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Properties of genetically-encoded indicators used for cytosolic and organellar Ca^{2+} measurements

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Abstract

For the last 15 years, there has been an explosion in the development of genetically-encoded biosensors that report enzyme activity, chemical transformation, or concentration of ions and molecules in living cells. Now, there are well over 120 biosensors of different cellular targets. As a general design principle, these sensors convert a molecular event, such as the binding of a molecule to a sensing domain or a signal-induced change in protein conformation, into a change in the sensors' fluorescence properties. In contrast to small-molecule sensors, genetically-encoded sensors are generated as cells, tissues, or organisms translate sensor-encoding nucleic acid sequences, which have been introduced by transgenic technologies. One of the best developed classes of biosensors is that of genetically-encoded Ca^{2+} indicators (GECIs). Here, we briefly summarize the properties of ratiometric GECIs that permit them to be used to quantify Ca^{2+} in specific cellular locations, such as the cytosol, nucleus, endoplasmic reticulum, and mitochondria. We also provide two protocols that describe in detail how to carry out such quantitative measurements in cultured mammalian cells.

Changes in intracellular Ca^{2+} concentrations are integral to important cellular processes, including cell division, growth, signaling, and death. GECIs have been optimized during the past few decades to monitor changes in Ca^{2+} in living cells. In contrast to small-molecule Ca^{2+} probes, GECIs can be targeted to subcellular organelles and to specific cell types, and they can be expressed for days, or longer, in cultured cells and transgenic organisms.

Fluorescent GECIs have been engineered to respond to Ca^{2+} in two different ways: the fluorescence intensity of *intensiometric* GECIs, or the absorption or emission spectrum of *ratiometric* GECIs change with fluctuations in Ca^{2+} concentration. Intensiometric GECIs, such as the GCaMP sensors, can be monitored using one fluorescence channel, and ratiometric GECIs are monitored by calculating the ratio of two different fluorescence emission intensities. Several excellent reviews outline the evolution, advantages, and disadvantages of the major GECI families, discuss important considerations when choosing a GECI for a specific application, and detail primary differences between GECIs and small molecule probes (Dean et al. 2012; Tian et al. 2012; Whitaker 2010).

Most ratiometric GECIs are designed in the following way¹ (see Figure 1): a donor fluorescent protein (FP) is attached to an acceptor FP by a linker containing a Ca^{2+} -binding domain. The GECI undergoes a conformational change upon Ca^{2+} binding, which alters the distance between the two FPs, the orientation of the FPs relative to each other, and, consequently, the efficiency of Förster resonance energy transfer (FRET) from the donor to the acceptor FP (reviewed in (Lakowicz 2006; Zhang et al. 2002)). The FRET ratio, which is the ratio of the acceptor FP emission intensity (also called the FRET intensity) to the donor FP emission intensity, is the metric used to monitor the change in FRET. For example, two images – a FRET image and a donor FP image – of a cell expressing a GECI can be used to calculate the FRET ratio, which is the cell's mean intensity in the FRET image divided by its mean intensity in the donor FP image. The FRET ratio can be converted to a Ca^{2+} concentration when the following are available: (1) the sensor's affinity, in terms of its dissociation constant (K_d'), (2) its empirical Hill coefficient (n) if cooperativity is involved, (3) the FRET ratio in the absence of Ca^{2+} (R_{free}), and (4) the FRET ratio when the sensor is saturated with Ca^{2+} (R_{bound}). The sensor's affinity and Hill coefficient can be measured *in vitro* or *in situ* (*i.e.* in cells) and is usually published in the literature, while R_{free} and R_{bound} are measured during a calibration procedure at the end of each experiment. Over the last decade, sensor calibrations have been optimized in several different cell types and organelles (Mccombs and Palmer 2008; Palmer and Tsien 2006; Pozzan and Rudolf 2009; Rudolf et al. 2006).

The protocols in this chapter focus on using ratiometric FRET-based GECIs for quantitative measurement of cytosolic, nuclear, mitochondrial, or ER Ca^{2+} . Table 1 highlights current, state-of-the-art, ratiometric GECIs. These GECIs are preferred over intensimetric GECIs for quantitative measurement of Ca^{2+} because their FRET ratios are independent of their concentration in each cell and the path length (*i.e.* the thickness of a cell), and hence Ca^{2+} can be estimated with greater confidence.

Perhaps one of the single greatest advantages of using ratiometric GECIs is that it is possible to rigorously define the intracellular concentration of the sensor and assess whether the sensor itself perturbs cellular Ca^{2+} . This can be done because the fluorescence intensity of the acceptor FP, upon direct excitation, is independent of the level of Ca^{2+} within the cell. For example, the fluorescence intensity of a yellowameleon sensor, using 495/10 nm excitation and 535/25 nm emission filters, is proportional to its concentration. Thus, a standard curve relating the acceptor FP intensity to the sensor concentration can be made by measuring the intensity of purified sensor protein, at a range of known concentrations, using a fluorescence microscope under conditions identical to a live cell imaging experiment. The standard curve can then be used to calculate the sensor concentration from a fluorescence image of an unknown sample (*i.e.* a cell). This method necessitates creating micro-cuvettes with volumes similar to that of cell, as has been done using the wedge method (Chen et al. 2005; Miyawaki et al. 1999), glass capillaries (Dittmer et al. 2009), and microwells

¹Ratiometric-pericam is an excitation-ratiometric GECI and is designed differently (Nagai et al. 2001). Ca^{2+} -sensor binding changes the absorption spectrum of ratiometric-pericam, and so the ratio of its fluorescence intensities upon excitation at two different wavelengths (415 nm or 494 nm) reports the proportion of Ca^{2+} -bound sensor. The excitation ratio can be used analogous to the FRET ratio in these protocols.

fabricated in polydimethylsiloxane (PDMS) (our work, unpublished). Having a means to quantify the sensor concentration in cells allows researchers to assess whether the measured Ca^{2+} concentration is independent of the sensor's concentration. We have found that for most sensors under control of a standard CMV promoter there is a wide range of sensor expression levels, typically varying from low μM to tens of μM (Dittmer et al. 2009; Qin et al. 2011), and hence there is natural variation of sensor concentration that enables researchers to examine estimated Ca^{2+} concentration as a function of sensor concentration. However, it is also possible to put the sensor under control of an inducible promoter to tightly control expression levels. For sensor concentrations ranging from low μM to tens of μM , we have observed little perturbation of resting Ca^{2+} measurement. In contrast, small molecule probes with acetoxymethyl (AM) esters are known to concentrate in cells at levels close to hundreds of μM (Dineley et al. 2002; Kao et al. 2010).

Ratiometric GECIs can be used to compare Ca^{2+} concentrations at subcellular resolution among different cell types, in cells with genetic or chemical changes, or using different instruments. For example, the ER-targeted D1ER probe enabled researchers to compare the resting levels of Ca^{2+} in the ER in cells expressing different mutant forms of the protein presenilin-1, a protein that is dysfunctional in inherited forms of Alzheimer's disease (Mccombs et al. 2010). D1ER also revealed altered ER Ca^{2+} related to Gaucher's disease and demonstrated that small molecule drugs that modulate ER Ca^{2+} partially relieve the disease associated proteostasis defect (Mu et al. 2008). The mitochondrial GECI 4mt-D3cpV allowed investigators to observe that knockdown of a putative regulator of mitochondrial Ca^{2+} uptake, *MICU1*, abolished mitochondrial Ca^{2+} uptake in single HeLa cells in response to thapsigargin or histamine (Perocchi et al. 2010). Lastly, in a particularly elegant example of the capabilities of ratiometric GECIs, Tay *et al.* fused TN-XL to the N-type Ca^{2+} channel ($\text{Ca}_v2.2$) and quantified Ca^{2+} in a nanodomain surrounding the channel (Tay et al. 2012).

The protocols that follow detail the experimental steps of using ratiometric GECIs to quantify Ca^{2+} in the cytosol, nucleus, mitochondria, or ER (Protocol 1). In addition, a protocol is presented for carrying out *in situ* titrations to determine the binding parameters (K_d and n) of sensors in particular cells and organelles (Protocol 2).

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FIGURE 1.

Design of genetically-encoded, ratiometric, FRET-based Ca^{2+} sensors. **(A)** A Ca^{2+} binding domain is sandwiched between a donor FP (usually cyan FP) and an acceptor FP (usually yellow FP). When Ca^{2+} ions reversibly bind to the sensor's binding domain, the sensor's conformation changes, which leads to a change in FRET efficiency. **(B)** Emission spectrum of a sensor in the presence and absence of Ca^{2+} . The FRET ratio of the unbound sensor (R_{free}) is distinct from that of the bound sensor (R_{bound}).

Selected ratiometric GECIs

This table presents the binding parameters of commonly-used, genetically-encoded, ratiometric GECIs.

Sensor family	Name	Ca ²⁺ -responsive elements	Ca ²⁺ -binding parameters ^a				Comments	Ref.
			F	K _d ^c	n			
Pericams	Ratiometric Pericam	CaM, M13p	...	1.7 μM	1.1		Excitation-ratiometric sensor; successfully targeted to the nucleus and mitochondria	(Nagai et al. 2001)
Yellow Cameleons	YC2.60	CaM, M13p	0.8	93.5 nM	2.7			(Horikawa et al. 2010; Nagai et al. 2004)
Yellow Cameleons	YC3.60	CaM E104Q, M13p	0.2	950.3 nM	1.0			
			0.3	215 nM	3.6		High dynamic range	(Horikawa et al. 2010; Nagai et al. 2004)
			0.7	779 nM	1.2			
Yellow Cameleons	YC-Nano50	CaM, M13p	0.8	52.5 nM	2.5		Optimized for detecting subtle cytosolic Ca ²⁺ transients in living organisms	(Horikawa et al. 2010)
			0.2	403.5 nM	1.0			
D-family Cameleons	D1	mCaM, mM13p	0.3	800 nM	1.2		Does not bind endogenous CaM; optimized for ER	(Palmer et al. 2004)
			0.7	60 μM ^b	1.7			
D-family Cameleons	D3cpV	mCaM, mM13p	...	600 nM	0.7		Does not bind endogenous CaM; optimized for cytosol & mitochondria	(McCombs and Palmer 2008; Palmer et al. 2006)
TroponinC family	TN-XXL	mTpC	...	800 nM	1.5		Optimized for imaging of neurons; fast response	(Mank et al. 2008)

Abbreviations: calmodulin (CaM); CaM-binding M13 peptide (M13p); mutant CaM and M13 peptide (mCaM and mM13p); modified EF hands I–IV from chicken skeletal muscle troponin C (mTpC); fraction (F); apparent dissociation constant (K_d^c); Hill coefficient (n)

^aIn cases where a two-site binding model has been used to fit the titration data, two sets of binding parameters are reported.

^bThe K_d^c of D1 has been reported to be 220 μM in the ER of HeLa cells (Rudolf et al. 2006).