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Developing Voltage-Sensitive FRET System to Image Neuronal Activity

A thesis submitted in partial satisfaction of the requirements for the degree Master in
Science

in

Biology

by

Peter Byongsoo Kim

Committee in Charge:

Professor Lei Wang, Chair
Professor Michael J. Sailor
Professor Milton H. Saier

2009

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Chair

University of California, San Diego

2009

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LIST OF ABBREVIATIONS

CFP: cyan fluorescence protein

DMEM: Dulbecco's Modified Eagle's Medium

FRET: fluorescence resonance energy transfer

MD-VSFP: maximized-dipole moment voltage-sensitive fluorescent protein

PCR: polymerase chain reaction

VSFP: voltage-sensitive fluorescent protein

YFP: yellow fluorescence protein

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ABSTRACT OF THE THESIS

Developing Voltage-Sensitive FRET System to Image Neuronal Activity

by

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Master of Science in Biology

University of California, San Diego, 2009

Professor Lei Wang, Chair

We report the design and generation of a fluorescence resonance energy transfer (FRET) reporter system tailored to sense changes in membrane potential. The FRET reporter consists of two fluorescence proteins: cyan fluorescence protein Cerulean with minimized dipole-moment and yellow fluorescence protein mCitrine with maximized dipole-moment. While Cerulean is membrane-localized by lipidating two opposite ends of the protein, mCitrine is lipidated only at its N-terminal. We hypothesize that in response to membrane potential change, mCitrine moves near and far from the plasma membrane, altering the amount of FRET ratio that provides a reasonable proxy for detecting changes in membrane potential.

Our preliminary tests suggest that our system is functional and that it could potentially be further optimized to report electrical activities in neuronal cells.

Introduction

Traditionally, functional studies in neurobiology have relied on electrophysiology using electrodes. However, classical electrophysiology, such as whole cell recording, perforated patch recording, and patch recording, presents several limitations. First, it is difficult to monitor a large population of neurons using multiple electrodes. Second, it is invasive to the cells. Third, it is very technically challenging, especially for studying neurons in intact animals. Therefore, many attempts have been made to develop alternative methods to track neuronal activities.

Voltage-sensitive dyes have been explored for more than 30 years as an alternative way to sense neuronal activities. They are capable of recording fast electrical signaling of neuronal cells while providing a relatively noninvasive methodology. Moreover, they allow study of thousands of nerve cells simultaneously (1). However, nonspecific and indiscriminate staining of both neuronal and non-neuronal cell types results in an undesirable effect of a poor signal-to-noise ratio. Moreover, whether for functional studies or for circuit studies, it is important to be able to distinguish different classes of neurons. In addition, these dyes have to be added exogenously.

With the advent of green fluorescence protein (GFP), genetically encoded-reporters can be constructed to target specific population of cells. Consequently, many groups have taken the challenge of designing optical electrophysiology technique via genetically encoded fluorescence probes (2-7).

In this paper, we describe a fluorescence resonance energy transfer (FRET)

reporter system tailored to sense change in membrane potential. This membrane-localized reporter consists of cyan fluorescence protein Cerulean with minimized dipole-moment and yellow fluorescence protein mCitrine with maximized dipole-moment. Cerulean is localized on the membrane by lipidating top and bottom ends of the protein; mCitrine is only lipidated at its N-terminal, allowing it to be able to translocate from near and far from the membrane. We hypothesized that upon maximizing dipole moment of mCitrine, it would sense the membrane potential change, and thus report a change in FRET when coupled with Cerulean. By localizing the FRET reporter to the surface of the membrane, we hope that a maximal movement of mCitrine in response to membrane potential change will result in a maximum FRET ratio change.

Our preliminary results suggest that our reporter is functional and that it could potentially be further optimized to report electrical activities in neuronal cells.

Chapter 1 Materials and Methods

1.1 Voltage-Sensitive Fluorescence Proteins Constructions

Initially, mCitrine and Cerulean were respectively inserted into a bacterial plasmid pLei using the following primers: a sense primer containing a *SpeI* restriction digest site and an anti-sense primer containing a *BglIII* restriction digest site.

Using these plasmids as templates, overlapping PCR was used to alter amino acids that modified dipole moments of each of the two fluorescence proteins: mCitrine was modified to maximize the dipole moment while Cerulean was modified to minimize its dipole moment.

Amino acids to be modified were identified with help of the McCammon group from UCSD, who calculated and identified amino acids that will maximize inherent dipole-moment of mCitrine and minimize the dipole-moment of Cerulean. mCitrine gene was mutated at the following sites: S28D, K43D, R118D, N145R, Y147R, N160R, D176R, and Y178R. Cerulean gene was mutated at the following sites: E32R and K162D.

To analyze whether the fluorescence of mutant proteins was intact after each amino acid substitution, we ligated each mutated fluorescence protein genes into bacterial plasmid pLei, and each resulting plasmid was transformed into DH5 α cells. The transformed colonies of each plasmid then were analyzed using a macro-imaging system for fluorescence. pLei carries chloramphenicol resistance gene, which was used to select for transformed colonies.

1.2 Membrane Lipidation

Modified versions of the fluorescence protein genes were cloned into a mammalian expression vector pCDNA3.0. Because pCDNA3.0 contains different restriction enzyme sites, PCR was used to create new restriction sites flanking the fluorescent protein genes. Additionally, lipidation motifs were added as well. PCR was performed using the following primers (bold nucleotides indicate restriction sites):

1.2.1 Cerulean Lipidation

In order to insert modified Cerulean into the pCDNA3.0 vector, PCR was performed with a sense primer containing a 5' *HindIII* site with Fyn lipidation sequence (5'-CCA**AAGCTT**ATGGGCTGCGTGCAAGGACAA GGAGGCCACCAAGCTGGTGAGCAAGGGCGAGGAGCTG-3') and an anti-sense primer flanked by *BamHI* sequence (5'-CG**GGATCCTT**ACTTGACAGCTCGTCCA-3').

Within Cerulean, between residues Ile-171 and Gly-174, a 13-residue internal lipidation motif (-GGTKKFCGLCACP-) derived from SNAP-25 (known as shortsnap-25) was introduced (6) using the following primers: sense tgccgctgtgcgctgccccGGCAGCGTGCAAGCTCGCC; anti-sense: ggggcaggcgccagggccgcagaacttcttggtgccgccGATGTTGTGGCGGATCTT

1.2.2 ss25 linker/lipidation

ss25 is a linker that connects Cerulean and mCitrine. It also contains

palmitoylation sequence and is membrane-localized upon palmitoylation.

ss25 linker was generated using Klenow extension using the following primers: sense primer containing *Bam*HI restriction site 5'-
CGGGATCCGGCGGCACCAAGAAGTTCTGCGGCCTGTG and anti-sense primer
containing *Eco*RI site GCGAATTCGGGGCAGGCGCACAGGCCGCAGAACTTC

1.2.3 mCitrine Lipidation

A sense primer containing *Eco*RI at 5' end (5'-GCGAATTCGTGAGC
AAGGGCGAGGAGCTG-3') and an anti-sense primer, 5'- GCCTCGAGTTACT
TGTACAGCTCGTCCATGCC, were used to create *Xho*I at the 3' end of mCitrine.
Cerulean's C-terminus and mCitrine's N-terminus are linked by a membrane lipidated
linker ss25.

1.3 Cell culture and transfection

HEK 293T cells were cultured in DMEM media supplemented with 20% fetal bovine serum and 5% penicillin/streptomycin. Cells were cultured in sterile 6-well tissue culture-treated plate at 37 °C. When the cells were 50% confluent, 293T cells were transfected (Lipofectamine 2000, Invitrogen) with about 5 µg of reporter plasmid. 24 hours after transfection, cells were split onto 3 cm dish with poly-D-lysine coated cover slips. About 48 hours post transfection, cells were tested and examined under confocal microscope (Olympus IX81) at around 22 °C.

1.4 Primary Screening

Our reporter system was evaluated whether they are functional as outlined by

Knopfel et. al (2). While FRET ratio is being calculated and fluorescence emissions are measured under the confocal microscope, HEK293T cells were switched from normal extracellular solution to “high-K⁺” solution to change membrane potential. CFP and YFP images were separately acquired to calculate the sensitized emission, or “FRET signal.” Subsequently, the signal is divided by donor emission to measure FRET efficiency.

$$\text{FRET Ratio is defined as } \frac{\text{Aceptor_Intensity}}{\text{Donor_Intensity}}$$

Cerulean is excited at 433 nm and mCitrine is excited at 516nm.

Chapter 2 Results and Discussion

2.1 General Scheme

Taking advantage of the fact that change in membrane potential indicates neuronal activity, we designed a reporter that alters FRET ratio upon voltage change (**Fig 2.1**). In our system, mCitrine is the voltage sensor, whose dipole moment is maximized in order to maximize sensitivity to change in membrane potential. Depending on the membrane potential, mCitrine will either be close to or far from the membrane due to its inherent dipole moment interacting with the electric field created by membrane potential (which ranges from $\sim -70\text{mV}$ to $+50\text{mV}$). Change in the membrane potential results in translocation of mCitrine due to altered electric field near the membrane. This translocation of mCitrine changes the average distance and/or orientation to the stringently membrane-localized Cerulean, thus changing FRET ratio, which can be used as a proxy for change in membrane potential. In order to do this, we designed a plasma membrane-localized FRET system of Cerulean and mCitrine. While Cerulean is localized to plasma membrane by lipidating opposite ends of the protein, mCitrine is only lipidated at one end of the protein that allows it to move around (**Fig 2.1**).

2.2 Localizing our FRET system to the membrane

In order for our system to work, the reporter system must be localized to the plasma membrane. At the same time, the dipole moment needs to be orthogonal to the membrane (**Fig 2.1**).

We used membrane-lipidation sequences to membrane localize our reporter system. We inserted Fyn sequence to the N-terminal of cerulean. This sequence is established to rapidly myristoylate and palmitoylate at cysteine-3 residue when added to N-terminus of a protein (**10**). Because we needed to restrict the movement of Cerulean relative to the membrane, we replaced a loop in Cerulean with ss25 internal lipidation motif (palmitoylation). Imaging under fluorescence microscope showed that Cerulean was indeed well localized on the membrane (**Fig 2.2**)

Two fluorescence proteins were linked by adding another ss25 lipidation sequence and deleting TAA sequence of cerulean. This ss25 lipidation sequence is also lipidated, and thus also acts as membrane localizing sequence of N-terminal side of mCitrine. After the ss25 sequence mCitrine was added without any additional lipidation sequence. Membrane localization of mCitrine was also confirmed under fluorescent microscope (**Fig 2.2**).

In order to do this, we designed a membrane-lipidated FRET system of cerulean and mCitrine. While cerulean is fixed by lipidating both ends, mCitrine is only lipidated at one end that allows it to move around. In order to maximize movement of “free” mCitrine, we identified sites within mCitrine that upon mutation will increase the inherent dipole moment of the fluorescent protein. Fig x shows which mutations retained fluorescent activity upon mutation. We identified S28D, N160R, Y176R, and Y178R to be used.

The chosen construct of cerulean and mCitrine were inserted into the mammalian vector pCDNA3.1. Fluorescent imaging indicated that this construct was targeted to plasma membrane.

2.3 Maximizing Dipole Moment of mCitrine and Minimizing Dipole Moment of Cerulean

After confirming that our FRET reporter is successfully membrane-localized, we changed amino acids in Cerulean and mCitrine that alters dipole moments. Amino acids to be modified were identified with help of the mCammon group from UCSD.

After each modification, whether fluorescence was abolished was checked under macro-imager. Results are shown on **Table 2.1**.

2.4 Initial Reporting of FRET ratio change

After VSFP FRET system was constructed, the reporter plasmid was transfected to HEK293T cells. 48 hours post-transfection, Cerulean and mCitrine fluorescence emissions from the transfected cells were measured while we changed media from the control media to the high-K⁺ media and back to the control media. **Graph 2.1** shows that upon membrane potential change, FRET ratio also changes.

Appendix

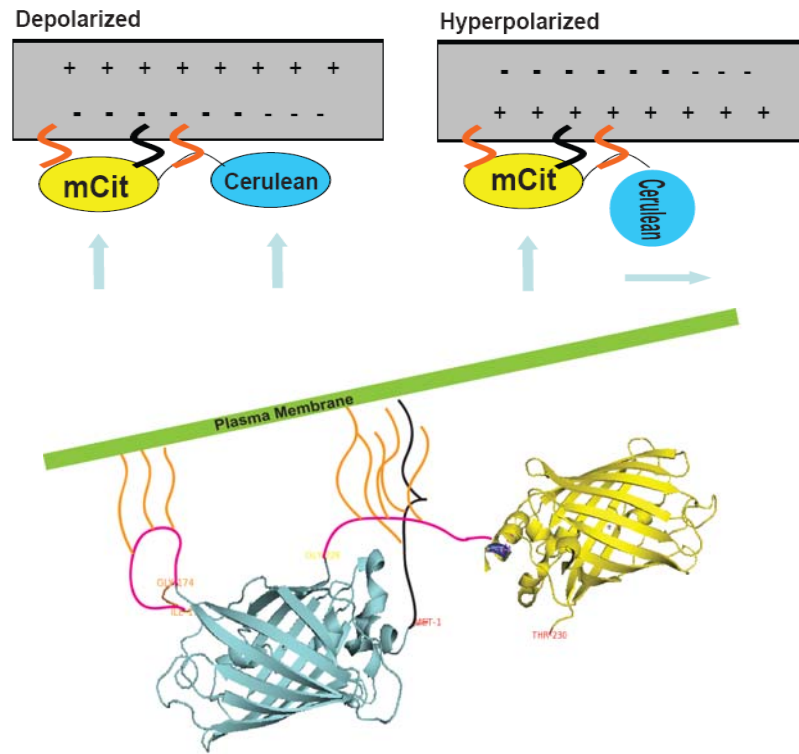


Fig 2.1: General Schematics. Arrows indicate dipole moment. During depolarization two proteins' dipole moments are parallel to each other, thus maximizing FRET signal. During hyperpolarization, membrane depolarization repels Cerulean and loss of FRET signal.

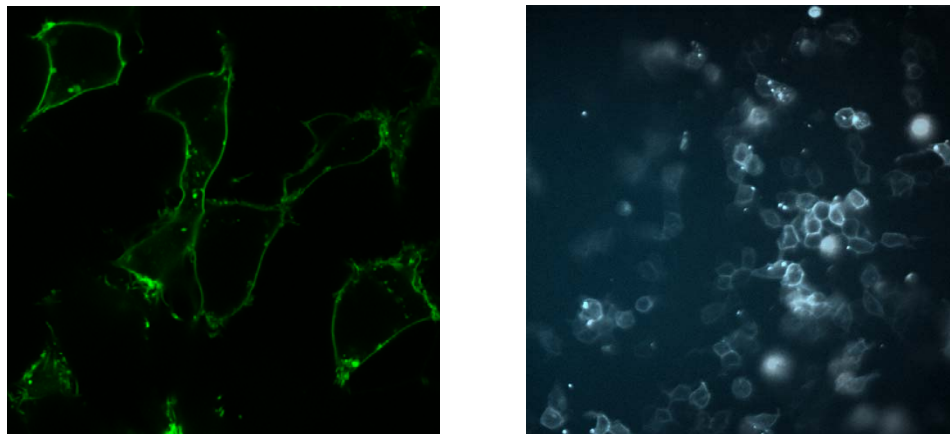


Fig 2.2: Membrane Lipidation imaged under a confocal microscope. mCitrine emission is on the left. Cerulean emission is on the right. Both fluorescence proteins are localized in the plasma membrane.

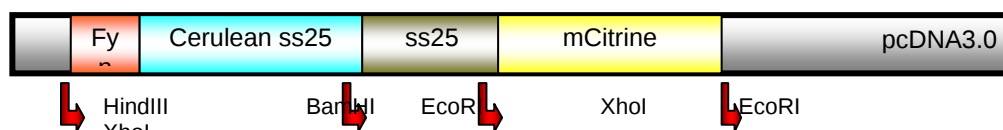


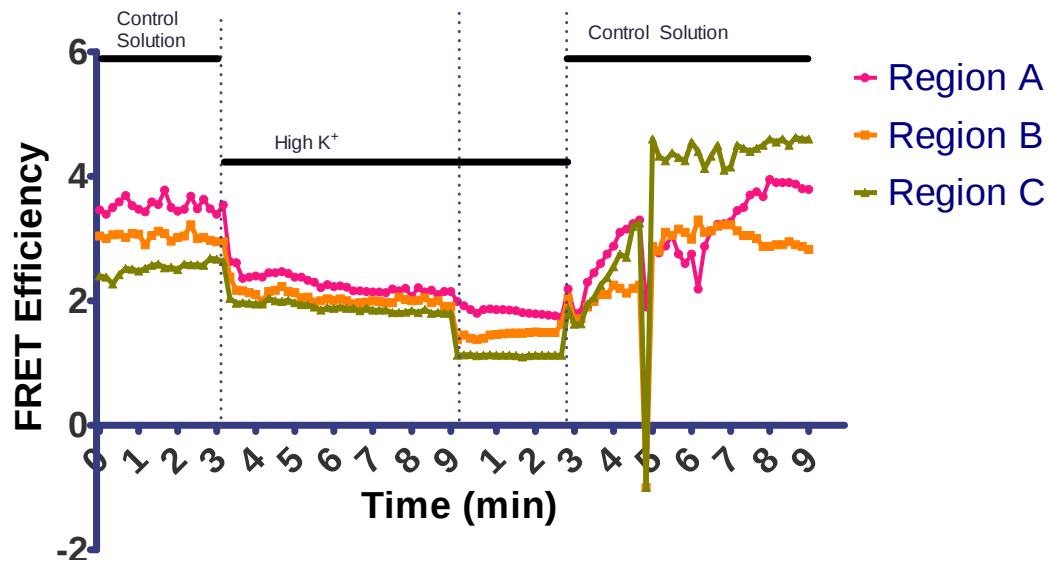
Fig 2.3: VFP Construct Scheme. TAA stop codon of cerulean is removed to allow the linker to connect two fluorescent proteins.

Table 2.1: Amino Acid Replacement and Fluorescence. Combination of individually fluorescent mutants does not necessarily result in fluorescence-intact protein.

<i>Mutation</i>	<i>Fluorescent</i>
L13D	-
S28D	Yes
K43D	No
R118D	Yes
N145R	Yes
N147R	Yes
N160R	Yes
D176R	Yes
Y178R	Yes

<i>Mutation</i>	<i>Fluorescent</i>
S28D, N160R	Yes
S28D, N160R, R118D	No
S28D, N160R, Y176R, Y178R	Yes
S28D, N160R, Y176R, Y178R, N145R	No
S28D, N160R, D176R, Y178R, Y147R	Weakly Fluorescent

Graph 2.1: Three random membrane regions were selected to measure FRET ratio over time. Background was subtracted. FRET ratio rapidly declines as high potassium solution is added. Upon aspirating potassium solution and control solution is added, FRET ratio slowly increased.



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