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Measuring calcium dynamics in living cells with Genetically Encodable Calcium Indicators

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Abstract

Genetically encoded calcium indicators (GECIs) allow researchers to measure calcium dynamics in specific targeted locations within living cells. Such indicators enable dissection of the spatial and temporal control of calcium signaling processes. Here we review recent progress in the development of GECIs, highlighting which indicators are most appropriate for measuring calcium in specific organelles and localized domains in mammalian tissue culture cells. An overview of recent approaches that have been undertaken to ensure that the GECIs are minimally perturbed by the cellular environment is provided. Additionally, the procedures for introducing GECIs into mammalian cells, conducting calcium imaging experiments, and analyzing data are discussed. Because organelle-targeted indicators often pose an additional challenge, we underscore strategies for calibrating GECIs in these locations.

Keywords

calcium; imaging; FRET; sensor; indicator; GECI; dynamics; localized;ameleon

1. Introduction

Calcium is one of the most important and versatile second messengers in cell biology; consequently there has been enormous effort devoted to developing tools to image Ca^{2+} in living cells. Cells organize Ca^{2+} into a diverse array of signals which regulate a host of critical processes, such as growth, proliferation, transcription, metabolism, exocytosis, contraction, and apoptosis (1). Berridge and coworkers eloquently refer to this phenomenon as the “calcium signaling toolkit” to describe the organization of calcium transients into signals that differ in space and time (2). Fluorescent indicators for calcium allow the researcher to monitor calcium signals in living cells and in real time, thus preserving temporal control of calcium signaling. Genetically encoded Ca^{2+} indicators (GECIs) are a subset of fluorescent indicators that offer the additional advantage of being able to monitor calcium dynamics in specific subcellular locations, thereby maintaining spatial heterogeneity of Ca^{2+} transients. GECIs (also sometimes referred to as Fluorescent Calcium Indicator Proteins, FCIPs) are defined as indicators that are produced by translation of a nucleic acid sequence. Therefore, these indicators are comprised solely of natural protein or peptide motifs, with no requirement for additional co-factors or chemicals. In order to convert a Ca^{2+} signal into an optical readout, GECIs consist of at least

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one light-emitting protein and a Ca^{2+} responsive element, such that Ca^{2+} binding changes the optical properties of the protein(s). These protein-based indicators are typically incorporated into cells by gene transfer techniques, as detailed in the Methods section.

There are three classes of GECIs that have been developed which differ in their overall architecture (schematized in Figure 1). The first class are bioluminescent reporters based on the aequorin photoprotein (3,4). These probes are inherently different than the fluorescent protein-based probes as light is generated by a chemical reaction that requires reconstitution of the indicator with a co-factor. The aequorin-based indicators are beyond the scope of this review, and therefore readers are referred to the following reviews that detail their applications (3,5,6). The second class is based on single fluorescent proteins. These indicators are composed of Ca^{2+} - responsive elements, such as calmodulin (CaM) or portions thereof that are inserted into a fluorescent protein, such that Ca^{2+} binding alters the protonation state, and hence spectral properties of the chromophore. These indicators include the camgaros (7,8), G-CaMPs (9–11), pericams (12), “Case” sensors (13), and grafted EF-hands (14). The last class of sensors are the “cameleon-type” in which Ca^{2+} -responsive elements are inserted between two fluorescent proteins so that upon Ca^{2+} binding, an alteration in the efficiency of fluorescence resonance energy transfer (FRET) occurs (15–23).

Comparison of GECIs and small molecule calcium indicators

While this review focuses on GECIs, given the preponderance and utility of small molecule calcium indicators, it is important to briefly highlight the strengths and preferred applications of these two classes of indicators. For a review of small molecule Ca^{2+} indicators, the reader is referred to (24). The advantages of using small molecule indicators over GECIs are the following. (i) Small molecule indicators exhibit greater dynamic range and increased sensitivity. Therefore these indicators are preferred when signal-to-noise is a limiting experimental factor. (ii) Small molecule indicators typically exhibit faster response kinetics; hence these indicators are more appropriate for measuring rapid Ca^{2+} transients where timing is a critical factor. (iii) Membrane-permeable small molecule indicators (such as acetomethoxy (AM)-ester versions) do not require gene transfer, and thus may be advantageous for cell lines that are difficult to transfect with plasmid DNA. However, difficult-to-transfect cell lines can often be virally transduced, thereby permitting incorporation of GECIs if experiments require. On the other hand, the strengths of GECIs include the following. (i) The localization of a GECI can be controlled by including a signal sequence as part of the indicator, thus GECIs are preferred for measuring Ca^{2+} in defined subcellular locations, such as the plasma membrane, ER, mitochondria, or Golgi. (ii) GECIs can be genetically fused to a protein of interest and therefore can measure Ca^{2+} in micro-domains in the immediate vicinity of a given protein. (iii) GECIs can be maintained within cells over days to weeks (in the case of stable incorporation). Hence, GECIs enable extended time-lapse experiments where small molecule indicators would slowly leak out, or be extruded, from the cell. (iv) The expression level, and thus concentration of GECIs can be controlled by incorporating the GECI into a plasmid with an inducible promoter. This feature enables the researcher to control, and vary, the indicator concentration. This is very difficult to do with small molecule indicators because the concentration of indicator within the cell depends on uptake, esterase activity, and pumps that extrude the indicator from the cell. Controlling the expression level may be important if a researcher wants to verify that the indicator is not perturbing the cellular environment (see Methods section). (v) GECIs can be transfected into cells along with other genes such that only the co-transfected subpopulation is monitored. (vi) GECIs can be used in nonvertebrate, but genetically tractable organisms, which do not permit use of small molecule indicators. (vii) Lastly, because GECIs can be incorporated into organisms by transgenic technologies, they can be used in cell- or tissue-specific expression in transgenic or virally transduced organisms.

2. Methods

This section details how to choose a GECI for a given experiment, how to incorporate the indicator into cells, and how to conduct a Ca^{2+} imaging experiment. For the experimental set up we focus on the use of a FRET-based cameleon-type sensor as single FP Ca^{2+} indicators have been detailed by others (10,12,25). Particular emphasis is placed on localized indicators as these often require special considerations.

2.1 Choosing the appropriate GECI

With the proliferation of GECIs in recent years, one of the biggest challenges for the researcher is how to choose the most appropriate sensor for a given application. The simple truth is that different applications will likely require different sensors. Table 1 summarizes recent advances in calcium indicators. This Table compares newly developed GECIs and therefore intentionally excludes GECIs which are considered outdated. It is important to note that some of these GECIs have only been tested in limited cellular scenarios (Case and Ca-G1 sensors). Therefore while these sensors show promise, researchers must ensure that their properties are not significantly altered in cells. Overall the sensors presented in Table 1 differ in how they sense Ca^{2+} (FRET vs. modulation of single FP properties), the dynamic range (minimum to maximum signal), the affinity for Ca^{2+} (K_d), and other important properties such as the rate at which they respond to changes in Ca^{2+} , the pKa, and brightness. In choosing which sensor to use, researchers must compare and contrast each of these features. For example, ratiometric indicators are often preferable to intensity-based indicators as they are more quantitative, more easily interpreted, and less subject to artifacts and pH interference. However, ratiometric indicators typically require more sophisticated instrumentation. For the FRET-based sensors, those that incorporate a circularly permuted (cp) version of the acceptor fluorescent protein (citrine or Venus) generally exhibit greater ratiometric sensitivity and expanded dynamic ranges (21,22,26). However, sensors with cpVenus do not express well in the ER and Golgi (McCombs and Palmer, unpublished observations) and therefore cp variants may not be appropriate for some localized sensors. Choosing a sensor with an appropriate affinity is particularly important for measuring Ca^{2+} in different subcellular domains. While measuring Ca^{2+} in the cytoplasm requires a high affinity indicator ($[\text{Ca}^{2+}]_{\text{cyt}} \sim 100 \text{ nM}$), the ER requires a low affinity indicator ($[\text{Ca}^{2+}]_{\text{ER}} \sim \text{hundreds of } \mu\text{M}$). For some applications, the most important criterion may be to choose a sensor with the fastest response kinetics, in which case GCaMP2.0 may be the most appropriate.

As mentioned previously, one of the advantages of GECIs is that they can be targeted to defined cellular locations. This is accomplished by attaching a signal sequence to either the N- or C-terminus of the sensor. Figure 2 displays images of a cameleon-type sensor expressed in the nucleus, ER, mitochondria, Golgi and plasma membrane.

2.2 Recent approaches for ensuring that GECIs are minimally perturbing

One concern about the original family of cameleons was that endogenous calmodulin could perturb them, leading to decreased Ca^{2+} sensitivity or even inversion of the FRET response. Three different approaches have been pursued to greatly reduce or eliminate this problem. In the first approach, Palmer et al. reengineered the Ca^{2+} responsive elements in the cameleons by generating mutated versions of calmodulin and calmodulin binding peptides (20,22). These selective or privileged calmodulin-peptide pairs were created by incorporating mutations into the peptide that inhibit binding to wild type calmodulin and then reconstituting binding by creating complementary mutations in calmodulin. The resulting sensors, designated the “D-family” (D1, D2, D3, and D4), no longer bind wild type calmodulin and function at the plasma membrane in neurons, which is notorious for high levels of endogenous calmodulin. Other calmodulin-based indicators such as G-CaMPs and pericams may also benefit from similar

mutations. The second approach employed by Griesbeck and coworkers was to replace calmodulin with a different Ca^{2+} responsive element, namely troponin C. These sensors do not appear to suffer any interference in either neurons (27) or cardiomyocytes (28). The third approach involves grafting Ca^{2+} binding sites directly onto a fluorescent protein and therefore bypasses any use of naturally occurring Ca^{2+} binding proteins (14). These sensors are low affinity and most appropriate for use in the ER or another domain expected to be high in Ca^{2+} . Although these sensors have not been directly tested for interference by calmodulin, perturbation seems unlikely given that they do not contain calmodulin binding elements.

2.3 Incorporation of GECIs into mammalian cells

Many methods are available for the incorporation of GECIs into mammalian cells, of which we will highlight the more commonly used. Some gene incorporation methods may be detrimental to cells and care should be taken when deciding which technique to use. In all cases, cell viability ought to be assessed post-gene incorporation. For each method, cells should be around 40–60% confluent (unless otherwise stated). Optimization of plating densities will lead to more efficient gene incorporation while yielding the desired confluency on the day of imaging (typically 36–72 hours after gene incorporation). All methods discussed here can be done either transiently or stably, as described at the end of this section.

One of the most straightforward DNA incorporation methods is lipofection, or transfection using cationic lipids, in which cells are incubated with DNA and transfection reagent over the course of a few days. Exact protocols depend on the cell line and reagent of choice, and are generally provided by the manufacturer. We suggest optimizing the ratio of DNA to transfection reagent to provide the most efficient GECI incorporation (50–70% of cells expressing the construct). Imaging can be conducted 36–72 hours after incorporation. Longer times result in loss of incorporation, and signals may be too weak if less than 36 hours have passed.

Calcium phosphate transfection relies on the precipitation of DNA with CaPO_4 and immediate addition to cells. Briefly, CaCl_2 and the GECI DNA are mixed with HEPES buffered saline before being placed on the cells to be transfected. This addition needs to occur immediately, as efficiency of transfection declines rapidly upon precipitation of the DNA and CaPO_4 . Media should be replaced after 3–5 hours, and cells may be imaged 1–6 days post-transfection. There are multiple methods for maximizing DNA uptake into cells, including addition of chloroquine with the DNA- CaPO_4 mixture, or treatment with 15% glycerol 30 s to 3 min after media has been replaced. With both of these methods, optimization of concentrations and treatment times may be necessary as both reagents are toxic to cells. Buffer pH may also be of concern, and usually is best between 6.9 and 7.1.

Another option for introducing a GECI into a mammalian cell is by viral transduction. Several types of viral vectors are available commercially, making it easy to find one that is suitable for cell type and experimentation needs. This method is particularly useful for non-dividing, primary cell lines such as neurons, as most other methods rely on division of cells for DNA incorporation. Protocols for making virus and using virus to infect mammalian cells are typically provided as part of manufacturer instructions (see Invitrogen, Imgenex, and others).

For cells that prove unresponsive to the above methods, biolistic delivery may be necessary. This involves shooting the cells with microparticles coated in the GECI DNA of interest. Microparticles are prepared by mixing with DNA, CaCl_2 , and spermidine, and then shot into cells using a gene gun. Exact protocols are provided by gene gun manufacturer. Cells are generally ready for imaging 1–4 days post-transfection.

Another method for DNA incorporation is the use of electroporation. Essentially, cells are harvested, washed, and re-suspended in growth medium or PBS. A small amount of this suspension is placed into an electroporation cuvette along with 10–30 μg DNA. Linearizing the DNA improves its cellular incorporation, and total reaction concentrations should be at 1–40 $\mu\text{g}/\text{ml}$. Electroporation is done immediately upon addition of DNA, with pulses generally lasting 20–100 ms. By letting cells sit for 1–2 minutes in the cuvette, transformation efficiency can be increased. The strength of field applied to cells needs to be high enough to alter the membrane to allow for passage of DNA, but not so high as to irreversibly damage the cells. For mammalian cells, this is usually between 250–750 V/cm. Imaging of cells may be done between 1–4 days post-electroporation. It is important to keep in mind that this method is not the most efficient as only 20–50% of cells survive so many samples will need to be processed to obtain adequate amounts of transfected cells.

Should more than one passage of cells be needed for experimentation, it is possible to make stable cell lines that express the GECI of choice from any of the above methods. After 48–72 hours post DNA incorporation, cells should be treated with an antibiotic based on the selection marker encoded in the vector used. Since transduction typically allows for more cells expressing the desired construct, we prefer this method for making stable cell lines. After a number of passages, expression of the construct may decrease from initial levels, due to promoter silencing or other effects. If this occurs, researchers may need to generate new stable cell lines.

2.4 Conducting a Ca^{2+} imaging experiment

The initial steps for carrying out a Ca^{2+} imaging experiment are to incorporate a GECI into cells, verify expression and localization, and conduct a calibration (see subsequent sections). The sensor calibration serves two purposes. First, it verifies that the sensor is functioning properly, and second it defines the dynamic range of a given sensor in a particular location in the chosen cell type. This experiment thus identifies the maximum signal achievable with a particular indicator. Once these steps are taken, the researcher is ready to measure Ca^{2+} dynamics of interest.

For a FRET-based GECI, two images must be collected: a direct CFP image (fluorescence intensity of the CFP donor emission upon CFP donor excitation, often referred to as the *CFP channel*) and a FRET image (fluorescence intensity in the YFP acceptor channel upon CFP donor excitation, i.e. the *FRET channel*). The FRET image is divided by the CFP image in order to obtain the FRET/CFP ratio. This ratio is proportional to the concentration of Ca^{2+} such that the higher the ratio, the higher the concentration. It may also be desirable to collect the direct YFP image (fluorescence intensity of the acceptor YFP emission upon acceptor YFP excitation, *YFP channel*) to monitor photobleaching. It is typically necessary to optimize parameters used for data collection to obtain images with a good signal to noise ratio while minimizing sensor photobleaching. This requires manipulation of the illumination light level and exposure time. Manipulation of the intensity of the excitation light (particularly when using arc lamp illumination) can be accomplished using neutral density filters. We typically use an ND1 filter which corresponds to 10% transmission. Additionally, we vary the excitation exposure times from 200–1000 ms. As a standard rule of thumb, we adjust the above parameters such that the fluorescence intensity (i.e. number of counts) of our weakest signal (typically the CFP channel) is *at least* a few thousand counts above background (using a 16-bit camera).

For imaging with an inverted fluorescence microscope, cells should be plated on coverslips or cell culture dishes with glass coverslip bottoms and imaged in an optically clear medium. The thickness of the coverslip should be chosen to match the Numerical Aperture (NA) of the objective (0.17 mm for a high NA objective). Our preferred medium is HHBSS (20 mM HEPES, 1 $\times$ Hanks Balanced Salt Solution (HBSS), 2 g/L D-glucose, pH 7.4). In terms of

selecting cells for analysis, it is advisable to choose cells with a medium level of fluorescence, i.e. not the brightest or the dimmest cells. The brightest cells are expressing large amounts of sensor protein which could buffer Ca^{2+} , whereas the dimmest cells may have too low a signal to noise to yield reliable FRET ratios. If Ca^{2+} buffering or other effects of indicator overexpression are of concern, the responses of cells of different brightnesses should be compared. If the GECI is under control of an inducible promoter (such as Tet-on or Tet-off), the expression level can be systematically varied by adjusting the concentration of the inducer. To assess buffering, resting ratios (which correspond to resting levels of Ca^{2+}) can be measured as a function of sensor concentration and extrapolated to obtain the resting Ca^{2+} at zero sensor concentration. If possible, researchers should independently verify that the desired Ca^{2+} phenotype is unperturbed by the GECI.

2.4.1 Calibration of indicator in cytoplasm

At the end of an experiment, it is important to determine the Ca^{2+} depleted state ($R_{\min}$) and Ca^{2+} saturated state ($R_{\max}$) for the GECI as these parameters are used to convert the observed FRET ratios into corresponding concentrations of calcium. We prefer to find the $R_{\min}$ first, as determination of $R_{\max}$ generally leads to cell death. To reduce cell calcium, cells should first be washed with Ca^{2+} -free HHBSS. Calcium is depleted from the cytoplasm using a Ca^{2+} chelator such as EGTA or BAPTA mixed with an ionophore such as ionomycin. Generally, ionomycin and EGTA are mixed in Ca^{2+} -free HHBSS and added to the cells in a final concentration of 3–5 μM ionomycin and 3–5 mM EGTA. Release of Ca^{2+} from internal stores will cause an initial increase in the observed cytoplasmic Ca^{2+} before being chelated by EGTA. Ensure that there is no further change in the ratio before determining the $R_{\max}$. This may take anywhere from 10–30 minutes, so acquisition times for calibration should be slowed to 20–30 seconds to minimize photobleaching. After obtaining $R_{\min}$, the chelator should be washed out and the media changed to standard HHBSS. Subsequently, the $R_{\max}$ is determined by loading the cells with an excess of Ca^{2+} to saturate the indicator. To do this, ionomycin and CaCl_2 are added to the cells in a final concentration of 3–5 μM ionomycin and 5–10 mM CaCl_2 . Optimization of the concentrations of each component is suggested to ensure saturation of the GECI occurs before cell death, which is apparent by membrane blebbing and change in size. The $R_{\max}$ has been reached when a plateau in the ratio is seen. The values obtained for $R_{\min}$ and $R_{\max}$ from these calibrations are then used along with the in vitro K_d' values to convert FRET ratios into Ca^{2+} concentrations (described in section 2.5.2).

2.4.2 Calibration of indicator within organelles

Calibrating indicators within organelles is often more challenging than calibrating the indicator in the cytoplasm because there is the additional hurdle of ensuring that the organelle is saturated in (or depleted of) Ca^{2+} . For example, at rest the ER is high (hundreds of μM) in Ca^{2+} concentrations. It is often difficult to increase ER Ca^{2+} further to obtain $R_{\max}$. Underestimating $R_{\max}$ because of insufficient saturation or overestimating $R_{\min}$ as a result of incomplete depletion will lead to inaccurate Ca^{2+} concentrations. Unfortunately, we have found that no single protocol works universally for all cell types so researchers are encouraged to optimize conditions accordingly. We have compiled a list of parameters that can be optimized and some important experimental considerations in Table 2. One control that can help determine if a localized probe is saturated/depleted is to compare the total dynamic range of the localized probe to that of the same probe located in the cytoplasm (e.g. D1ER compared to D1) in the same cell type and on the same microscope system. However there are reports that localized probes may exhibit reduced dynamic ranges (25), so this control is not always effective.

2.4.3 Anticipated results

Representative examples of experiments using FRET-based GECIs targeted to the ER and mitochondria are shown in Figure 3 in a plot of FRET ratio as a function of time. For the ER targeted cameleon (D1ER), a baseline in the FRET ratio, which corresponds to the resting Ca^{2+} , was observed first. Subsequently, thapsigargin was added. Thapsigargin inhibits the SERCA pump and results in a slow leak of Ca^{2+} from the ER, as is evident from the decrease in FRET ratio. Once steady state values in the ratio were reached, treatment of the cell with EGTA and ionomycin fully depleted the ER of Ca^{2+} , thereby obtaining an $R_{\min}$ that was below the starting level for the sensor in this experiment. Similarly, for the mitochondrially-targeted cameleon (4mtD3cpv), addition of ATP caused an increase in the FRET ratio as Ca^{2+} released from the ER is taken up into the mitochondria. Once the FRET ratio returned to baseline, cells were washed and calibration of the sensor was performed. Cells were treated with ionomycin and EGTA to obtain the $R_{\min}$, which causes an initial increase in the ratio as more Ca^{2+} is released from the ER before being chelated by the EGTA. The ratio again reached values well below the starting baseline, indicating that mitochondria contain a small but measurable pool of Ca^{2+} at rest. Upon treatment with ionomycin and Ca^{2+} , the $R_{\max}$ was obtained, with ratio levels higher than those observed for either ATP or ionomycin/EGTA addition.

2.5 Important Experimental Considerations

2.5.1. Microscope setup

FRET-based GECIs require rapid collection of both the FRET channel and CFP channel. These channels use the same excitation filter and dichroic mirror (see below for suggested filters), but a different emission filter. Therefore ratio imaging requires a means to either (i) rapidly change the emission filter or (ii) simultaneously collect 2 emission wavelengths. The first can be accomplished using an external filter wheel controlled by a Lambda 10-2 or Lambda 10-3 controller (Sutter Instruments) while the second can be accomplished using a beamsplitter (dual-view, Optical Insights). We use a Zeiss Axiovert 200M inverted fluorescence microscope equipped with motorized dichroic turret, and 10-position excitation and emission filter wheels. Equivalent microscope systems from other manufacturers (Olympus, Nikon) are available. The microscope should be equipped with a high sensitivity CCD camera, such as CoolSNAP or Cascade 512B (Photometrics, Roper Scientific Inc.), or a comparable camera from another manufacturer.

For a microscope using arc lamp excitation (Xe or Hg), selective excitation of CFP and separation of emission from CFP and YFP is accomplished using excitation and emission filters along with a dichroic mirror. These are available from Chroma Technology Corp or Semrock. We prefer to use sputter-coated (ET) filter sets because they have increased transmission (93–97 %), resulting in better signal to noise. For sensors based on FRET between CFP and a variant of YFP (EYFP, citrine, Venus, cp-citrine, or cp-Venus) recommended filter sets include: CFPx 425/20, YFPx 495/10, CFPm 480/40, YFPm 525/20, CFP and FRET dichroic 450, YFP dichroic 515 (29).

2.5.2 Data analysis

In order to monitor changes in a FRET-based GECI signal (i.e. the ratio of intensity in the FRET channel divided by the intensity in the CFP channel; FRET/CFP) over time, it is useful to draw a region of interest (ROI) in specific locations on individual cells. An ROI should also be collected to determine the background signal. Ideally a background ROI should be placed on an untransfected cell to allow subtraction of cellular autofluorescence. However, it can sometimes be hard to distinguish autofluorescence from fluorescence of cells expressing low levels of sensor protein; therefore, an alternative is to select a background region in the field of view that does not contain any cells (i.e. an unoccupied region of the coverslip). The

fluorescence intensity in the ROI can be either averaged or summed. We typically average the fluorescence intensity for cytoplasmic, nuclear, ER, and Golgi-localized GECIs and sum the fluorescence intensity for mitochondrial- and plasma membrane-localized GECIs. If summing the fluorescence intensity instead of averaging, it is critical that the background ROI be the *same exact size* as the ROI on the cell. The data acquisition program used (METAFLUOR, ImageJ, or an equivalent program) should allow the averaged or summed fluorescence intensity for each channel and each ROI to be exported in an ASCII or delimited format. These data can then be imported into a data processing program to enable background correction.

All FRET ratio data should be background corrected, i.e. $RATIO = (FRET_{CellROI} - FRET_{BKGND ROI}) / (CFP_{Cell ROI} - CFP_{BKGND ROI})$, where $FRET_{Cell ROI}$ and $CFP_{Cell ROI}$ are the intensity of the FRET and CFP channels in the cellular ROI and $FRET_{BKGND ROI}$ and $CFP_{BKGND ROI}$ are the intensity of FRET and CFP channels in the background ROI. The background corrected ratio can be represented as a pseudo-color image or as a time course (ratio vs. time). To create a background corrected ratio image, a program such as METAFLUOR or ImageJ can be used. To plot a time course for an ROI, a data processing program such as excel is sufficient.

As discussed above, the R_{min} and R_{max} values can be used in conjunction with the *in vitro* K_d values to convert FRET ratios to Ca^{2+} concentrations. It is important to note that some FRET-based GECIs exhibit monophasic Ca^{2+} binding (D3, D4, TN-XL), whereas others exhibit biphasic binding (D1, D2), and different equations must be used for mono- vs. bi-phasic responses. Researchers are also cautioned that the *in situ* (i.e. within cells) K_d values may differ from the *in vitro* values due to environmental changes. Therefore all conversions to Ca^{2+} should note the exact parameters used. It is preferable to report time traces of both FRET ratio changes and $[Ca^{2+}]$. The first step in the calculation is to convert background corrected ratio data to “% FRET ratio of max” (abbreviated % ΔR). Briefly, in excel calculate % $\Delta R = (R - R_{min}) / (R_{max} - R_{min}) * 100\%$, where R is the background corrected ratio in a particular ROI at a given time and R_{min} and R_{max} are the minimum and maximum ratios (respectively) within the same ROI. Finally, use the *in vitro* parameters and relevant equation (Table 3) to determine the Ca^{2+} concentration at each time point.

3. Concluding remarks

After the initial introduction of GECIs in 1997 (15,16), there has been a concerted effort to build upon initial designs to generate more robust and reliable indicators of cellular Ca^{2+} . Recent efforts have led to improvement of the dynamic range, minimization of interference by and perturbation of the cellular environment, and optimization indicator properties for use in more sophisticated cellular and *in vivo* systems. The utility of these sensors has been expanded by the generation of GECIs with varying affinities for Ca^{2+} , more rapid response kinetics, and the localization of GECIs to specific subcellular compartments. As a result, researchers now have a wide range of tools from which to choose. The challenge is to select the appropriate GECI for a given application as each sensor has both advantages and disadvantages.

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(a) Aequorin

(b) Single FP sensors

M13	cpGFP	CaM	G-CaMP
M13	cpYFP (T203F)*	CaM	Pericam/Case

(c) Grafted sensors

GFP (1-172)	CaM-EIIF	GFP (173-238)	Ca-G1
YFP (1-144)	CaM	YFP (145-238)	Camgaroo

(d) FRET-based sensors

CFP	CaM	M13	YFP	Cameleon
CFP	TnC	YFP		TroponinC-based

Figure 1.

Models of the three classes of GECIs. (a) The aequorin photoprotein is shown in complex with coelenterazine. Upon binding of Ca^{2+} , the aequorin undergoes a conformational change, releasing coelenteramide and emitting blue light. (b) Single FP sensors employing the Ca^{2+} -responsive element CaM and a CaM binding peptide attached to a circularly permuted FP. On binding Ca^{2+} , CaM executes a conformational change, interacting with the peptide and altering the protonation state of the chromophore, thus changing the fluorescence intensity of the protein. Note the Case sensors are built from a cpYFP with a T203F and a few other mutations. For details see reference (13). (c) Grafted sensors utilizing EF hands or portions of CaM inserted into a fluorescent protein. Binding of Ca^{2+} causes a change in protein conformation and a shift in the protonation state of the chromophore. (d) FRET-based sensors having a Ca^{2+} binding domain located between two fluorescent proteins. As Ca^{2+} binds, the Ca^{2+} binding domain undergoes a conformational change, interacting with its binding peptide. This brings the two FPs closer together, increasing the efficiency of FRET. Below each model are maps for the various available families of GECIs.

Figure 2.

Fluorescence images of localized GECIs. (A) nucleus, (B) Golgi, (C) mitochondria, (D) Plasma membrane, and (E) ER. Localization was accomplished by addition of a nuclear localization sequence (PKKKRKVEDA at the C-terminus); fusion to the 81-amino acids at N-terminus of human galactosyltransferase type II; incorporation of 4 repeats of the cytochrome C oxidase signal sequence at the N-terminus; addition of the lyn kinase myristoylation-palmitoylation sequence (MGCIKSKRKDNLNDDGVDMKT) at the N-terminus; and incorporation of both the calreticulin signal sequence at the N-terminus and a KDEL ER-retention tag at the C-terminus.

Figure 3.

Representative examples of experiments employing cameleon-type GECIs. Plots are given as FRET ratio versus time for (a) the ER-targeted cameleon D1ER and (b) the mitochondria-targeted cameleon 4mtD3cpv. Changes in Ca^{2+} over time are easily observable upon addition of $4\mu\text{M}$ thapsigargin and $5\mu\text{M}$ EGTA/ 5mM ionomycin for D1ER (a) or $5\mu\text{M}$ ATP, $5\mu\text{M}$ ionomycin/ 5mM EGTA, and $5\mu\text{M}$ ionomycin/ 5mM Ca^{2+} for 4mtD3cpv(b).

Table 1
Summary of the properties of recently developed genetically encoded Ca^{2+} Indicators

Type of sensor	Name of sensor	Dynamic range ^d	K_d for Ca^{2+}	Other information	Cellular localization and construct name	ref
FRET-based	D1 ^a	2× ratio	0.8, 60 μM	Not perturbed by CaM. k_{on} : $3.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ k_{off} : 250 s^{-1}	ER: D1ER	(20)
	D2cpv ^b	5× ratio	0.03, 3 μM	Not perturbed by CaM.	Mito: 2-, 4-, 6-mtD2cpv	(22)
	D3cpv ^b	5× ratio	0.6 μM	Not perturbed by CaM. Replaces YC2, YC3, and YC3.60 as a high affinity, high dynamic range, all-purpose sensor.	PM: lynD3cpv Mito: 2-, 4-, 6-mtD3cpv	(22)
	D4cpv ^b	5× ratio	64 μM	Not perturbed by CaM.	Mito: 2-, 4-, 6-mtD4cpv	(22)
	TN-XL ^c	4× ratio	2.5 μM^e	Fast response kinetics: $\tau_{diss} = 430 \text{ ms}$ $\tau_{diss} = 240 \text{ ms}$ Kinetics reported for in vivo (Drosophila NMD).		(21)
Single FP	GCaMP2.0 ^e	5× intensity	0.146 μM	Fast kinetics: $\tau_{assoc} = 14 \text{ ms}$ $\tau_{diss} = 75 \text{ ms}$ pH sensitive, pKa N/A		(11)
	Case-12	12× intensity	1.0 μM	pH sensitive, pKa = 7.2 Properties are for in vitro sensors.		(13)
	Case-16	16.5× intensity	1.0 μM	pH sensitive, pKa = 7.2 Properties are for in vitro sensors.		(13)
	Ca-G1	1.1× ratio	800 μM	pH sensitive, pKa = 7.45 Reported dynamic range is for ER-targeted sensorin cells.	ER: Ca-G1-ER	(30)
	Ca-G1-37	1.8× ratio	440 μM	pH sensitive, pKa = 7.45 Reported dynamic range is in vitro.		(30)
	Ratiometric pericam	10× ratio	1.7 μM		Mito pericam mt Nucleus: pericam-nu	(12)

(a) D1 contains citrine as the acceptor FP and therefore only has a dynamic range of 2×. When the acceptor FP is changed to cpv, the dynamic range increases to 5×. However the cpv variant does not express well in the ER and Golgi. Therefore a low affinity D1cpv variant is available, but not targeted to organelles.

(b) Versions of D2, D3, and D4 which contain citrine instead of cpVenus are also available. When citrine is the acceptor FP instead of cpv, the dynamic range decreases to 2×. Details can be found in ref (22).

(c) An improved Troponin-C based sensor will be available soon: TN-XXL has a higher affinity for Ca^{2+} ($K_d = 700 \text{ nM}$) but retains the large dynamic range of TN-XL (Oliver Griesbeck, personal communication).

(d) The dynamic range is expressed as the fold change upon Ca^{2+} binding. Sensors are either ratio metric or intensity-based, as indicated.

^(e) Improved GCaMPs with increased dynamic range will be available soon (Loren Looger, personal communication).

Abbreviations used: cpv, circular permuted venus; mito, mitochondria, PM; plasma membrane; calmodulin, CaM.

Table 2Parameters to optimize when calibrating Ca^{2+} indicators.

	Chemicals used ^a	Typical concentrations	Experimental considerations and comments
$R_{\min}$	EGTA with ionomycin	1 – 5 mM EGTA 2 – 10 μM ionomycin	Cells should be in Ca^{2+} -free HHBSS. Full chelation can take anywhere from 10 – 30 minutes so data acquisition should be slowed to minimize photobleaching. These conditions are effective for cytoplasmic as well as mitochondrial and ER sensors.
	BAPTA with ionomycin	0.5 – 1 mM BAPTA 2 – 10 μM ionomycin	BAPTA is a Ca^{2+} specific chelator, is less pH dependent, and acts faster than EGTA, however it is more costly.
	BAPTA-AM	5 – 50 μM	BAPTA-AM is membrane permeable and does not require ionomycin. AM-esters have limited solubility and should be prepared with Pluronic F-127. Cells must be treated with BAPTA-AM according to manufacturer instructions, washed and given sufficient time for AM-ester cleavage, thus this $R_{\min}$ should be done at the end of an experiment.
$R_{\max}$	High Ca^{2+} with ionomycin	5 – 20 mM Ca^{2+} 2 – 10 μM ionomycin	Ionomycin will facilitate Ca^{2+} entry across all membranes (i.e. plasma membrane as well as internal membranes).
	High Ca^{2+} with digitonin	5 – 20 mM Ca^{2+} 10 – 25 μM digitonin	At low concentrations, digitonin should selectively permeabilize the plasma membrane but not internal membranes. Therefore this approach allows endogenous mechanisms to pump elevated cytoplasmic Ca^{2+} into intracellular organelles.
	High Ca^{2+} with digitonin, ATP, and Mg^{2+}	5 – 20 mM Ca^{2+} 10 – 25 μM digitonin 1 mM ATP 1 mM Mg^{2+}	These conditions are intended to permeabilize the plasma membrane and add the necessary components to allow the SERCA to pump Ca^{2+} into the ER in order to obtain $R_{\max}$ of an ER-localized sensor. Conditions should be optimized so that ER Ca^{2+} does not decrease upon permeabilization.

(a) Details on how to prepare the relevant solutions and other experimental considerations not covered in this review are available in the excellent review by Kao(24).

Table 3

In vitro calibration parameters for the D-family cameleon sensors

Sensor	In vitro equation ^a	Method for obtaining [Ca ²⁺]	Parameters obtained from in vitro fit ^b
D1	$\% \Delta R = \frac{\Delta R_{\max 1} ([Ca^{2+}]^{n1})}{(K'_d)^{n1} + [Ca^{2+}]^{n1}} + \frac{\Delta R_{\max 2} ([Ca^{2+}]^{n2})}{(K'_d)^{n2} + [Ca^{2+}]^{n2}}$	Provide an initial guess for the [Ca ²⁺] and iteratively vary this value until the calculated $\Delta R\%$ converges to the experimentally determined $\Delta R\%$. ^c	K' _{d1} (0.58) K' _{d2} (56.46) $\Delta R_{\max 1}$ (0.28) $\Delta R_{\max 2}$ (0.72) n ₁ (1.18), n ₂ (1.67)
D2cpv	$\% \Delta R = \frac{\Delta R_{\max 1} ([Ca^{2+}]^{n1})}{(K'_d)^{n1} + [Ca^{2+}]^{n1}} + \frac{\Delta R_{\max 2} ([Ca^{2+}]^{n2})}{(K'_d)^{n2} + [Ca^{2+}]^{n2}}$	Same as for D1.	K' _{d1} (0.097) K' _{d2} (7.67) R _{max1} (65.9) R _{max2} (33.53) n ₁ (1.34), n ₂ (0.77)
D3cpv	$\% \Delta R = \frac{\Delta R_{\max} ([Ca^{2+}]^n)}{(K'_d)^n + [Ca^{2+}]^n}$	$\% \Delta R = \frac{\Delta R_{\max} ([Ca^{2+}]^n)}{(K'_d)^n + [Ca^{2+}]^n}$	K' _d (0.76) R _{max} (105.3) n (0.74)
D4cpv	$[Ca^{2+}] = \left[\frac{K'_d{}^n (R - R_{\min})}{(R_{\max} - R)} \right]^{1/n}$	$[Ca^{2+}] = \left[\frac{K'_d{}^n (R - R_{\min})}{(R_{\max} - R)} \right]^{1/n}$	K' _d (49.68) R _{max} (98.04) n (1.35)

^(a) Where $\% \Delta R = (R - R_{\min}) / (R_{\max} - R_{\min})$ and $\Delta R_{\max} = R_{\max} - R_{\min}$.

^(b) K'_d reported in μM and for a one-site Hill model, the apparent dissociation constant (K'_d) is related to the true Ca²⁺ dissociation constant K_d by the equation (31) $K'_d = K_d (S_{f2}/S_{b2})^{1/n}$, where (S_{f2}/S_{b2}) is the ratio of emission intensities of the Ca²⁺-free cameleon to Ca²⁺-bound cameleon, measured over the denominator wavelength passband, i.e. the FRET-excited YFP emission band. For cameleons incorporating cpVenus, (S_{f2}/S_{b2}) is about 0.47.

^(c) This can be readily accomplished using the SOLVER function in Microsoft excel.