[
pw90=pw
\]

Adjust the gain \( ga \)

jexp5 and load the power sequence: pg_sled_jg (apply pre-saturation to suppress the water)

\[
pw90=pw
\]

Adjust the gain