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Genetically Encoded Calcium Indicators as Probes to Assess the Role of Calcium Channels in Disease and for High-Throughput Drug Discovery

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Abstract
The calcium ion (Ca^{2+}) is an important signaling molecule implicated in many cellular processes, and the remodeling of Ca^{2+}homeostasis is a feature of a variety of pathologies. Typical methods to assess Ca^{2+} signaling in cells often employ small molecule fluorescent dyes, which are sometimes poorly suited to certain applications such as assessment of cellular processes, which occur over long periods (hours or days) or in vivo experiments. Genetically encoded calcium indicators are a set of tools available for the measurement of Ca^{2+} changes in the cytosol and subcellular compartments, which circumvent some of the inherent limitations of small molecule Ca^{2+} probes. Recent advances in genetically encoded calcium sensors have greatly increased their ability to provide reliable monitoring of Ca^{2+} changes in mammalian cells. New genetically encoded calcium indicators have diverse options in terms of targeting, Ca^{2+} affinity and fluorescence spectra, and this will further enhance their potential use in high-throughput drug discovery and other assays. This review will outline the methods available for Ca^{2+} measurement in cells, with a focus on genetically encoded calcium sensors. How these sensors will improve our understanding of the deregulation of Ca^{2+} handling in disease and their application to high-throughput identification of drug leads will also be discussed.

Abbreviations
$[Ca^{2+}]_{CYT}$ resting cytosolic free calcium ion concentration
AM acetoxymethyl
ATP adenosine triphosphate
BAPTA 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid
Ca^{2+} calcium ion
ER endoplasmic reticulum
FLIPR fluorescence imaging plate reader
FRET Förster resonance energy transfer
GECI genetically encoded calcium indicator
GFP green fluorescent protein
IP_3 inositol 1,4,5-trisphosphate
IP_3R 1,4,5-trisphosphate-activated receptor
NFAT nuclear factor of activated T cells

PS1 presenilin-1
RyR ryanodine receptor
SR/ER sarcoplasmic/endoplasmic reticulum
TRP transient receptor potential

1 Introduction

The calcium ion (Ca^{2+}) is an important intracellular second messenger, the movement of which is responsible for the regulation of a variety of cellular processes. These include proliferation, excitation/contraction coupling, cell death, gene transcription, and cell motility (Berridge, Bootman, & Roderick, 2003; Clapham, 2007). Simultaneous regulation of these and other Ca^{2+}-dependent processes is achieved via a suite of Ca^{2+} channels, pumps, exchangers, and regulators. Together, they function to manipulate the temporal and spatial aspects of the Ca^{2+} signal (Berridge, 2000; Berridge et al., 2003; Prevarskaya, Ouadid-Ahidouch, Skryma, & Shuba, 2014). Plasma membrane Ca^{2+} pumps are responsible for preserving a large plasmalemmal concentration gradient for Ca^{2+}, which consists of a resting cytosolic free Ca^{2+}concentration ($[Ca^{2+}]_{CYT}$) of approximately ~ 100 nM. This is in stark contrast to the high concentration of free Ca^{2+} in the extracellular space (~ 1–2 mM) (Berridge, 2000; Clapham, 2007). Fig. 1 outlines the key classes of Ca^{2+}channels located in mammalian cells, with the approximate free Ca^{2+} levels for the cytosol, sarcoplasmic/endoplasmic reticulum (SR/ER), and mitochondria.

Alterations in Ca^{2+} signaling have been linked to the pathophysiology of several diseases including cardiovascular disease, neurological disorders, and cancer (Brini, Cali, Ottolini, & Carafoli, 2014; Fearnley, Roderick, & Bootman, 2011; Monteith, McAndrew, Faddy, & Roberts-Thomson, 2007). Targeting regulators of Ca^{2+} signaling therefore may represent an area of opportunity for the identification of new therapies for such diseases. Modulating Ca^{2+} signaling has already been demonstrated to have clinical relevance. Examples include the L-type voltage-gated Ca^{2+} channel blockers such as amlodipine and nifedipine, which have been widely used for hypertension (Elliott & Ram, 2011; Godfraind, 2014). Other examples include the N-type Ca^{2+} channel blocker ziconitide which can be used in the treatment of severe chronic pain (Schmidtko, Lotsch, Freynhagen, & Geisslinger, 2010) and the clinical trial of an inhibitor of the Ca^{2+} ion permeable transient receptor potential V4 (TRPV4) channel (GSK2798745) in congestive heart failure patients (GlaxoSmithKline, 2000).

Studies investigating alterations in Ca^{2+} signaling in disease often focus on changes in expression of Ca^{2+} channels, pumps, or their regulators. Some examples include the identification of increased expression of inositol 1,4,5-trisphosphate (IP_3)-activated receptors (IP_3Rs) in cardiomyocytes isolated from spontaneously hypertensives rats, a model of cardiac hypertrophy (Harzheim et al., 2009), and the down regulation of plasma membrane Ca^{2+} ATPase four levels in colon cancers (Aung et al., 2009). While studies reporting altered expression of calcium channels and pumps have improved our understanding of many diseases, another important consideration is changes to the Ca^{2+} signal itself. The significance of assessment of Ca^{2+} levels is evident when one considers Ca^{2+} permeable ion channels. Overexpression of a plasmalemmal ion channel per se is unlikely to alter calcium signaling in disease if the channel is not active due to the absence of appropriate stimuli or a lack of appropriate trafficking to the plasma membrane.

Changes in Ca^{2+} signaling in disease is almost always likely to be the result of a symphony of changes, not only due to changes in expression but also due to alterations in protein localization or changes in posttranslational modifications (Stewart, Yapa, & Monteith, 2015). One way to measure the sum of these changes and their impact on disease, is via the measurement of the Ca^{2+} signal directly. Advances in our knowledge of such changes have been catalyzed by improvements in the methods to measure intracellular Ca^{2+}. These improvements have been the development of tools (e.g., small molecule Ca^{2+}-sensitive fluorescent dyes and genetically encoded calcium indicators (GECIs)) and their use with advanced imaging methodologies. As will be outlined in this review, the recent expansion of GECIs continues to diversify the tools available to measure Ca^{2+} changes in disease. With the range of sensors now available, it is often now just a matter of the thoughtful selection of the correct probe for the specific application. This review will focus on GECIs and how these tools can be applied to the study of Ca^{2+} signaling in disease and in high-throughput screening for drug discovery.

2 The Calcium Signal in Disease

Remodeling of intracellular Ca^{2+} signaling is a key component of several diseases (Brini, Ottolini, Cali, & Carafoli, 2013; Missiaen et al., 2000; Roderick & Cook, 2008). Research into pathological changes of Ca^{2+} signaling in neurodegenerative disease, cardiovascular disease, and cancer predominate. In Alzheimer's disease, resting cytosolic free Ca^{2+} has been reported to be elevated (Kuchibhotla, Lattarulo, Hyman, & Bacskai, 2009) and this remodeling of intracellular Ca^{2+} signaling is thought to be the result of several mechanisms (Berridge, 2014). In presenilin (PS1) mutation models of familial Alzheimer's disease, upregulation of ryanodine receptor (RyR) Ca^{2+} channels (Chan, Mayne, Holden, Geiger, & Mattson, 2000) and increased activity of IP_3Rs may enhance Ca^{2+} store release from the SR/ER (Cheung et al., 2008). Given the role of Ca^{2+} signaling in apoptosis and necrosis, this dysregulation of Ca^{2+}present in Alzheimer's disease and other neurodegenerative disorders is speculated to contribute to characteristic neuronal cell death associated with a variety of pathologies (Mattson & Chan, 2003). Changes in the handling of Ca^{2+}have also been identified in cardiac hypertrophy and heart failure. For example, the $Ca_v3.2$ voltage-gated calcium channel has been implicated in cardiac hypertrophy, in mice, $Ca_v3.2$ knockout was found to be protective against induction of cardiac hypertrophy, a process thought to be the consequence of reduced calcineurin/nuclear factor of activated T cells (NFAT) activation (Chiang et al., 2009). The 1,4,5-triphosphate receptor, type 3 (IP_3R3) Ca^{2+} channel, while normally having minimal expression in cardiac tissue, is found to be upregulated in patients with heart failure (Go et al., 1995) and it has been proposed that the result of this upregulation is the sensitization of RyRs to increase Ca^{2+} store release (Harzheim et al., 2009).

As discussed earlier, calcium signaling is integral to normal cell physiology and has an important role in a variety of processes ranging from proliferation and hormone secretion to cell death (Berridge et al., 2003). In cancer, many of these cell functions are altered which can contribute to disease progression (Hanahan & Weinberg, 2011). It is therefore unsurprising that the dysregulation of Ca^{2+} signaling is a reported feature of some cancers. Alteration of Ca^{2+} signaling has been identified in cancers of the prostate, breast, colon, and ovaries (Monteith, Davis, & Roberts-Thomson, 2012; Prevarskaya, Zhang, & Barritt, 2007; Roderick & Cook, 2008). As an example, the Ca^{2+} channel TRPV6 can be upregulated in prostate cancer

(Peng et al., 2001) and this is thought to function as a mechanism to increase $[Ca^{2+}]_{CYT}$ to promote proliferation (Lehen'kyi, Flourakis, Skryma, & Prevarskaya, 2007). Indeed, levels of TRPV6 have been suggested as a marker of prostate cancer progression (Fixemer, Wissenbach, Flockerzi, & Bonkhoff, 2003). TRPV6 can also be overexpressed in breast cancer (Bolanz, Hediger, & Landowski, 2008; Dhennin-Duthille et al., 2011) and has been associated with the aggressive estrogen receptor-negative subtype (Peters et al., 2012). Several other calcium channels and pumps have been linked to breast cancer, these include other TRP channels such as TRPC1 (Dhennin-Duthille et al., 2011) and TRPM7 (Guilbert et al., 2009), members of the store-operated Ca^{2+} entry family ORAI1 (McAndrew et al., 2011) and ORAI3 (Faouzi et al., 2011), and pumps that are responsible for Ca^{2+} efflux across the plasma membrane such as PMCA2 (Lee, Roberts-Thomson, & Monteith, 2005).

The diversity of disorders associated with altered Ca^{2+} signaling has propelled the need for tools to improve our understanding of the contribution of these changes to disease pathophysiology. With recent advancements, we also now have the capabilities and measurement tools to identify compounds able to disrupt or prevent Ca^{2+} alterations in some diseases which are currently not effectively treated. Therefore, monitoring the Ca^{2+} signal is an increasingly important method for drug discovery.

3 The Calcium Signal as a Tool in Biomolecular Screening

The concept of Ca^{2+} signaling assessment as an endpoint for biomolecular screening in drug discovery is not new; however, recent advances in Ca^{2+} measurement methods including those related to genetically encoded Ca^{2+} indicators will allow further progress in this field. Likewise improvements in high-throughput screening instrumentation are further contributing to the development of this area.

Assays investigating alterations in the Ca^{2+} signal not only provide a platform to better identify the mechanism of Ca^{2+} changes in disease, but they also allow the screening of compounds able to alter intracellular Ca^{2+} and the nature of the Ca^{2+} signal. For example, in neuroscience, where Ca^{2+}oscillations can be a surrogate for activity (Smetters, Majewska, & Yuste, 1999), assessment of $[Ca^{2+}]_{CYT}$ may allow the identification of compounds able to alter neuronal activity and synaptic transmission (Woods & Padmanabhan, 2012). Another example is the screening of compounds potentially able to promote cancer cell death induced by chemotherapeutic drugs, through assessment of the high cytosolic or mitochondrial Ca^{2+} levels associated with cell death. Screening for Ca^{2+} changes could allow the identification of lead compounds that are not capable of inducing cell death alone, but able to augment free Ca^{2+} levels to a sufficient extent to promote the effectiveness of existing therapies.

The above examples of assessment of Ca^{2+} levels as a biomolecular screen are easily applied to plate reader devices, such as the fluorescence imaging plate reader ($FLIPR^{TETRA}$; Molecular Devices, Sunnyvale, CA, USA) for high-throughput assays (Monteith & Bird, 2005). An increasing area of interest is the development of cell-based assays involving high content screening (Mattiazzi Usaj et al., 2016). Instead of a single measurement from a population of cells (such as produced in plate reader assays), high content screening allows dynamic visualization of cells at a single-cell level often with simultaneous assessment of other cellular functions through other fluorescence probes with distinct fluorescence spectra (Zanella, Lorens, & Link, 2010). For example, this has been used in a genome-wide siRNA screen for regulators of Parkin, a gene important in mitochondrial damage in Parkinson's disease

(Hasson et al., 2013). Candidate genes were identified by examining Parkin translocation in HeLa cells, determined by loss of nuclear localized green fluorescent protein (GFP)-tagged Parkin fluorescence (Hasson et al., 2013). This was identified by measuring Parkin-GFP colocalization with spectrally distinct Hoechst 33342 fluorescence, with some hits discarded based on mitochondrial depletion identified by a third fluorescence channel (Hasson et al., 2013). High content screening drastically increases the depth of information uncovered from a bioassay and is well suited to investigation of the nature of Ca^{2+} alterations induced by compounds or extracts from screening libraries. Some examples include the identification of heterogeneity in the response between individual cells and cell populations; changes in subcellular Ca^{2+} levels (e.g., mitochondria), and how these relate to changes in the cytosol; Ca^{2+} oscillation, and Ca^{2+} wave rates; and simultaneous assessment of Ca^{2+} changes with other events (e.g., morphology or transcription factor translocation). When developing such phenotypic measurements of Ca^{2+} signaling success requires streamlined analysis, including features such as automated cell detection and cell tracking. These requirements are common for other high content imaging assays, such as those investigating cellular migration, proliferation, or protein colocalization (Boutros, Heigwer, & Laufer, 2015; Mattiazzi Usaj et al., 2016).

The success of cell-based assays when applied to Ca^{2+} signaling is critically dependent on the method for measuring intracellular calcium. The next focus of this review will be to highlight some of the methods to measure intracellular calcium levels and how these may influence their application to studying disease processes or biomolecular screening. There will be an emphasis on GECIs and how recent developments in this field can be leveraged for these types of assays.

4 Methods to Measure Cytosolic Calcium

The intricacies of intracellular Ca^{2+} signaling are progressively becoming unraveled with advances in the methods to measure intracellular free Ca^{2+}. Development of such methods has highlighted the importance of spatial and temporal aspect of the Ca^{2+} signal for differential regulation of Ca^{2+}-dependent functions (Rudolf, Mongillo, Rizzuto, & Pozzan, 2003). Ca^{2+} indicators are often classified into one of two groups: small molecule fluorescent Ca^{2+} dyes or protein-based GECIs.

4.1 Small Molecule Fluorescent Dyes for the Assessment of Ca^{2+} Signaling

Small molecule fluorescent calcium indicators are organic molecules capable of undergoing a change in fluorescence in response to Ca^{2+} binding. These compounds were developed over 30 years ago (Grynkiewicz, Poenie, & Tsien, 1985; Tsien, Pozzan, & Rink, 1982) and continue to be widely used for Ca^{2+} measurement. Their extensive use is related to their high performance (high fluorescence intensity, large dynamic range, fast kinetics, linear responses) (Lock, Parker, & Smith, 2015; Mank & Griesbeck, 2008), established protocols (Takahashi, Camacho, Lechleiter, & Herman, 1999), and the now vast array of variants available—including a range of Ca^{2+} affinities and emission wavelengths.

Small molecule Ca^{2+} indicators may be intensiometric (or single wavelength) or ratiometric. Intensiometric probes only undergo an increase in fluorescence intensity on

binding Ca^{2+}; in contrast, ratiometric probes also exhibit a shift in fluorescence spectra upon Ca^{2+} binding. With appropriate calibration ratiometric dyes can be used for accurate quantitative assessment of absolute free Ca^{2+} (Bootman, Rietdorf, Collins, Walker, & Sanderson, 2013). Small molecule Ca^{2+} dyes are mostly modeled on the structure of the calcium chelator 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) coupled to a fluorescent reporter group (Grynkiewicz et al., 1985). This BAPTA structural element has a high affinity for Ca^{2+}, though this structure can be modified to adjust Ca^{2+} affinity to allow detection at a range of Ca^{2+}concentrations (Oheim et al., 2014). Probes are most commonly used in an acetoxymethyl (AM) ester form, allowing free passage of the otherwise highly charged dye across the phospholipid bilayer (Paredes, Etzler, Watts, Zheng, & Lechleiter, 2008). Once the AM ester form has crossed the plasma membrane, intracellular esterases generate the Ca^{2+} sensitive ionized moiety (Thomas et al., 2000).

Some applications for Ca^{2+} measurement are poorly suited to the use of small molecule fluorescent dyes. One example is long-term Ca^{2+} monitoring. Dye leakage is a common occurrence (Palmer & Tsien, 2006; Thomas et al., 2000) and can occur within 30 min of dye loading dependent on cell types and/or experimental conditions (Paredes et al., 2008). This limitation can be particularly evident in some cancer cell lines that express multidrug-resistance proteins that can transport many of these probes (Homolya et al., 1993). Over time, fluorescent dyes also have the tendency to sequester into subcellular Ca^{2+}stores such as the endoplasmic reticulum (ER) (Mank & Griesbeck, 2008) where Ca^{2+} levels may saturate probes appropriate for $[Ca^{2+}]_{CYT}$ measurements. Together, these limitations can reduce the reliability of these indicators for long-term Ca^{2+} measurements.

A further weaknesses of small molecule calcium dyes is their inability to be readily targeted to specific tissues, cell types, or subcellular locations (Demaurex, 2005). Dyes can accumulate in subcellular regions (Oheim et al., 2014), though it is often not without background fluorescence in other areas such as the cytosol (Petrou et al., 2000). One example are members of the rhodamines that can accumulate in the mitochondria that has allowed probes such as Rhod-2 to be used to assess mitochondrial Ca^{2+} changes in mammalian cells (Babcock, Herrington, Goodwin, Park, & Hille, 1997). Fluorescent dyes are also poorly suited to in vivo studies (Helmchen & Waters, 2002; Whitaker, 2010). This is related to challenges in dye loading in vivo, including the inability to load specific cell types, loss of the probe due to efflux mechanisms, and probe sequestration. Although there are some examples where in vivo measurements have been achieved (Helmchen, Svoboda, Denk, & Tank, 1999; Stosiek, Garaschuk, Holthoff, & Konnerth, 2003), such approaches are unsuited to most in vivo applications.

4.2 GECIs for the Assessment of Ca^{2+} Signaling

GECIs can overcome some of the issues associated with small molecule fluorescent dyes. One of the principle advantages of GECIs is that these sensors enable long-term, repeat $[Ca^{2+}]$ measurement. Instead of experiments over minutes or hours, GECIs can allow repeated measurement over an extended period, up to many weeks or months (Aramuni & Griesbeck, 2013). This has led to these sensors being successfully introduced into several organisms for in vivo intracellular Ca^{2+} studies, from *Drosophila* (Tian et al., 2009) to mice (Hasan et al., 2004), to even primates (Santisakultarm et al., 2016).

Arguably, the development and application of GECIs has predominantly been driven by neuroscience research, where the disadvantages of small molecule Ca^{2+} probes are particular evident (Mank & Griesbeck, 2008; Tian, Hires, & Looger, 2012). Unlike small molecule fluorescent Ca^{2+} dyes, GECIs can be readily targeted to specific tissues, cell populations, or subcellular location through the addition of an appropriate promoter or targeting sequence. For example, GECIs allow Ca^{2+} measurement of astrocytes among a population of neurons (Shigetomi, Kracun, Sofroniew, & Khakh, 2010), or can be targeted to presynaptic neurons (Jackson & Burrone, 2016) or even a specific interneuron subtype (Hinckley & Pfaff, 2013). This targeting feature also opens up the investigation of Ca^{2+} handling in particular organelles such as the ER or mitochondria, through the introduction of a targeting sequence (recently reviewed by Suzuki, Kanemaru, & Iino, 2016).

The basic structure of most GECIs consists of a Ca^{2+} sensing element coupled to one or two fluorophores, capable of an alteration in fluorescence with Ca^{2+} binding. Calmodulin is a common binding domain for GECIs. An alternative approach uses troponin C as a binding domain, a protein suggested to have less potential for endogenous interaction (Heim & Griesbeck, 2004).

As outlined later, genetically encoded calcium sensors use either fluorescent or luminescent proteins to report changes in calcium signaling.

4.2.1 Aequorin-Based GECIs

Aequorin is a bioluminescence-based indicator first isolated from the *Aequorea victoria* jellyfish (Shimomura, Johnson, & Saiga, 1962). In the presence of an external cofactor (coelenterazine), aequorin undergoes an irreversible reaction on binding Ca^{2+} to produce a photon of light (Brini, Pinton, Pozzan, & Rizzuto, 1999). Aequorin and its derivatives can be successfully targeted to subcellular locations (Robert, Pinton, Tosello, Rizzuto, & Pozzan, 2000) and represented the first advance in protein-based indicators of Ca^{2+} appropriate for use in the study of Ca^{2+} levels in subcellular organelles. However aequorin-based indicators are dim relative to fluorescent GECIs and for this reason are not ideal for single-cell Ca^{2+} measurements (Ottolini, Cali, & Brini, 2014). Because the probe is consumed during the reaction (Bonora et al., 2013), these indicators are also poorly suited to long-term Ca^{2+} monitoring. This limits the application of aequorin-based indicators, compared with their fluorescent counterparts. A new class of genetically encoded calcium sensors named GAP (GFP-Aequorin Protein) are capable of both Ca^{2+}-dependent luminescence or fluorescence (Rodriguez-Prados, Rojo-Ruiz, Aulestia, Garcia-Sancho, & Alonso, 2015). The ability to perform mean cell population luminescence in conjunction with fluorescence measurements may aid the identification of between-experiment variability (Rodriguez-Prados et al., 2015).

4.2.2 Förster Resonance Energy Transfer-Based GECIs

Fluorescent GECIs were first developed more than 15 years ago (Miyawaki et al., 1997; Romoser, Hinkle, & Persechini, 1997) and exploited the phenomenon of Förster resonance energy transfer (FRET). This occurs when excitation of a donor fluorophore enables nonradiative energy transfer to allow fluorescence of a closely linked second acceptor fluorescent protein (Zhang, Campbell, Ting, & Tsien, 2002). FRET-based Ca^{2+} sensors often link two fluorophores with overlapping excitation/emission spectra via a Ca^{2+}-binding domain.

Measurement of Ca^{2+} can be achieved by monitoring the change in FRET signal as a result of a conformational change with Ca^{2+} binding (Romoser et al., 1997). The first major family of FRET-based indicators was termed cameleons, consisting of a blue and green fluorescent protein linked by calmodulin fused to M13, a myosin light chain kinase-binding peptide (Miyawaki et al., 1997). Early versions of cameleons suffered from pH sensitivity, low signal, and photobleaching (Demaurex, 2005; Mank & Griesbeck, 2008), subsequent versions reduced some of these limitations (Palmer, Qin, Park, & McCombs, 2011).

One advantage of FRET-based genetically encoded calcium sensors is the possibility of ratiometric imaging (and thus a superior ability to approximate absolute free Ca^{2+} concentrations) (Rose, Goltstein, Portugues, & Griesbeck, 2014). Ratiometric measurements with FRET sensors can be used to resolve motion artifacts or variations in sensor expression among a tissue population, which can be an advantage for in vivo experiments (Lutcke et al., 2010; Rose et al., 2014). However, these indicators generally have a lower signal-to-noise ratio, decreased brightness and slower kinetics compared to single fluorophore GECIs (Ai, 2015; Tian, Akerboom, Schreiter, & Looger, 2012; Tian, Hires, et al., 2012). FRET-based GECIs have a large spectral bandwidth, limiting capacity to multiplex with fluorescent indicators targeting other parameters (Rose et al., 2014; Tian, Akerboom, et al., 2012).

4.2.3 Single-Wavelength Fluorescent GECIs

Single fluorescent protein calcium indicators were first developed following the observation that GFP can have proteins inserted in its beta-barrel structure and still maintain the ability to fluoresce (Baird, Zacharias, & Tsien, 1999). This led to the development of the Camgaroo family of indicators, consisting of a single fluorophore (circular permutation of yellow fluorescent protein) modified to contain a calmodulin Ca^{2+}-binding domain within its beta-barrel structure (Baird et al., 1999). Although this series formed the basis for future GECI classes, Camgaroos suffered from sensitivity to pH and low brightness (Mank & Griesbeck, 2008; Whitaker, 2010). The Pericams were a subsequent family of single-fluorophore indicators, although these offered some improvements over Camgaroo sensors, pH sensitivity, and low brightness limited use (Mank & Griesbeck, 2008; Pologruto, Yasuda, & Svoboda, 2004; Whitaker, 2010).

First developed at a similar time to the Pericams, another class of single fluorescent protein GECIs are termed GCaMP. These Ca^{2+} sensors consist of a circular permutation of GFP linked to a M13 fragment of myosin light chain kinase at the N-terminus and calmodulin-binding domain at the C-terminus (Nakai, Ohkura, & Imoto, 2001). Like other early GECIs, the first series of GCaMP suffered issues with low brightness, temperature sensitivity, poor expression, and pH sensitivity (Tallini et al., 2006; Whitaker, 2010). An advance came in 2009 with the development of GCaMP3, with several improvements including increased protein stability and dynamic range (Tian et al., 2009). The subsequent GCaMP5 indicators were developed with further improvements in brightness, dynamic range, and affinity for Ca^{2+} (Akerboom et al., 2012). The most recent version of this series, GCaMP6, was developed through screening variants with point mutations from the GCaMP5G structure (Chen et al., 2013). Three of these were selected, named GCaMP6f (fast), GCaMP6m (medium), and GCaMP6s (slow), distinguished by their response kinetics in detecting Ca^{2+} transients (Chen et al., 2013). Though GCaMP6s has slower kinetics than the other GCaMP6 variants, it is the

most sensitive of the three sensors (Chen et al., 2013). In contrast, the fast kinetics of GCaMP6f is likely the preferred variant for the measurement of rapid Ca^{2+} oscillations, like those observed in neurons.

Compared with FRET-based indicators, single fluorescent protein indicators have the advantage of generally more favorable dynamic range, in part due to their typically lower basal fluorescence. This also reduces the potential for photobleaching in long-term assays. However, given measurements of fluorescence intensity is influenced by sensor expression, single fluorescent protein indicators are arguably still best suited to relative measurements of intracellular free Ca^{2+} (Abdul, Ramlal, & Hoosein, 2008; Whitaker, 2010).

5 New GECIs

In recent years, there have been considerable gains in performance for genetically encoded Ca^{2+} indicators. A significant improvement came from the development of the GCaMP6 indicators, as for the first time, these indicators had comparable performance (Chen et al., 2013) with regards to sensitivity and kinetics compared with leading small molecular Ca^{2+}-sensitive dyes (e.g., Fluo-4). GCaMP6 indicators have widespread use (Lee, Huang, & Fitzpatrick, 2016; Montijn, Meijer, Lansink, & Pennartz, 2016; Sidik et al., 2016; Theis et al., 2016) and several variants on the original series have since been developed, targeted to various locations. Table 1 outlines the properties of some of these indicators, along with several other GECIs discussed in this review.

Recently, there has also been considerable interest in developing high-performance GECIs capable of fluorescence emission in longer wavelength hues (Zhao et al., 2011), allowing the potential for multicolor Ca^{2+} imaging. This has a major advantage in studies seeking to investigate the Ca^{2+} signaling interactions between two cell populations, or between two cellular compartments (e.g., cytosolic vs mitochondrial Ca^{2+} levels; Li et al., 2014). One example includes the GECO series of indicators, which are available with red, blue, or green intensiometric emission (Zhao et al., 2011). Red-shifted indicators have the added advantage of greater tissue penetration, reduced phototoxicity, and less light scattering in vivo (Oheim et al., 2014; Pendin, Greotti, Filadi, & Pozzan, 2015; Rodriguez et al., 2017). Newly developed cytosolic red Ca^{2+} indicators have significantly improved performance relative to previous red indicators (Dana et al., 2016; Inoue et al., 2015). These include R-CaMP2 (Inoue et al., 2015), jRGECO1a (Dana et al., 2016), jRCaMP1a, and jRCaMP1b (Dana et al., 2016). R-CaMP2 and jRGECO1a are derived from the mApple fluorophore, a protein capable of photoswitching (Shaner et al., 2008), which may limit the use of these indicators in certain protocols (Dana et al., 2016). jRCaMP1a and jRCaMP1b have a different fluorophore, mRuby, and were derived by screening mutational variants of RCaMP1h (Dana et al., 2016). These sensors have distinct properties which may make them suitable for different applications; jRCaMP1a has a higher affinity for Ca^{2+} (K_d 214 nM), while jRCaMP1b has a greater dynamic range (Dana et al., 2016).

Other developments in GECIs include the Fast-GCaMPs (Sun et al., 2013), later improved to combine the properties of brightness associated with GCaMP6f (Badura et al., 2014). A GCaMP8 (Ohkura et al., 2012) indicator is also available, though the alternative numbering structure employed by this group should not suggest superiority to GCaMP6 (Broussard, Liang, & Tian, 2014). It has also recently been identified that some GCaMP variants can be photoconverted from green to red, by prolonged exposure with blue-green

light at 450–500 nm (Ai et al., 2015). Importantly, these indicators remain Ca^{2+} sensitive (Ai et al., 2015). This can allow the "highlighting" of selective cells among a population in vivo, potentially useful for repeated measurements or distinguishing individual cell morphology (Berlin et al., 2015; Hoi, Matsuda, Nagai, & Campbell, 2013). Differentiating individual cells could also be achieved by the newly developed photoactivatable GCaMP6 series, where cells expressing the Ca^{2+} sensor can be selectively highlighted via excitation at 405 nm (Berlin et al., 2015). Further diversity has come from the targeting of several modern GECIs to various intracellular organelles as described later. In many cases, this has been possible through the development of indicators with lower affinities for Ca^{2+} ions, thus avoiding saturation in organelles with high free Ca^{2+} levels in their lumen (e.g., the ER).

6 Targeting GECIs

One of the advantages of all GECIs is their ability to be targeted to specific tissues, cell populations, subcellular regions, or organelles. The concept of targeting GECIs has been around for some time; however with recent advances, new efforts have been made to develop sensors that are capable of reliable Ca^{2+} measurement at various cellular sites. GECIs have been targeted to the mitochondria, ER, nucleus, Golgi apparatus, endosomes, peroxisomes, and the subplasmalemmal domain (Demaurex, 2005; Suzuki et al., 2016; Williams, Monif, & Richardson, 2013). Given there is a large variation in the resting-free Ca^{2+} levels between different subcellular organelles (e.g., cytosol ~ 100 nM vs the ER ~ 500 μM (Carafoli, 1987; Vandecaetsbeek, Vangheluwe, Raeymaekers, Wuytack, & Vanoevelen, 2011)), care needs to be taken when selecting a GECI to measure Ca^{2+} changes, with an appropriate K_d required for the expected Ca^{2+}level (to avoid indicator saturation). Fig. 2 highlights some examples of targeted GECIs for various intracellular organelles and subcellular locations. Environmental differences such as pH can also vary between organelles and this must also be considered in indicator selection (Perez Koldenkova & Nagai, 2013). The effect of the addition of a targeting sequence likewise needs to be carefully considered, given the possibility of obstruction of normal sensor activity (Suzuki et al., 2016). Low-affinity indicators have been developed for measurement of free Ca^{2+} in the ER lumen. One example are the CEPIA series, which have three variants; red (R-CEPIA1*er*), green (G-CEPIA1*er*), or the ratiometric blue/green (GEM-CEPIA1*er*) (Suzuki et al., 2014). The dual emission of GEM-CEPIA1*er* enables the ability to normalize for factors such as variations in sensor expression (Suzuki et al., 2014). GAP Ca^{2+} indicators have also been targeted to the ER and Golgi apparatus (Navas-Navarro et al., 2016; Rodriguez-Garcia et al., 2014) and likewise several FRET-based cameleons have been targeted to various organelles successfully (Demaurex & Frieden, 2003). Aside from organellar targeting, it can be advantageous to monitor Ca^{2+} levels at other locations within the cell, such as the plasma membrane. Indeed localized Ca^{2+} microdomains located near the plasma membrane can have signaling functions independent of global $[Ca^{2+}]_{CYT}$ changes (Rizzuto & Pozzan, 2006). Several GECIs have been targeted to this location (Akerboom et al., 2012; Heim & Griesbeck, 2004; Nagai et al., 2004; Shigetomi et al., 2010). A recent study also described single channel recording of STIM1/ORAI1 interaction in HEK-293A cells with three different GECI-ORAI1 fusions (Dynes et al., 2016).

The increasing array of GECIs available continues to expand the choices available for Ca^{2+} measurement. However, indicators need to be selected based on the specific

experimental requirements. Consideration must be given to factors such as the importance of absolute quantitation, impact of phototoxicity, location of Ca^{2+} changes to be measured, and the need for multicolor imaging.

7 Application of GECIs in the Assessment of Calcium Homeostasis in Disease

The application of GECIs has often been focused on studies investigating neuronal physiology. Such work often takes advantage of the ability of GECIs to be targeted to specific cell populations or cell locations, in addition to the suitability of GECIs for chronic imaging (a significant limitation of small molecule fluorescent indicators). There are numerous examples of GECIs being used to better understand the nature of Ca^{2+} signaling changes in disease using both in vitro and in vivo models. Some exemplar studies are outlined later which provide insights into how other areas of biomedical research may utilize GECIs to better understand the role of Ca^{2+} homeostasis remodeling in disease.

In astrocyte neuronal death induced by oxygen deprivation, the plasma membrane targeted Lck-GCaMP6s sensor was able to identify spontaneous Ca^{2+}transients, accompanying mitochondrial disruption (O'Donnell et al., 2016). These were of two distinct phenotypes. The first, a fast transient propagating between mitochondria, while the latter, a newly identified localized Ca^{2+}oscillation at plasma membrane immediately adjacent to mitochondria (O'Donnell et al., 2016). In pluripotent stem cell-derived cardiomyocytes, GCaMP5G was expressed to identify proarrhythmic disruption of Ca^{2+} signaling induced by pharmacological agents (Shinnawi et al., 2015). By coexpressing with a genetically encoded voltage indicator, this model provides the ability to screen compounds that disrupt normal Ca^{2+} dynamics or membrane depolarization (Shinnawi et al., 2015).

GECIs have also been used to characterize Ca^{2+} changes associated with viral infection (Perry, Ramachandran, Utama, & Hyser, 2015). By expressing both R-CEPIA1*er* (targeting the ER) and the cytosolic GCaMP5G (or GCaMP6s) sensor, Perry et al. observed attenuated adenosine triphosphate (ATP)-induced Ca^{2+} transients at the cytosol and ER of both MA-104 and HeLa cells following infection with rotavirus or poliovirus (Perry et al., 2015). The authors also demonstrated the GECIs could be used to monitor Ca^{2+} changes during live cell imaging of viral infection measured over 16 h, identifying increased Ca^{2+}oscillations with rotavirus infection, which could be attenuated with inhibitors of Ca^{2+} influx (Perry et al., 2015). Application of GECIs has extended to other research areas. The expression of GCaMP indicators in somatosensory neurons of live mice enabled the observation of differential neuronal responses dependent on the type of painful stimuli, suggesting future use of this GECI-based model could help elicit the neuronal mechanisms of distinct pain pathways (Emery et al., 2016). Another example used expression of GCaMP indicators in *Toxoplasma gondii* to screen compounds capable of disrupting Ca^{2+} signaling, enabling the authors to identify novel antiparasitic compounds (Sidik et al., 2016).

As already discussed, there is an increasing awareness of the importance of the nature of calcium signaling changes rather than changes in expression of calcium channels in cancer (Stewart et al., 2015); however, GECIs remain relatively underutilized in this research area. Of the few studies that have used GECIs in cancer cells, most have been in breast cancer cells. Indeed over 10 years ago, D1ER (an ER-targeted GECI) was used to monitor ER Ca^{2+} changes

because of Bcl-2-mediated ER stress in MCF7 cells (Palmer et al., 2004). The authors identified that Bcl-2 reduced Ca^{2+} levels in the ER by increasing Ca^{2+} leak from internal stores (Palmer et al., 2004) likely via IP_3Rs. A more recent example is the use of a mitochondrial targeted GCaMP6f indicator to identify that mitochondrial calcium uniporter silencing attenuates mitochondrial calcium uptake in MDA-MB-231, MDA-MB-468, and BT-549 breast cancer cells (Tosatto et al., 2016).

Arguably one of the greatest advantages for GECIs is their ability to be used in chronic measurements of Ca^{2+}, a property that favors the use of these sensors in vivo. Repeated Ca^{2+} measurement can be achieved through intravital microscopy (Pittet & Weissleder, 2011). This typically involves the implantation of a window in the model organism allowing the visualization of Ca^{2+} changes (Karreman, Hyenne, Schwab, & Goetz, 2016). For example, implantation of a cranial window was used in an Alzheimer's disease mouse model (APP/PS1 mice) to image neurons expressing the GCaMP6m sensor (Liebscher, Keller, Goltstein, Bonhoeffer, & Hubener, 2016). This enabled the identification of various neuronal Ca^{2+} alterations in this model, including a reduction in the magnitude of neuronal responses in response to visual and motor cues (Liebscher et al., 2016). Expression of the cameleon indicator YC3.6 in neurons of APP mice (another Alzheimer's disease model) also made use of intravital imaging, finding increased Ca^{2+} levels in the dendrites and axons adjacent to amyloid-β plaques with associated morphological changes in part the result of these increases (Kuchibhotla et al., 2008). YC3.6 has also been used in a mouse model of familial hemiplegic migraine type 1 (Eikermann-Haerter et al., 2015). In vivo imaging was achieved via a cranial window allowing the identification of Ca^{2+} overload associated with changes in $Ca_v2.1$ channels (Eikermann-Haerter et al., 2015). Given the role of calcium signaling in the heart, there is also potential for use of GECIs in vivo in this context (see recent review, Kaestner et al., 2014) and protocols have been published outlining intravital imaging of the heart in live mice (e.g., Vinegoni, Aguirre, Lee, & Weissleder, 2015). One example used the expression of GCaMP3 in human embryonic stem cell-derived cardiomyocytes to confirm functional activity of these cells following transplantation to regenerate myocardium tissue damage in guinea-pigs (Shiba et al., 2012).

Intravital imaging is also technique to be exploited in in vivo cancer models, where the ability to chronically image cancer cells could further our understanding of tumor progression and metastasis (Ellenbroek & van Rheenen, 2014). An example of this potential is seen by the use of an intravital chamber in p53 knockout mice to measure the disruption of Ca^{2+} dynamics during apoptosis, using the small molecule dye fura-2 (Giorgi et al., 2015). Given the recent progress in both GECIs and intravital imaging, there is likely to be an increase in the use of these sensors to investigate Ca^{2+} signaling changes in cancer in vivo.

8 GECIs and Biomolecular Screening

Only a few published studies have used GECIs in the context of high-throughput screening in drug discovery; however, there has been wide use of small molecule Ca^{2+} indicators in a variety of G-protein-coupled receptors and ion channel-based biomolecular screens (Behrendt, Germann, Gillen, Hatt, & Jostock, 2004; Herington et al., 2015; Wang et al., 2015). This is a strong indicator of the utility of GECIs in biomolecular screening, given the recent advances in GECIs as discussed throughout this review.

One example, where GECIs have been used in a cell-based screen, was described by Honarnejad et al. (2013). This involved the stable expression of the FRET-based cameleon YC3.6 in HEK-293 cells expressing a PS1 gene mutation associated with Alzheimer's disease. PS1 mutation can augment ER calcium homeostasis, evidenced by a reduction of the magnitude Carbachol-induced ER calcium release (Honarnejad et al., 2013). This group screened 20,000 compounds and was able to identify 53 hits capable of causing recovery of the wild-type ER calcium release, by examining those with the highest FRET signal (Honarnejad et al., 2013). RCaMP1h, a red fluorescent GECI, has recently been used to screen genes capable of altering synaptic vesicles using the model organism *Caenorhabditis elegans* using high content imaging (Wabnig, Liewald, Yu, & Gottschalk, 2015). By incorporating RCaMP1h into postsynaptic body wall muscle, RNA interference allowed identification of genes able to alter synaptic transmission following stimulation of cholinergic neurons. This assay was not without challenges; nematodes were imaged in microwells made from agar and despite identifying five target genes capable of altering Ca^{2+}, this did not translate to a strong phenotype of altered synaptic vesicle trafficking (Wabnig et al., 2015). It is likely that some GECIs are already part of biodiscovery projects within pharmaceutical companies and have not yet been described in journal articles, as has been done with small molecule-based probes (Herington et al., 2015). However, it is clear that further application of GECIs in academia and industry will help accelerate the discovery of a new generation of pharmacological agents capable of modulating calcium signaling in disease.

9 Conclusion

The advances in GECIs have been exponential over recent years. Each year incremental improvements are made and new GECIs released. Further development in the properties of fluorescent proteins (including far-red and infrared protein indicators) will enhance this diversity of available Ca^{2+} sensors (Rodriguez et al., 2017). This will assist researchers to avoid spectral overlap, an increasingly important consideration for simultaneous cytosolic/organellar Ca^{2+} measurements (Rose et al., 2014). Advancement will also come from improvements in instrument capabilities and enhancement of software platforms for streamlining analysis.

Conflict of Interest

None to declare.

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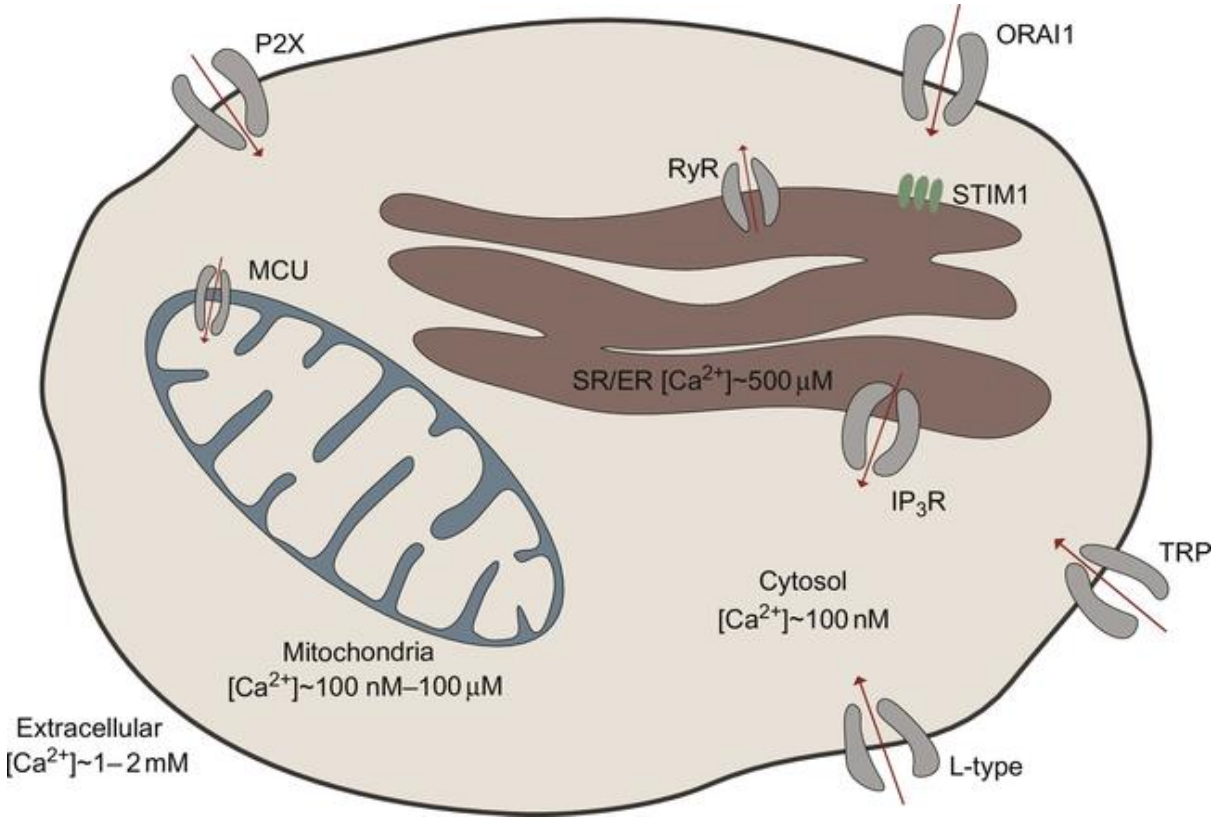

Fig. 1 Schematic representation of a mammalian cell, highlighting some of the main Ca^{2+} channel families. Ca^{2+} channels in conjunction with Ca^{2+} pumps, exchangers, and other regulators are responsible for intracellular calcium signaling. Through the action of these proteins, Ca^{2+} concentration gradients are apparent across the plasma membrane and at intracellular organelles such as the endoplasmic reticulum, which can be altered by these channels.

Adapted from Stewart, T. A., Yapa, K. T., & Monteith, G. R. (2015). Altered calcium signaling in cancer cells. Biochimica et Biophysica Acta, 1848(10), 2502–2511.

Table 1 Properties in vitro of Widely Used Genetically Encoded Calcium Indicators and Those Discussed in This Review

Adapted from Perez Koldenkova, V., & Nagai, T. (2013). Genetically encoded Ca(2 +) indicators: Properties and evaluation. Biochimica et Biophysica Acta, 1833(7), 1787–1797; Rose, T., Goltstein, P. M., Portugues, R., & Griesbeck, O. (2014). Putting a finishing touch on GECIs. Frontiers in Molecular Neuroscience, 7, 88; Suzuki, J., Kanemaru, K., & Iino, M. (2016). Genetically encoded fluorescent indicators for organellar calcium imaging. Biophysical Journal, 111(6), 1119–1131.

Indicator		Class	Fluorophore(s)	Max Dynamic Range	K_d (nM)	Excitation (nm)	Emission (nm)	References
4mtD3cpv		FRET	ECFP/cpVenus	5.1	760	433	475,528	Palmer et al. (2006)
CEPIA1*er*	G-CEPIA1*er*	Intensiometric	cpEGFP	4.7	672,000	497	511	Suzuki et al. (2014)
	GEM-CEPIA1*er*	Ratiometric	cpEGFP	21.7	558,000	391	462,510	Suzuki et al. (2014)
	R-CEPIA1*er*	Intensiometric	cpmApple	8.8	565,000	562	584	Suzuki et al. (2014)
D1ER		FRET	ECFP,citrine	—	810, 60,000	433	475,529	Palmer, Jin, Reed, and Tsien (2004)
D4ER		FRET	ECFP,citrine	—	195,000	435	475,540	Ravier et al. (2011)
Fast-GCaMPs	Fast-GCaMP3-RS09	Intensiometric	cpEGFP	9.5	690	497	512	Sun et al. (2013)
	Fast-GCaMP6f-RS06	Intensiometric	cpEGFP	18.7	320	488	512	Badura, Sun, Giovannucci, Lynch, and Wang (2014)
	Fast-GCaMP6f-RS09	Intensiometric	cpEGFP	25	520	488	512	Badura et al. (2014)

Indicator		Class	Fluorophore(s)	Max Dynamic Range	K_d (nM)	Excitation (nm)	Emission (nm)	References
GAP	goGAP1	Ratiometric/bioluminescent	GFP variant/aequorin	—	12,000	403,470	510	Rodriguez-Garcia et al. (2014)
	erGAP3	Ratiometric/bioluminescent	GFP variant/aequorin	3	489,000	405,470	535	Navas-Navarro et al. (2016)
GCaMP3		Intensiometric	cpEGFP	12	840	485	510	Tian et al. (2009)
GCaMP5G		Intensiometric	cpEGFP	32.7	460	485	510	Akerboom et al. (2012)
	Lck-GCaMP5G	Intensiometric	cpEGFP	—	—	485	510	Akerboom et al. (2012)
GCaMP6	GCaMP6f	Intensiometric	cpEGFP	51.8	375	497	515	Chen et al. (2013)
	GCaMP6m	Intensiometric	cpEGFP	38.1	167	497	515	Chen et al. (2013)
	GCaMP6s	Intensiometric	cpEGFP	63.2	144	497	515	Chen et al. (2013)
	2mtGCaMP6m	Intensiometric	cpEGFP	—	167	474	515	Hill et al. (2014)
	4mtGCaMP6f	Intensiometric	cpEGFP	—	—	497	515	Tosatto et al. (2016)
	Lck-GCaMP6s	Intensiometric	cpEGFP	—	144	497	515	O'Donnell, Jackson, and Robinson (2016)
	sPA-GCaMP6f	Intensiometric	cpEGFP	—	681	480	513	Berlin et al. (2015)

Indicator		Class	Fluorophore(s)	Max Dynamic Range	K_d (nM)	Excitation (nm)	Emission (nm)	References
GCaMP8		Intensiometric	cpEGFP	37.5	200	488	—	Ohkura et al. (2012)
GECO	G-GECO1	Intensiometric	cpEGFP	25	749	496	512	Zhao et al. (2011)
	GEM-GECO1	Ratiometric	cpEGFP	110	340	390	455,511	Zhao et al. (2011)
	R-GECO1	Intensiometric	cpmApple	16	482	561	589	Zhao et al. (2011)
	ER-LAR-GECO1	Intensiometric	cpmApple	10	24,000	561	589	Wu et al. (2014)
	mito-LAR-GECO1.2	Intensiometric	cpmApple	8.7	12,000	557	584	Wu et al. (2014)
	NLS-R-GECO1	Intensiometric	cpmApple	16	482	561	589	Zhao et al. (2011)
	Orai-G-GECO1	Intensiometric	cpEGFP	25	749	496	512	Dynes, Amcheslavsky, and Cahalan (2016)
	Orai-G-GECO1.2	Intensiometric	cpEGFP	23	1150	498	513	Dynes et al. (2016)
go-D1cpv		FRET	ECFP,cpVenus	—	—	425	480,520	Lissandron, Podini, Pizzo, and Pozzan (2010)
NLS-GCaMP2		Intensiometric	cpEGFP	—	—	480	508	Bengtson, Freitag, Weislogel, and Bading (2010)

Indicator		Class	Fluorophore(s)	Max Dynamic Range	K_d (nM)	Excitation (nm)	Emission (nm)	References
jRCaMP1a		Intensiometric	cpmRuby	3.2	214	570	600	Dana et al. (2016)
jRCaMP1b		Intensiometric	cpmRuby	7.2	712	570	600	Dana et al. (2016)
jRGECO1a		Intensiometric	cpmApple	11.6	148	570	600	Dana et al. (2016)
RCaMP1h		Intensiometric	cpmRuby	10.5	1300	571	594	Akerboom et al. (2013)
R-CaMP2		Intensiometric	cpmApple	4.8	69	565	583	Inoue et al. (2015)
TN-XXL		FRET	ECFP,cp174Citrine	—	800	430	480,535	Mank et al. (2008)
Twitch3		FRET	ECFP,cp174Citrine	7	250	432	475,527	Thestrup et al. (2014)
Twitch4		FRET	ECFP,cp174Citrine	6	2800	432	475,527	Thestrup et al. (2014)
YC3.6		FRET	ECFP,cp173Venus	5.6	250	430	480,530	Nagai, Yamada, Tominaga, Ichikawa, and Miyawaki (2004)
YC-Nano50		FRET	ECFP,cp173Venus	12.5	52.5	430	480,530	Horikawa et al. (2010)

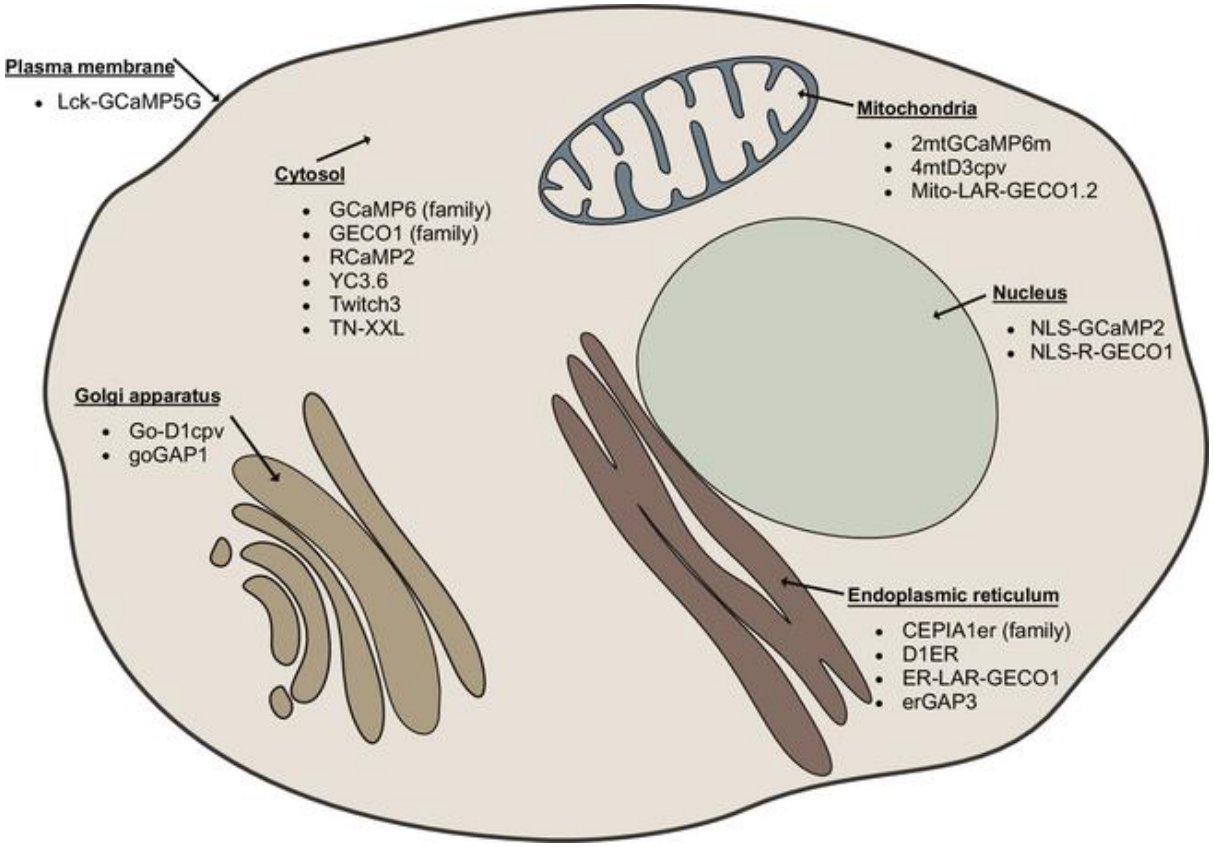

Fig. 2 Examples of genetically encoded calcium indicators targeted to intracellular organelles, subcellular locations, or with cytosolic localization. While not a complete list, this figure highlights some of the commonly applied genetically encoded calcium indicators for Ca^{2+} measurements at various cell locations.