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Review

Genetically encoded Ca^{2+} indicators: Properties and evaluation ☆

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ABSTRACT

Genetically encoded calcium ion (Ca^{2+}) indicators have become very useful and widely used tools for Ca^{2+} imaging, not only in cellular models, but also in living organisms. However, the *in vivo* and *in situ* characterization of these indicators is tedious and time consuming, and it does not provide information regarding the suitability of an indicator for particular experimental environments. Thus, initial *in vitro* evaluation of these tools is typically performed to determine their properties. In this review, we examined the properties of dynamic range, affinity, selectivity, and kinetics for Ca^{2+} indicators. Commonly used strategies for evaluating these properties are presented. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

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1. Introduction

Calcium ion (Ca^{2+}) is a common second messenger in cellular signal transduction by which many biological phenomena such as muscle contraction, neuronal transmission, fertilization and hormonal secretion are regulated [1]. Ca^{2+} imaging is the powerful technique to investigate how Ca^{2+} exerts its action on these phenomena. To monitor Ca^{2+} dynamics in living cells, optical Ca^{2+} indicators are indispensable. Thus, developing methods for Ca^{2+} measurement is still the subject of a growing number of studies.

The first experiments visualizing intracellular Ca^{2+} changes [2] were conducted using the Ca^{2+}-sensitive bioluminescent protein aequorin [3]. These and subsequent experiments were performed by directly injecting purified protein from the jellyfish *Aequorea* into the cells examined since the method of introduction of exogenous genes into eukaryotic cells was established more than a decade later [4].

The development of Ca^{2+}-sensitive dyes such as Quin2 (characterized by a dim fluorescence) [5] and fura-2 [6] as well as the use of their acetoxymethylated ester forms for non-invasive cell loading [7] were later breakthroughs that allowed much broader use of Ca^{2+}-imaging.

The next leap in Ca^{2+}-imaging technology, the introduction of genetically encoded Ca^{2+} indicators, GECIs, was preceded by three important steps: the discovery of green fluorescent protein (GFP, reported in the same study in which aequorin was presented [3]), the creation of GFP color variants, which are suitable for Förster resonance energy transfer (FRET) experiments [8], and the biochemical study of Ca^{2+} binding through a fusion of calmodulin with its binding peptide, M13 derived from the myosin light chain kinase [9].

These achievements lead to the studies regarding development of FRET-based Ca^{2+} indicators, FIP-CB_{SM} [10] and cameleon [11]. FIP-CB_{SM} was composed of the M13 peptide sandwiched between two fluorescent proteins, BGFP and RGFP. The FRET signal of FIP-CB_{SM} was reduced under interaction with endogenous Ca^{2+}-calmodulin in the cell. In the case of cameleon, two fluorescent proteins were fused directly to calmodulin and M13 peptide; in this indicator FRET signal was increased upon Ca^{2+} binding. These two pioneering works established the basis for further development of genetically encoded indicators.

Next, other improvements in GECIs included development of single-fluorescent protein Ca^{2+}-sensors (camgaroos [12], pericams [13], GCaMP [14]), implementation of circularly permuted fluorescent proteins (FPs) to improve the dynamic range of FRET-based indicators (YC 2.60 and YC3.60 [35]), replacement of the Ca^{2+}-sensing moiety calmodulin by troponin C (TN-indicators family [15]), or structure-guided remodeling of the calmodulin-M13 interface (DcpV-indicators families

Abbreviations: [Ca^{2+}], free calcium ion concentration; BRET, bioluminescence resonance energy transfer; cDNA, complementary DNA; cp, circularly permuted; *D*, dynamic range; EDTA, 2-({2-[Bis (carboxymethyl) amino] ethyl} (carboxymethyl) amino) acetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; *F*, fluorescence intensity or FRET ratio; F_0, fluorescence intensity or FRET ratio in resting conditions (*in vivo*); FP, fluorescent protein; FRET, Förster resonance energy transfer; GECI(s), genetically encoded Ca^{2+} indicator(s); GFP, green fluorescent protein; *I*, fluorescence intensity; K_d, dissociation constant; k_{on}, k_{off}, rate constants of Ca^{2+} binding and unbinding reactions, respectively; *n*, Hill constant; *N*, number of photons; *R*, FRET ratio; *SNR*, signal-to-noise ratio; YC, yellow cameleons; ΔF, fluorescence intensity or FRET ratio change; τ, time constant of dissociation of the Ca^{2+}-indicator complex; Θ, fractional saturation

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[16]) to allow reduction of non-specific interactions in the cytoplasm. More recent improvements involving large-scale screening after random (GECOs [17]) or semi-rational mutation (GCaMP5-indicator family [18]), have led to color and high dynamic range variants of single fluorescent protein-based indicators. Other important modifications included the complete redesigning of the Ca^{2+}-sensing moiety by duplicating the troponin C C-terminal EF hands in the sensor TN-XXL [19] and the significant increase in Ca^{2+}-affinity in the YC-Nano indicators [20]. Interestingly, a completely new type of fluorescent Ca^{2+}-indicator, CatchER has been recently developed by replacement of 5 residues to create a low-affinity Ca^{2+}-sensing motif in EGFP, becoming the smallest GECI to date [21].

This quickly increasing diversity of fluorescent GECIs has been described in many recent reviews [22–25].

Ectopic expression of aequorin in eukaryotic cells by gene transfection was employed in the 1990s for Ca^{2+}-imaging experiments, although a dim chemiluminescence prevented its wide usage for monitoring intracellular Ca^{2+} dynamics. It was not until aequorin and fluorescent proteins were fused that the use of aequorin expanded [26], but by then, it had to compete with fluorescent Ca^{2+} indicators. Recently, other types of bioluminescent indicators such as BRAC [27] and Nano-lantern (Ca^{2+}) [28] were also developed by using *Renilla* luciferase derivatives. These indicators showed excellent performance under conditions in which external light excitation was undesirable, such as in highly autofluorescent plant tissues [27], or when coexpressed with the light-sensitive optogenetic tool channelrhodopsin-2 [28].

Despite increases in the number of indicators, little is known regarding the details underlying their functions, and schematic representations are still primarily used to explain observed properties. Thus, to further understand the mechanisms by which the indicators can work, we reviewed the composition and working principles of available fluorescent and bioluminescent GECIs (Section 2) and have briefly described the selection strategies used for fast preliminary evaluation of indicator variants (Section 3). Properties of interest for any GECI, including dynamic range, affinity, selectivity, and kinetics, are described in Section 4, together with other approaches aimed to obtain a detailed picture regarding the functioning of these indicators.

2. Reporting Ca^{2+} with GECIs

2.1. Genetically encoded fluorescent Ca^{2+} indicators

Genetically encoded fluorescent Ca^{2+} indicators can be categorized in two classes according to the number of fluorescent proteins present in the indicator: some GECIs contain a single fluorescent protein (non-ratiometric with some exceptions, Fig. 1) and some contain two fluorescent proteins (ratiometric probes, Fig. 2).

Single fluorescent protein-based GECIs typically share a common principle of action involving a change in fluorescence intensity upon Ca^{2+} binding. Ca^{2+}-chelating properties in most available indicators are provided by the calmodulin moiety, which is fused with the fluorescent protein and the calmodulin-binding peptide M13. An exception, which contains only a calmodulin fragment sandwiched between the two halves of a split GFP molecule, is the Camgaroo indicator family [12] (Fig. 1). Binding of Ca^{2+} promotes an intramolecular rearrangement that alters the chromophore protonation state and its associated fluorescent properties [29,30]. A completely different mechanism underlies the low-affinity Ca^{2+} sensor CatchER, in which the Ca^{2+}-binding motif formed by the introduction of five negatively charged residues in front of the tyrosine-derived hydroxyl group of the chromophore affects the chromophore electron density distribution, reducing the background fluorescence of this protein [21]. Bound Ca^{2+} shields this negative charge, allowing the recovery of fluorescence intensity.

In all of these cases, the chromophore chemical structure is not modified during conformational rearrangement, and therefore, the change in fluorescence is associated with an increase or decrease in the absorption spectra (or change in fluorescence quantum yield, in the case of inverse-pericam [13]), leading to a change in the resulting emitted fluorescence intensity. Since only a single wavelength is measured, these types of indicators are known as intensiometric or non-ratiometric (as opposed to the ratiometric indicators, described below).

Monitoring Ca^{2+} dynamics using GECIs containing two fluorescent proteins takes advantage of the concept of FRET. During FRET, one of the fluorescent proteins acts as a donor that transfers absorbed energy to the second fluorescent protein, the acceptor, when excited (Fig. 2). The efficiency of energy transfer depends on several factors, including the overlap of donor emission and acceptor absorption spectra, donor–acceptor distance, donor lifetime, and relative orientation of the donor and acceptor transition dipole moments [31].

FRET efficiency is highly dependent on the distance between the donor and the acceptor moieties [31], and therefore, it can only be used at distances of 1–10 nm. In our case, GECIs are designed in such a way that after Ca^{2+} binding the FRET efficiency increases, either due to approaching the two fluorescent protein moieties each other or due to changing in their relative orientations.

FRET-based techniques can be used to assess different aspects of donor–acceptor interactions [32,33], but for detecting changes in Ca^{2+} levels, a simple donor–acceptor emission ratio is typically used:

$$R = I_A / I_D, \tag{2.1}$$

where R is the FRET ratio, I_A and I_D are the peak emission intensities of the donor and the acceptor, respectively. An oppositely directed intensity change at two emission wavelengths allows reductions in intensity fluctuations associated with varying sensor concentrations (to which single-wavelength indicators are more sensitive). Because the FRET ratio value provides information regarding Ca^{2+} binding, these indicators are known as ratiometric indicators.

The first intensiometric indicators were developed on the basis of *Aequorea* GFP-derivatives and showed spectral characteristics similar to those of GFP, but more recently developed non-ratiometric probes employ other GFP color variants or are based on other fluorescent proteins [17], thus allowing simultaneous multicolor Ca^{2+} imaging in different intracellular compartments, the combined use of Ca^{2+}-imaging with fluorescent-tag labeling, or simultaneous Ca^{2+} imaging with optogenetic manipulation.

Several ratiometric indicators composed of different FRET pairs, such as blue-green [10,11], or green-orange [34], have been examined; however, the cyan-yellow pairs of fluorescent proteins provide the highest ratio change upon Ca^{2+} binding. Thus, most currently available ratiometric GECIs use a combination of these proteins, primarily an enhanced cyan fluorescent protein such as ECFP [8] as a donor, and a yellow fluorescent protein such as Venus [35] or Citrine [36,15] as an acceptor.

Brighter variants of cyan fluorescent proteins, such as Cerulean [37] and mTurquoise2 [38], were also tested as FRET donors; however, these sensors showed a lower dynamic range than those based on ECFP [19 and personal observations].

2.2. Genetically encoded bioluminescent Ca^{2+} indicators

As described in the Introduction, aequorin was the first chemiluminescent protein employed to detect Ca^{2+} changes *in vivo*. Targeting of recombinant aequorin allowed monitoring of Ca^{2+} changes in several intracellular organelles, including the mitochondria, nucleus, sarcoplasmic reticulum, endoplasmic reticulum, Golgi, secretory granules, and gap junctions [39,40]. However, the low turnover rate of luminescent reaction [41] and low quantum yield with its associated difficulties in signal detection prevented widespread use of aequorin for Ca^{2+} imaging. The low signal level of aequorin was overcome through fusion with its natural counterpart, GFP (resulting in the "GA" sensor [26], Fig. 3, Table 3), which exploits the same bioluminescent resonance

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