REVIEW

Recent progress in the development of genetically encoded Ca^{2+} indicators

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Abstract : Genetically encoded calcium indicators (GECIs) are powerful tools to monitor the dynamics of calcium ion (Ca^{2+}) in living cells and organisms. With the help of GFP technology and DNA engineering, a dozen sets of GECIs have been developed so far. Their application has been widely extended into the analysis at the subcellular local, single and population of cell. In the past decades, GECIs have been dramatically improved in their performance and are becoming more and more useful for live imaging. In this review, the progress in the development of GECIs is discussed by introducing the history and emerging GECIs, which would help the selection of the appropriate GECI for a given application. J. Med. Invest. 62 : 24-28, February, 2015

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1. INTRODUCTION

Intracellular calcium ion (Ca^{2+}) is a universal second messenger that controls both physiological and pathological phenomena. Examples include fertilization, muscle contraction, cell death, wound healing and so on. Accumulating knowledge of Ca^{2+} homeostasis has been mainly brought by Ca^{2+} imaging, in which Ca^{2+} indicators play a role. Currently over one hundred of chemically synthesized and genetically encoded indicators (GECIs) are available. All of them share the principle both in its design and mode of action, *i.e.*, indicators are the hybrid molecule of Ca^{2+} binding molecules decorated with chromophore(s) whose absorption or emission property changes depending on Ca^{2+} binding.

The most commonly utilized are chemical indicators such as fura-2, indo-1, fluo-4 and Calcium Green-1 being the derivative of Ca^{2+} selective chelator, called BAPTA (1, 2). The easiness of intracellular loading of acetoxymethyl esterated dyes facilitated Ca^{2+} imaging not only in cultured cells but also in living animals (3). While this technical feasibility, chemical dyes can not be localized to subpopulations of cells and subcellular compartments like Ca^{2+} storing endoplasmic reticulum (ER), being the important research target in Ca^{2+} homeostasis. Once loaded, dyes also suffer from deleterious compartmentalization and poor retention in several hours of observation.

Genetically encoded Ca^{2+} indicators (GECIs) are alternative tools that overcome these limitations. In broader sense, GECI are the luminescent or fluorescent proteins (FPs) having Ca^{2+} binding ability, so as to changes the absorption or emission property upon Ca^{2+} binding. In addition to the naturally existing Aequorin (4), genetically engineered indicators such as cameleon and camgaroo are the members of this family (5, 6). GECIs can be localized to subcellular locals such as nucleus, ER and mitochondria by genetic tagging. Targeted and long-term expression in a given cellular network has been successfully demonstrated in a number of model organisms.

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2. GENETICALLY ENCODED Ca^{2+} INDICATOR

GECIs are categorized into two groups based on the molecular design, i.e., two-FP based and single-FP based one as is discussed in many reviews (Table1, 2) (7, 8).

Table 1 *In vitro* properties of two-FP GECIs, by family.

Indicator	Em, nm	*D*	K_d, μM	references
YC-2	480(530)	1.8	-	(5)
YC-3	〃	-	4.4	〃
YC-4	〃	-	0.083/700	〃
YC2.60	480(530)	6.6	0.04	(14)
YC3.60	〃	6.6	0.25|0.22/0.78	(14)|(15)
YC4.60	〃	4.6	0.06/14.4	(14)
YC-Nano140	〃	14	0.14/0.75	(15)
YC-Nano65	〃	14	0.06/1.4	〃
YC-Nano50	〃	13.5	0.05/0.4	〃
YC-Nano30	〃	13.5	0.03/0.2	〃
YC-Nano15	〃	15.5	0.016/0.3	〃
D2cpV	480(535)	5.3	0.03/3.0	(24)
D3cpV	〃	5.1	0.6	〃
D4cpV	〃	3.8	64	〃
TN-L15	〃	2.4	1.2	(25)
TN-XL	〃	5	2.5	(26)
TN-XXL	〃	3.3	0.8	(27)
Indicator	Em, nm	*D*	K_d, μM	references

Em : emission wavelength, *D* : dynamic range, K_d : dissociation constant, Values from different references are separated by vertical bar |. Modified from ref (8)

Table 2 *In vitro* properties of single-FP GECIs, by family.

Indicator	Backbone	Ex/Em (Ca^{2+} free)	Ex/Em (Ca^{2+} saturated)	*D*	K_d, μM	references
camgaroo	splitEYFP (Y145)	-	490	7	7	(6)
F-Pericam	cp145EYFP(V68L/Q69K)	488*/514	494*/514	8	0.7	(17)
I-Pericam	〃	505*/515	490*/513	6.7	0.2	〃
R-Pericam	〃	415/511	494/517	10	1.7	〃
GCaMP1 (G85)	cp149EGFP (144)	488*/510	487*/510	4.3	0.235	(18, 20)
GCaMP1.6	〃	489*/510	488*/509	4.9	0.146	(19, 20)
GCaMP2	〃	491*/511	487*/508	4.9	0.146|0.545	(20)|(22)
GCaMP3	〃	505/517	497/515	13.5|12	0.345|0.54	(23)|(28)
GCaMP5G	〃	〃	〃	32.7|45.4	0.46|0.447	(22)|(23)
GCaMP6f	〃	〃	〃	51.8	0.375	(23)
GCaMP6m	〃	〃	〃	38.1	0.167	〃
GCaMP6s	〃	〃	〃	63.2	0.144	〃
G-GECO1	GCaMP3	496*/512	496*/512	26	0.75	(28)
G-GECO1.1	GCaMP3 x mApple	496*/512	496*/512	27	0.62	〃
G-GECO1.2	GCaMP3 x f-pericam x mApple	498*/513	498*/513	24	1.15	〃
B-GECO	GCaMP3 x f-pericam x mApple	378*/446	378*/446	8	0.16	〃
R-GECO	cp146mApple	577*/600	561*/589	17	0.48	(28)|(29)
GEX-GECO1	GCaMP3 x f-pericam x mApple	397*/512	390*/ 506	27	0.32	(28)
GEM-GECO1	GCaMP3 x f-pericam x mApple	397*/ 511	390*/455	111	0.34	〃
BCaMP1c	cpBFP	-	-	2	0.5	(29)
CyCaMP1b	cpCFP	-	-	2.6	0.42	〃
YCaMP1b	cpYFP	-	-	9.2	0.8	〃
RCaMP1h	cp159mRuby	575*/602	571*/594	10.5	1.3	〃
Indicator	Backbone	Ex/Em (Ca^{2+} free)	Ex/Em (Ca^{2+} saturated)	*D*	K_d, μM	references

Ex/Em : excitation and emission wavelength, D : dynamic range, K_d : dissociation constant, * for Ex is a peak absorption. Values from different references are separated by vertical bar |. Modified from (8)

2.1 Two-FP indicator

Two-FP GECIs report Ca^{2+} dynamics based on Förster resonance energy transfer (FRET) mechanism, being an opto-physical phenomenon that occurs on closely positioned donor and acceptor pairs within 10 nm (9). What observed is the emission spectrum change caused by non-radiative energy transfer of the excited state energy of donor to acceptor. The efficiency of FRET is sensitive to their positional parameters including the distance and orientation between two chromophores, in addition to their spectral property. The spatial scale less than 10 nm is in a close proximity of the size of protein (ex. Φ=3 nm for GFP), FRET emission change had been often utilized to monitor the conformational change of proteins labeled with a pair of fluorescent molecule as the donor and acceptor.

Cameleon is one of the first reported FRET-based GECI, being a hybrid protein consisting of Ca^{2+} sensing motif sandwiched with two GFP variants (5). Ca^{2+} concentration is measured in the form of FRET emission ratio that reflects the conformational state of Ca^{2+} sensing motif, calmodulin (CaM) and its binding peptide (M13). Upon Ca^{2+} binding, CaM-M13 moiety drastically changes its overall structure from relaxed to compact form (6). This repositions the donor and acceptor closely, which eventually causes low to high FRET emission change. The molecular design of cameleon standardized the way to develop FRET-based indicators as has been employed to detect diverse signaling events (10). In the proof-of-concept demonstration, original cameleon successfully reported the cellular and subcellular Ca^{2+} dynamics in cultured cell.

For long term and reliable imaging, enough brightness and photochemical stability are needed for GECIs. Substituting FPs with enhanced version was shown to be effective. While CFP is relatively insensitive to pH fluctuation, original YFP with a p*K*a around 7.0 was sensitive to small pH fluctuations in the physiological condition. V68L/Q69K mutations on EYFP reduces p*K*a to 6.1 and yellow cameleon (YC2.1) incorporating EYFP-V68L/Q69K as acceptor was less pH-sensitive, although its folding efficiency at 37°C remained suboptimal (11). Better solution was brought by other YFP variants such as Citrine-Q69M and Venus. Citrine was developed to show efficient folding at 37°C and chloride resistance,

and Q69M mutation further lowered the p*K*a to 5.7 (12). Venus, the most commonly utilized YFP variant, is characterized with the highest brightness among this group, fast maturation and improved environmental insensitivity (13). Cameleons harboring Citrine-Q69M or Venus (YC2.3 or YC2.12, respectively) are less artifact-prone than parental versions (12, 14).

The most challenging optimization of FRET-type indicator had been to increase the dynamic range. As the FRET efficiency strongly depends on the positional parameters, *i.e*., the distance and orientation between donor and acceptor, systematic change in these parameters should be effective. This was achieved by replacement of donor or acceptor with its circularly permuted one. Among examined, replacing Venus acceptor with cp173Venus, dramatically expanded the dynamic range of YC as large as 560% (14). FRET efficiency at the ON-Ca^{2+} state was increased as high as 93% while that of OFF-Ca^{2+} state was left to 43% (15). Resulting YC2.60 and YC3.60 had been successfully utilized in *in vivo* applications including the fiber optics-assisted neuronal imaging in freely moving mice (16).

2.2 Single-FP indicator

GECIs consisting single FP instead of two have been available. This group reports Ca^{2+} dynamics with changes in the emission intensity brought by Ca^{2+} dependent modulation of chromophore state. The first reported single FP-based indicator is camgaroo, in which N- and C- terminally split EYFP was linked with full length CaM (6). Ca^{2+} dependent intra-molecular reconstitution of split YFP restores the fluorescence emission while it is non-fluorescent in the absence of Ca^{2+}. Poor emission recovery of camgaroo was improved in "pericam series" which utilize a circularly permuted YFP as its backbone (17). Original YFP (and GFP) has double absorption peak at 400 and 490 nm, each corresponding to chromophre state with or without its protonation. Although Ca^{2+} dependent modulation of the chromophore state was not efficiently achieved in original YFP, the breakthrough was brought by the use of circularly permuted YFP as a backbone. cp145EYFP_V68L/Q69K in which M13 and CaM_E104Q was fused to its N- and C-terminus displayed Ca^{2+} dependent brightness change. Additional mutations on EYFP and the linker optimization generated three variants having distinct property. Mutation Y203H resulted in Flash-Pericam displaying 8-fold increase of emission upon Ca^{2+} binding. Another mutation Y203F and H148T resulted in Inverse Pericam, being a bright indicator in the absence of Ca^{2+} displayed 15% decrease of emission intensity upon Ca^{2+} binding. H148D/F203F with modified linker at the center of cpEYFP yielded Ratiometric Pericam, whose emission intensity can be measured by alternating excitation with 415 and 494 nm.

GFP is also utilized to independently develop a series of GCaMPs. GCaMP1, consisiting cp149EGFP in which M13 and CaM were fused to N- and C-terminus, displayed 4.5-fold increase of emission intensity in response to Ca^{2+} addition (18). Its low brightness was serially improved by introducing mutations on cpEGFP moiety. V163A/S175G and D180Y/V93I increased the brightness both at the chelating and saturating Ca^{2+} condition, yielding GCaMP1.6 and GCaMP2, respectively (19, 20). M153K/T203V on cpEGFP moiety in combination with affinity modulating mutation on CaM (N60D) resulted GCaMP3, whose dynamic range was 2.5 times increased to GCaMP2 (21). The linker sequences connecting M13/CaM to the cpEGFP, which were identified to be the critical interface to tune the response properties, were also optimized to generate a series of GCaMP5 (5A, 5D, 5G, 5K and 5L) and GCaMP6 (6f, 6m, 6s) (22, 23). Improved dynamic range (38- to 63-fold signal change) and slow to fast kinetic variants for off-reaction were identified by neuronal expression screening and successfully utilized in *in vivo* functional imaging in zebrafish, flies and mice (23).

3. PARAMETER TUNING

Affinity

Because the $[Ca^{2+}]_{rest}$ and the amplitude of Ca^{2+} concentration change differs significantly among subcellular locals, celltypes and organisms, low to high affinity variants of GECIs covering K_ds of nM to mM are essential. Wildtype CaM contains four Ca^{2+} binging motif, called EF-hand. EF hand consists 12 amino acids of consensus motif and 7 of these are involved in Ca^{2+} chelating. Site directed mutagenesis on these chelating residue achieves tuning Ca^{2+} affinities of YCs. While YC-2 harboring wildtype CaM was reported to have a biphasic Ca^{2+} dependency with K_d's of 70 nM and 11 μM, substitution of glutamate 104 with glutamine in the third EF-hand resulted in the indicator YC-3 having reduced affinity (K_d of 4.4 μM). E31Q mutation on the second EF-hand motif further lowered affinities to K_d's of 83 nM and 700 μM in YC-4 (5). Rearrangement of the overall design also affects Ca^{2+} affinity. Substituting EYFP-V68L/Q69K acceptor to cp173Venus increased Ca^{2+} affinity from 1.5 μM to 0.25 μM (YC 3.1 and YC3.60, respectively) (5, 14).

While developing YCs with moderate and low affinity variants (K_d> 0.1 μM) had been successful, systematic way to engineer high affinity variants had been waited. *In vitro* analysis reported much higher Ca^{2+} affinity of free CaM and M13 (K_d of 20 nM) than that of fusion peptide of CaM-M13 linked with two amino acid linkers (K_d of 80 nM) (6). This suggested steric hindrance might prevent efficient interaction of Ca^{2+}-CaM with M13 in YCs. This possibility was examined by serial increment of the linker length from 2 to 5 amino acids. As a result, YCs with increased Ca^{2+} affinity were obtained. The length of flexible linker 3 and 4 yielded K_d's of 60 nM and 30 nM and plateaued for 5 amino acid linker with K_d of 15 nM, being the highest Ca^{2+} affinity reported so far. These affinity variants, named YC-Nano enabled the detection of the subtle Ca^{2+} change at nM in chemotaxing cells, which had not been performed by conventional GECIs (15).

Interference

The functional interference with GECIs and endogenous proteins could be a problem. CaM of YCs potentially trans-activates endogenous CaM targets being abundant in the cell. *In vitro* analysis reported excess amounts of CaM does affect the dynamic range of conventional YCs in a dose-dependent manner (24). To avoid these side-effects, computational re-designing of Ca^{2+} sensing motifs was performed. Modified binding interface of the newly designed CaM and its target should prevent intermolecular interaction. Resulting YCs, named D2/3/4cpV, have been demonstrated to be insensitive to large excesses of CaM with keeping K_d of 0.03 to 64 μM and large dynamic ranges from 3.8- to 5.3-fold (24).

Alternative way to avoid the uncontrolled interaction of GECI with endogenous proteins is to employ different Ca^{2+} binding motif than CaM. While CaM has a variety of downstream targets, Toroponin C (TnC), a skeletal and cardiac muscle specific Ca^{2+} binding protein is known to have limited interaction just with Troponin I and Troponin T. Indicators incorporating TnC from avian skeletal muscle or human cardiac muscle were generated based on similar molecular design of cameleon. Resulting TN-L15 and TN-hTnC displayed moderate Ca^{2+} affinity (K_d's of 1.2 and 0.47 μM) at the expense of a low specificity to Ca^{2+} (due to the cross reactivity with Mg^{2+}) and small signal change (due to the lack of its binding peptide which enhances the conformational change of sensor motif) (25). As performed on YCs, further improvements have been introduced to TN-L15. Mg^{2+} reactivity was eliminated by site directed mutagenesis on TnC, and dynamic range was increased by replacing Citrine accepor to its cp174variant, eventually yielded TN-XL (26). Low affinity of TN-XL(K_d of 2.5 μM) was improved in TN-XXL (K_d=0.8 μM) by replacing TnC moiety with concatenated its high affinity C-lobe. Although the *in vitro*

dynamic range of TN-XXL was small (3.3-fold), its *in vivo* performance was acceptable suggesting the advantages of TnC with reduced interference (27).

4. NEW GECIs COMPATIBLE WITH OPTOGENETIC TOOLS

In life science a paradigm shit has been brought by optogenetics. Channel rhodopsin (ChR) and halorhodopsin (HR), being a light-gated ion channel and pump, allows the spatially and temporally localized control in the activity of neural circuits through the light driven activation or silencing. Recently, demands for a combinatorial application of optogenetic tools with Ca^{2+} imaging have been rapidly growing and challenges for developing color variants of GECIs have been attempted. As ChR and HR is activated by blue (400-500 nm) and yellow (500-600 nm) light, respectively, the development of blue-and red-shifted GECIs are needed. GECOs, are the first reported color variants of single-FP GECIs based on cpGFP and cpmApple (28). By utilizing bacterial peri-plasmic expression system, large-scale screening identified blue and red color variants in addition to the green and ratiometric ones. Structure guided evolution of GCaMP also expanded its color hue, yielding BCaMP1c, CyCaM1a, YCaMP1b and a series of RCaMPs (29).

Bioluminescent-based Ca^{2+} imaging is an ideal strategy highly compatible with optogenetic. As bioluminescent indicator requires no excitation light, observation can be performed free from undesired functional crosstalk with optogenetic tools. Although the problem had been the small signal of Ca^{2+} sensitive Aequorin, increased emission signal was achieved in BRAC and Nano-lantern(Ca^{2+}), being the latest version of bioluminescent-based GECIs (30, 31). Both BRAC and Nano-lantern(Ca^{2+}) are the cameleon like FRET-type indicator harboring CaM-M13 moiety with *Renila reniformis* derived improved luciferase (RLuc8) and Venus as a donor and acceptor. BRAC, in which Venus and RLuc8 are connected with CaM-M13, displays Ca^{2+} dependent FRET emission change. Nano-lantern(Ca^{2+}), consisting with directly fused Venus with RLuc8 in which CaM-M13 was inserted into RLuc8 moiety. In this case, luciferase activity was recovered by Ca^{2+} dependent fashion, thus the emission signal enhanced with energy transfer to Venus was observed in the form of intensity change. In the state-of-art demonstration by using cultured hippocampal neurons, Ca^{2+} transient triggered by photo-activated ChR2 can be imaged as fast as at 10 Hz with high SNR, demonstrating the good compatibility of bioluminescent imaging with optogenetics (31).

5. CONCLUSION AND PERSPECTIVE

Although GECIs are advantageous over synthetic Ca^{2+} dyes in their targetability and reliability for chronic imaging, there still remains the room for further improvement on several parameters. Suboptimal kinetic property of FRET-type GECIs, pH sensitivity of single-FP based indicator should be overcame. The future of GECIs development is no doubt promising because the most notable feature of GECIs is its evolvability. As have been seen in the past and recent attempts, emerging GECIs would contribute to deepen our understanding of Ca^{2+} signaling.

FOOTNOTE

Part of this manuscript was modified from "Genetically encoded Ca^{2+} indicators ; expanded affinity range, color hue and compatibility with optogenetics", published in Front. Mol. Neurosci., 25 November 2014.

CONFLICT OF INTEREST

The Author declared no conflict of interest.

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