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Review of development of fluorescent protein sensor for monitoring calcium signalling

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Abstract: *Synaptic transmission monitoring has been problematic due to several shortcomings from measuring instruments and the miniature nature of nerve terminals. The advent of fluorescent protein calcium sensors has improved our understanding, especially of lighting neural circuit and monitoring calcium transients. Notably among them is the GCaMP family of fluorescent sensors. Subsequent development of numerous types of GCAMP's has improved our understanding of neural circuitry and mechanism behind numerous neurodegenerative diseases. The attachment of a vesicular protein synaptophysin to GCaMP2 which led to SyGCaMP2 has two major advantages over existing cytoplasmic GECI in detecting electrical activities of neurones; (a) it identified the locations of synapses and (b) had a linear response over a wider range of spike frequencies [23]. Here we explored the various GCaMP fluorescent sensors, their advantages and disadvantages.*

Keywords: *Synaptic transmission, fluorescent sensor, GCaMP family, synaptophysin and neurodegenerative diseases.*

I. Introduction

There are many different ways of measuring network dynamics. Methods include those that are able to measure global activities of large brain regions such as functional magnetic resonance imaging (fMRI), positron emission tomography (PET), field potential analysis (FPA) and intrinsic imaging. Single cell activity can be measured with extracellular single or multi-cell electrode arrays or with intracellular recording methods including current clamp and voltage clamp techniques. Optical recording methods using fluorescence dyes and, more recently, fluorescent proteins can be used to assess neuronal activity, in many cases with both high spatial and temporal resolution. An ever increasing range of dynamic sensors have been developed including voltage, sodium, pH and calcium sensors. Amongst these, it is the calcium sensors that are arguably the best developed in terms of their sensitivity, dynamic range and speed of response to biological signals.

II. Synthetic fluorescent calcium indicators

In order to understand the complex nature of the brain we need technologies that allow observation and measurement of neural activities with both high spatial and temporal resolution. Hence the development of fluorescent indicators goes a long way in resolving these puzzles though with its own biases. Various dyes have emerged, such as FURA- 2 [1, 2] Fluo-1, 2, 3 [3], Fluo-4 [4] and Indo group, notable among them are acetoxymethyl (AM) ester derivatives [5, 6].

In the past two decades, Ca^{2+}-chelating dyes have been used as a mode of measuring the changes in Ca^{2+} levels in living organism in response to stimuli. Free salt versions are membrane impermeant and so need to be injected into cells. The AM forms of the dyes are cell permeable and can cross the cell plasma membrane after a period of incubation. De-esterification of the dyes occurs inside the cell, to form less permeable forms, which are maintained inside the cell for the duration of the experiment. In most cases the emission wavelengths of the dyes changes in contact with Ca^{2+} providing means of detecting the change in Ca^{2+} levels. A light source is needed to excite the dye in the cells. It is this difference in the fluorescent emission when the dye is bound versus when the dye is unbound that gives the measure of the change in $[Ca^{2+}]$ (Graham 2010). Their major drawbacks include in vivo implementation and consequent hindrance in targeting specific sub-cellular compartment although there are few examples where people have been very successfully applying AM-versions of the dye in vitro and in vivo to produce loaded cell. Hence the engineering of genetically encoded calcium sensors throws more light in studying calcium signalling dynamics in the central nervous system. See table I-1 for comparisons of some calcium indicators.

III. The fluorescent proteins

The use of photo-proteins as a method for [Ca^{2+}] detection originated with the discovery of bioluminescent proteins such as aequorin, which naturally emit light with changes in [Ca^{2+}] [7-9]. The core distinction between the use of these proteins and the fluorescent dyes comes from the fundamental difference between fluorescence and bio- or chemoluminescence. Because the light emitted from bioluminescent elements comes from an inherent property of the molecule, no exothermic chemical reaction is necessary to create the signal. This also means that there is a much higher background signal. This results in a low signal to noise ratio (SNR), which also makes detection of smaller signal difficult. In the case of chemoluminescence however, the light signal emitted is a consequence of a supplementary chemical reaction and consequently yields a much higher SNR because of the absence of a substantial background signal [10]. The discovery of the green fluorescent protein (GFP) and its derivatives has thrown more light in the study of calcium signalling and in resolving most of the limitations of previous dyes.

GFP derived from the jelly fish Aequorea victoria is a 238 amino acid protein with an apparent molecular weight of 27-30kDa [7, 8]. Various forms of fluorescent protein based calcium sensor have been developed, ranging from the Cameleons [11, 12], Camgaroo [13-15], Pericams [16, 17] and the GCaMP family [18-20].

Table Error! No text of specified style in document.-1 Comparison of some calcium indicators

Sensors	Design/name	Apparent Kd for Ca^{2+} (nM)	Advantages	Disadvantages
Synthetic Dye	Stil-1	132-200		Invasive and difficult to target subcellular compartments
	Stil-2	224		
	Indo-1	250		
	Fura-1	107	High affinity, can sense resting values of Ca^{2+}. Better selectivity for Ca^{2+} than Mg^{2+}	
	Fura-2	135-224		
	Fura-3	140		
	Quin-2		High affinity, can sense resting values of Ca^{2+}	Not suitable for sensing elevated Ca^{2+} as it saturates quite easily and loses resolution (Grynkiewicz et al 1985). More sensitive to other divalent ions such as Mg^{2+}, Mn^{2+}, and Zn^{2+}
	Fluo 1-4		High and low affinity sensors	Not suitable for specific compartment localization
Fluorescent Proteins				
(a) Cameleons	YC2	(1-300μM)	Suitable for fast Ca^{2+} change detection	Poor dynamic range Sensitive to pH and Cl^-
	YC3			
	YC4			
	YC3.6 Venus	250	Wider and better dynamics (Nagai et.al 2004)	
(b)Camgaroo	Camgaroo	7μM		
	Camgaroo-2	5.3μM-8μM		
(c)Pericam	Flash pericam	700		No suitable for detecting slow Ca^{2+} dynamics of the residual calcium activity
	Inverse pericam	200-900		
	Ratiometric pericam	2.1uM		

Sourced (Grynkiewicz et.al 1985, Demaurex 2005, Reiff et.al 2005, Kotlikoff 2007, Hendel et.al 2008)

Development of GCaMP family of sensors Various genetically encoded calcium sensors have been developed ranging from single chromophores, circularly permutated single chromophores to double chromophores. The
single and circularly permuted single GECI rely on a calcium-dependent rearrangement of the green fluorescent proteins (GFP) conformation, whereas the double chromophore GECIs are based on calcium-dependent changes in the efficacy of fluorescence resonance energy transfer (FRET) between two spectral GFP variants [21].

Table Error! No text of specified style in document.-2 Comparison of GCaMP family of calcium indicators

Sensors	Design /name	Apparent Kd for Ca^{2+} (nM)	Advantages	Disadvantages
GCaMP family	GCaMP1	235	bright	Unstable and low signal to noise ratio
	GCaMP1.6	146-150	Increased brightness	
	GCaMP2	146-188 (545±32nm)	Stable and brighter than version 1	Low signal to noise ratio
	GCaMP3	(405±9)	More stable and brighter than GCaMP 1 and 2 versions	Inability to measure 1-2AP due to low signal to noise ratio
	SyGCaMP2*	183 ± 6.1	More stable and brighter and sub-cellular targeting	Low signal to noise ratio
	GCaMP5B	(307±12)	Improved brightness	Subcellular targeting impossible
	GCaMP5D	(730±13)	Improved brightness	Subcellular targeting impossible
	GCaMP5G	(460±11)	Improved brightness	Subcellular targeting impossible
	GCaMP5K	(189±5)	Improved sensitivity/brightness	Subcellular targeting impossible
	GCaMP5L	(390±18)	Improved brightness	Subcellular targeting impossible
	GECO	340 - 1140		Same as GCaMP3
	GCaMP6	??	Better than GCaMP5	Unknown Kd

Sourced from Reiff et.al 2005, Kotlikoff 2007, Hendel et.al 2008, Akerboom et.al 2012, those in bracket are sourced from Akerboom et.al 2012, while that in asterisks was obtained in our laborator

The mode of action of the GCaMP family is based on the effect of a conformational change through calmodulin interacting with M13 light chain kinase in the presence of Ca^{2+}, which subsequently alters the conformation of the fluorophore of an attached FP leading to an increase in brightness [18]. The emergence of genetically encoded calcium indicators goes some way in addressing some of the shortcomings of synthetic dyes and fluorescent proteins such as low signal to noise ratio [11, 22] and subcellular compartment targeting [23, 24]. Despite certain limitations of GECIs (reduced dynamic range, limited range of affinities), they have certain distinct advantages and these include an ability to target the sensor to particular sub-cellular compartments by fusing the sensor to a protein of interest..

(a) GCaMP1 basic structure and principle of operation GCaMP1 comprises the circularly permuted enhanced green fluorescent protein (cpEGFP) attached to N-terminal of calmodulin (CaM) (Calcium binding protein) and a M13 myosin light chain kinase (MLCK) attached to the N-terminus of the cpEGFP (See Figure 1) [18, 25].

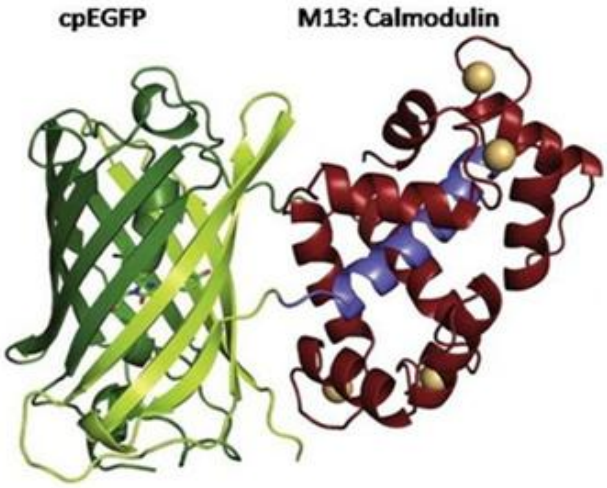

Figure 1 Structural basis of GCaMP based sensors

GCaMP ribbon structure showing the cpEGFP on the left and the Calmodulin + M13 molecules on the right (Wang et.al 2008)

When Ca^{2+} binds to calmodulin a conformational change induces another conformational change in the cpEGFP that enhances fluorescence intensity [18, 25, 26]. Nakai et al (2001)

Showed that GCaMP1 has an apparent dissociation coefficient (Kd) of about 235 nM and a Hill coefficient of 3.3 [18]. GCaMP1's basal fluorescence was very low and could not fluoresce well at 37^{o}C and above [25, 27]. These limitations led others towards developing a more thermal stable indicator with a higher affinity for calcium.

(b) GCaMP1.6

GCaMP1 fluoresces at 28^{o}C but not at 37^{o}C [18] and its fluorescence is dim at baseline calcium levels. Mutations at Glu-11/Phe-12, Glu-14/Ala-15/Phe-16 and Glu-67/Phe-68 in the CaM, and also V163A and S175G led to the emergence of GCaMP1.6 [25]. This improved brightness by 40 times compared to GCaMP1 and also affinity for Ca^{2+} with a Kd of about 146 nM. However, it was not thermally stable and had little or no fluorescence increase at 37^{o}C [19, 27].

(c) GCaMP2

Subsequent mutation at A206K and the addition of a plasmid leader sequence RSET to the N-terminal of the M13 peptide prevented dimerisation and improved thermal stability respectively and produced GCaMP2 [19]. Also, brightness was improved by mutating D180Y and V93I. The Kd for the purified protein was similar to GCaMP1.6 (146nM) but higher when measured in vitro 188nM. GCaMP2 was 200 times and 6 times brighter than GCaMP1 and GCaMP1.6 respectively. GCaMP2 was not suitable for detecting transient Ca^{2+}signals due to its low signal-to-noise ratio and low baseline fluorescence. In addition, GCaMP2 cannot detect single action potentials reproducibly [26, 27].

(d) GCaMP3

Further mutation at T116V, M66K and N363D (Calmodulin N60D) increased the fluorescence change for small Ca^{2+} transients - GCaMP3, which had a 3-fold increase in fluorescence and greater dynamic range than the GCaMP2. It has a 1.3 times higher affinity for calcium than GCaMP2 and was more photostable [20]. GCaMP3 is apparently brighter than GCaMP2 yet unable to detect single to double action potentials [28].

(e) GCaMP5 and its derivatives

Modification of GCaMP3 through protein structure determination, targeted mutagenesis, high-thoroughput screening, and a battery of in vitro assays, has led to several fold increase in the dynamic range of GCaMP3, creating a family of "GCaMP5" sensors. Signal-to-noise ratio was improved by at least 2- to 3-fold [29]. They reported that in the visual cortex, two GCaMP5 variants detected twice as many visual stimulus-responsive cells as GCaMP3. By combining in vivo imaging with electrophysiology Akerboom et al (2012a) and group showed that GCaMP5 fluorescence provides a more reliable measure of neuronal activity than its

predecessor GCaMP3 [29]. Mutations at D380Y and R392G yielded their best and highly sensitive sensor GCaMP5K with a Kd of about 189 ± 5nm yet single AP detection was still elusive.

Few GCaMP based sensors has been developed with improved sensitivity towards detecting single AP such as GCaMP6 [30] while the GECO did not differ from its parent sensor GCaMP3 [31, 32].The Kd for GECO ranges from 340 - 1150 nm, which is insensitive to residual Ca^{2+}. Currently the Kd for GCaMP6 is not spelt out although it is better than GCaMP5 as reported by Cheng et.al (2011). A schematic diagram illustrating the level of GCaMP development with their various achievements is shown in figure 2.

(f) Membrane and cytoplasmic protein attachments to GCAMP2

Targeting specific sub-cellular compartment was then paramount in detecting reproducibly, even small Ca^{2+} signals and also increasing the usefulness of existing calcium sensors. Attachment of specific proteins to the GCaMP2 led to improved monitoring of sub-cellular compartment Ca^{2+} signalling such as plasma membrane proteins; α1(f)GCaMP2 and α2(f)GCaMP2 (Lee et.al 2006), vesicular membrane proteins - synaptophysin; SyGCaMP2 (Dreosti et.al 2009), isoforms of adenylate cyclase; GCaMP2-AC8 (Willoughby et.al 2010), and the N-terminal domain of Lck, a Src tyrosine kinase Lck-GCaMP2 [33].

Willoughby et al 2010 fused GCaMP2 to adenylate cyclase 2 and 8 (GCaMP2-AC2 and GCaMP2-AC8) to detect capacitance calcium entry, which showed that these sensors can target discrete micro-domains [24]. Tethering GCaMP2 to membrane domain Lck has been used to monitor Ca^{2+} signalling in astrocytes [33]. The attachment of the 4 transmembrane domain vesicular protein synaptophysin to GCaMP2 led to the development of SyGCaMP2, which targets the pre-synaptic terminal [23]. They reported a linear response to change in intracellular calcium concentration over a wide range of stimulus number and frequencies. And also detects brief Ca^{2+} transient passing through the pre-synaptic compartment since it is localised at the synaptic vesicles hence very close to voltage gated Ca^{2+} channels [23]. Using a model to simulate calcium signalling within presynaptic terminals, they showed that placing the GCaMP2 within 50 nm of the active zone would generate signals that differed little from averages obtained over the entire bouton. These simulations suggest that the best combination of SNR and temporal resolution will be obtained by distributing GCaMP2 molecules throughout the pre-synaptic compartment, hence the fusion of GCaMP2 to C-terminal of synaptic vesicular protein - synaptophysin (Dreosti 2009).

Dreosti et.al (2009) reports that by targeting GCAMP2 to presynaptic terminals, SyGCaMP2 has an improved sensitivity to measurement of single action potentials because it is located in a compartment where calcium increases to a high level [23]. SyGCaMP2 is well positioned to provide a good SNR and it can even detect single action potential, which its parent (GCaMP2) cannot. However, it is not very bright which makes detection of synapses difficult. See a schematic diagram (Figure 2) showing GCaMP family tree for more illustration. Employing ratiometric methods to targeted sensors would bring a number of additional advantages over single wavelength fluorescent sensors. These include better identification of the targeted compartment, reduction in motion artefacts via wavelength ratioing [20].

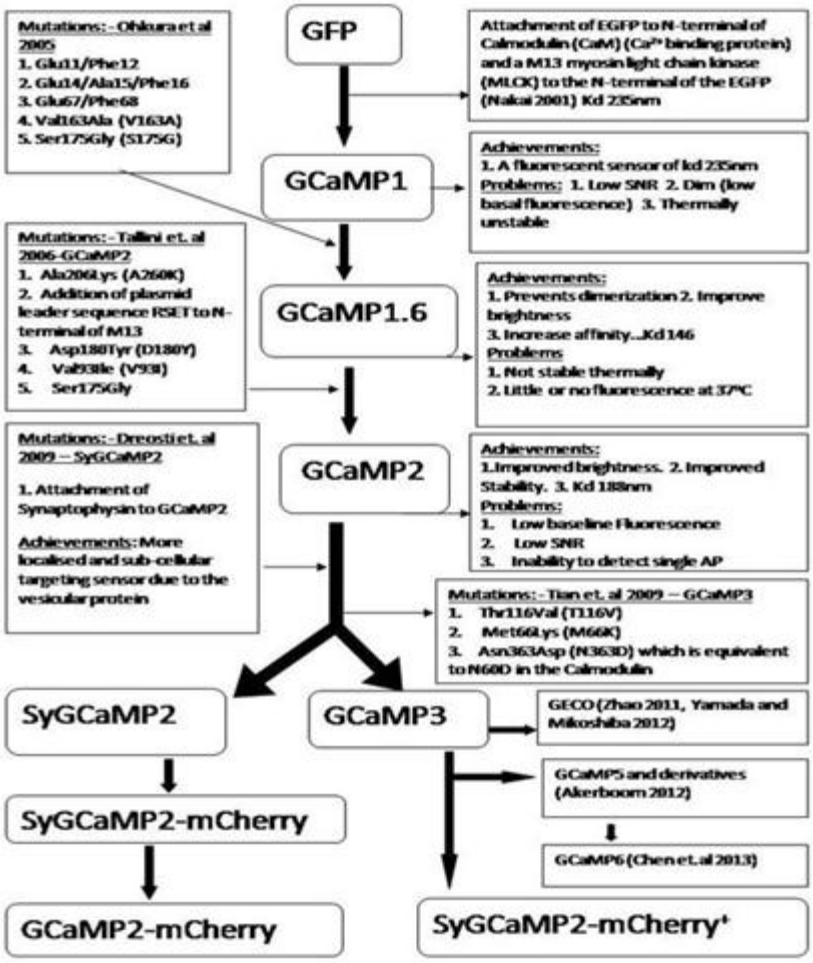

Figure 2 Development of the GCaMP family fluorescent protein calcium sensors schematic diagram

IV. Conclusion

Monitoring calcium dynamic especially at the level of the synapse has been a herculean task. The development of fluorescent calcium sensors has brought succour to our understanding of calcium kinetics in health and in disease in the nervous system. GFP and its derivatives has been of intense studies and modification of the GCaMP family as explored in this study shows a good direction towards better understanding cum visualisation of numerous neurodegenerative disease and calcium measurement even residual calcium.

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