Genetically Encoded Ca\textsuperscript{2+} Indicators in Cardiac Myocytes

Lars Kaestner, Anke Scholz, Qinghai Tian, Sandra Ruppenthal, Wiebke Tabellion, Kathrina Wiesen, Hugo A. Katus, Oliver J. Müller, Michael I. Kotlikoff, Peter Lipp

Abstract: Genetically encoded Ca\textsuperscript{2+} indicators constitute a powerful set of tools to investigate functional aspects of Ca\textsuperscript{2+} signaling in isolated cardiomyocytes, cardiac tissue, and whole hearts. Here, we provide an overview of the concepts, experiences, state of the art, and ongoing developments in the use of genetically encoded Ca\textsuperscript{2+} indicators for cardiac cells and heart tissue. This review is supplemented with in vivo viral gene transfer experiments and comparisons of available genetically encoded Ca\textsuperscript{2+} indicators with each other and with the small molecule dye Fura-2. In the context of cardiac myocytes, we provide guidelines for selecting a genetically encoded Ca\textsuperscript{2+} indicator. For future developments, we discuss improvements of a broad range of properties, including photophysical properties such as spectral spread and biocompatibility, as well as cellular and in vivo applications. (Circ Res. 2014;114:1623-1639.)

Key Words: calcium ■ fluorescence resonance energy transfer ■ gene transfer techniques ■ myocytes, cardiac

Conceptual Considerations

Excitation–contraction coupling is an essential mechanism responsible for the blood-pumping function of the heart. In cardiac myocytes, repetitive Ca\textsuperscript{2+} cycling translates electric excitation (the action potential) into force generation (contraction). Altered Ca\textsuperscript{2+} handling has been related to many cardiac diseases, such as cardiac hypertrophy and heart failure. Therefore, the elucidation of the initiation and progression of cardiac diseases via monitoring Ca\textsuperscript{2+} handling, either at the cellular level or in tissue, is of great interest.

Fluorescence-based intracellular Ca\textsuperscript{2+} imaging is the most versatile method for monitoring Ca\textsuperscript{2+} concentrations in living cells or tissues. Fluorescence-based Ca\textsuperscript{2+} imaging typically uses Ca\textsuperscript{2+}-sensitive fluorescent dyes, such as small organic molecules and, to a lesser extent, genetically encoded Ca\textsuperscript{2+} indicators (GECIs). GECIs represent a new generation of Ca\textsuperscript{2+}-sensing molecules. The first exogenous expression of green fluorescent protein (GFP) in living cells led to the proposed use of GFPs in genetically encoded biosensors. They offer a multitude of advantages over small molecule indicators, including the following: (1) almost no unintended compartmentalization; (2) homogeneous expression in cells from tissues, enabling imaging at the tissue/organ level; (3) cell-type–specific Ca\textsuperscript{2+} mapping through promoter-driven expression; (4) subcellular targeting by the expression of fusion proteins; and (5) the possibility for extended imaging over days and weeks, enabling the investigation of chronic treatments.

The basic initial concept of the GECI is the coupling of 2 fluorescent proteins of distinct colors to a Ca\textsuperscript{2+}-binding domain such that the emission spectrum of the so-called donor and the excitation spectrum of the acceptor protein overlap. A conformational change in the Ca\textsuperscript{2+}-binding domain after Ca\textsuperscript{2+} binding changes the steric arrangement between the 2 proteins. If the donor is excited, part of its energy can be transferred to the acceptor in a distance-dependent manner by a process called Förster Resonance Energy Transfer (FRET). This idea was realized quickly by several groups, and subsequently, genetically encoded Ca\textsuperscript{2+} sensors based on such FRET pairs were introduced. A second design approach of GECI is based on circular permuted fluorescent proteins (CPFPs) that lead to the simultaneous development of several of such sensors. Here, the Ca\textsuperscript{2+}-sensing domain is the same as for FRET sensors. The particular differences in the properties of FRET sensors and CPFP-based sensors will be described in the following sections.

Depending on the incorporated fluorescence protein(s) and the Ca\textsuperscript{2+}-sensing domain, available GECIs exhibit different properties (eg, signal:noise ratio, Ca\textsuperscript{2+} affinities, or other general properties, such as the Ca\textsuperscript{2+} detection principle) and require particular read-out modes. Furthermore, a recent report highlighted that some indicators may display cell-type–specific behaviors; therefore, results obtained in one cell system may not necessarily be transferable to other cell types. Here, we provide an overview of the concepts, experiences, state of the art, and ongoing developments in GECI, with particular emphasis on applications in cardiac cells and heart tissue. We also address numerous fundamental concerns, such as obtaining the expression of GECI in cardiac myocytes, comparing...
the properties of GECI with those of small molecule dyes and to each other in cardiac myocytes, and describing the major pitfalls and challenges of using GECI in cardiac myocytes. The literature discusses such questions insufficiently. To address these aspects specifically, we also include results from dedicated experiments.

Delivery of Genetically Encoded Ca\(^{2+}\) Sensors to Cardiac Myocytes

GECIs require the genetic information encoding the sensor to be transferred into the cardiac myocyte. The not only most elegant but also most elaborate method is to generate a transgenic animal line as for GCaMP-based indicators in the hearts of zebrafish\(^{17}\) and mice.\(^{18}\)

Alternatively, the DNA plasmid can be transfected. Popular chemical transfection methods, such as those that catalyze DNA cross-membrane transport through the use of Ca\(^{2+}\)-phosphate, polycations, or dendrimers, and physical transfection approaches (including electroporation, microinjection, or particle guns), provide low transfection rates in end-differentiated cardiac myocytes and, therefore, have been overtaken by biological transfection methods. Nevertheless, although biological transfection methods (such as the popular lipofection, which when compared with chemical methods leads to a 5- to 100-fold increased transfection rate) may work in neonatal cardiac myocytes, they are insufficient in adult cardiac myocytes.\(^{19}\) Viral gene transfer is the transduction method of choice for end-differentiated cells, such as cardiac myocytes.\(^{20-22}\) In this context, the integration of the indicator DNA into the viral genome can be challenging. On the basis of our own observations, we found that FRET-based sensors with 2 variants of the same fluorescent protein, for example, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) and variants thereof, flanking the protein sensor, may result in inefficient transposition because of the similarities in structure of the genes encoding the 2 fluorescent proteins.

Viral transduction can be performed in vivo or in isolated cells. The latter approach benefits from improvements in cell culture techniques, such as those recently introduced, for example,\(^{23}\) to maintain the morphological and functional properties of cardiac myocytes for several days.

In preparation for constructing viruses for viral transduction, we evaluated various viral systems, including the Semliki-Forest virus,\(^{24}\) lentivirus,\(^{25}\) and adenovirus.\(^{26}\)

In our hands, the transduction of cardiac myocytes with Semliki-Forest virus led to a low transduction rate (<5%). By contrast, lentiviral gene transfer was much more efficient (transduction rate, >97%), but protein expression required ≈ 1 week to reach detectable levels. However, third-generation lentivirus vectors with a conditional packaging system may perform much better.\(^{27}\) This timeframe is far too long for established culture systems of cardiac myocytes.\(^{21,28,29}\)

Therefore, lentiviruses may be appropriate for in vivo gene transfer but not for gene transfer in primary cultured cardiac myocytes. When using gene transfer with adenoviruses, protein expression of the genetically encoded fusion proteins or biosensors reached optically detectable levels within 24 hours.\(^{30,31}\) Adenovirus-mediated expression is rather stable during the time course of 1 week, and we did not observe adverse effects caused by the viral transduction or induced gene expression during that period.\(^{32,33}\) Therefore, adenovirus-mediated gene transfer seems to be the most suitable method for the expression of proteins in cultured adult cardiomyocytes. Alternatively, herpes virus transduction has also been successfully used to express a GECI in adult cardiomyocytes.\(^{34}\)

For whole-heart and in vivo imaging, the transduction and expression of the GECI must be performed not only in isolated cells but also in the living animal. As mentioned above, the production and breeding of transgenic animal lines are a serious undertaking, and the encoded sensor might be superseded by a better GECI by the time of successful generation. An alternative approach to generating transgenic animals is to transduce the sensor into individual animals, again with the help of a viral vector. Vectors can be applied directly to the myocardium/pericardial space by local injection or via the vascular system. Although direct injections may lead to a patchy transduction pattern, transvascular or systemic application approaches have the advantage of a more homogeneous cardiac transduction but face additional obstacles, such as neutralizing antibodies, binding to plasma proteins in the circulation, and effective passage through the vascular wall.\(^{35}\) Furthermore, transduction efficiency depends on successful uptake into cardiomyocytes. Finally, sustained expression of the transgene depends on the ability of the vector to prevent clearance of its vector genome in the cell and the lack of an immune response against vector epitopes.

Although there are reports of adenovirus-mediated gene silencing in the heart,\(^{36}\) in our hands expression of the sensor in the heart of rodents was at or below the detection limit when we injected neonatal or adult hearts of rats or mice directly. This result is likely because of the strong immune responses of the animals against the adenoviral capsid,\(^{37}\) elucidating an innate immune response against the vector that rapidly cleared the adenoviral vector. The low number of reports applying adenovirus-mediated gene transfer in vivo seems to support this notion. A method that circumvents the immune response is gene painting,\(^{38}\) in which virus suspensions are mixed with trypsin and directly applied to the excised heart. This is a popular method to transduce proteins into the atria\(^{39}\) of swine or sinoatrial node\(^{40}\) of mice; however, studies on gene painting in the ventricles or in other species are lacking. Therefore, we and others have focused on adenov-associated viruses (AAVs) as an alternative approach for gene delivery.\(^{21,41,42}\) AAV is a nonpathogenic parvovirus that enters the target cell via receptor-mediated endocytosis. Its low immunogenicity enables...
a sustained gene transfer in rodents.\textsuperscript{35} Certain naturally occurring AAV serotypes, such as serotype 9 vectors, resulted in uniform and extensive cardiac transfer in adult mice after intravenous injections.\textsuperscript{42–46} Although further modifications of the AAV-vector surface (educated guess or library-based approaches) could extend the tropism of gene transfer, for example, to vessels or increase specificity, AAV9 vectors still remain the vector of choice for systemic cardiac gene transfer in rodents.\textsuperscript{21,47} Because AAV9 vectors enable a highly efficient but not specific cardiac gene transfer, specificity of expression can be increased using a tissue-specific promoter or an appropriate microRNA-target site into the AAV-genome.\textsuperscript{48,49}

The role of a tissue-specific promoter is increasingly acknowledged in the development of gene therapy\textsuperscript{50–52} but has also been demonstrated for the cardiac-specific expression of fluorescent proteins.\textsuperscript{53,54} Another important question is the mode of virus delivery, which we addressed by comparing virus delivery via direct injection into the neonatal heart, injection into the tail vein of adult mice, and injection into the jugular vein of neonatal mice (<1 week). Injection into neonatal mice has the advantage of achieving higher ratios of AAV serotypes, such as serotype 9 vectors, resulted in uniform and extensive cardiac transfer in adult mice after intravenous injections.\textsuperscript{42–46} Although further modifications of the AAV-vector surface (educated guess or library-based approaches) could extend the tropism of gene transfer, for example, to vessels or increase specificity, AAV9 vectors still remain the vector of choice for systemic cardiac gene transfer in rodents.\textsuperscript{21,47} Because AAV9 vectors enable a highly efficient but not specific cardiac gene transfer, specificity of expression can be increased using a tissue-specific promoter or an appropriate microRNA-target site into the AAV-genome.\textsuperscript{48,49}

Injection into the jugular vein of neonatal mice was the best approach that resulted in the most rapid and highest expression of YC3.6, whereas the other methods were either associated with high variability in expression (direct injection into the neonatal cardiac muscle) or displayed a rather mediocre expression rate over time (tail vein injection of adult mice). Figure 1C depicts that the fluorescence distribution throughout the cardiac issue seems rather homogeneous. However, the single-cell analysis presented in Figure 1D reveals a broad variation in fluorescence intensities. Both, the image (Figure 1Da) and the histogram of the fluorescence intensities (Figure 1Db) indicate that in approximately one third of the cells the expression is too low to allow reliable measurements based on the fluorescence intensity. However, whole-heart measurements are not affected (compare Figure 1C) and for single-cell measurements the number of cells with adequate expression is sufficiently high for reliable measurements.

Properties of GECI in Cardiac Myocytes When Compared With Those of Fura-2

There are numerous reviews on GECI in general,\textsuperscript{57–62} and a broad subset of sensors has also been applied in cardiac investigations, which are summarized in Figure 2. The left 4 columns in Figure 2 provide the basic properties of the sensors, including a scheme for the principle of operation. It is almost impossible to compare different GECI based on the literature because their properties have not been determined by standardized methods.\textsuperscript{16} Many parameters, such as Ca\textsuperscript{2+} sensitivity, potential toxicity, and targeting, may depend on the experimental conditions and, in
<table>
<thead>
<tr>
<th>Ca(^{2+}) sensor</th>
<th>scheme of principle (taken from (^{39}))</th>
<th>spectral properties ([\text{nm}])</th>
<th>(K_d) ((\mu\text{M}))</th>
<th>targeted versions (fusion proteins)</th>
<th>special property</th>
<th>application in cardiac myocytes</th>
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<tr>
<td>aequorin</td>
<td>no exitation</td>
<td></td>
<td>1.5, 10, 40</td>
<td>subunit VIII of cytochrome c oxidasen (mitochondria)(^{38})</td>
<td>based on bioluminescence allowing detections between 0.1 and 100 (\mu)M limited use for fast repetitive kinetics</td>
<td>mitochondrial expression in neonatal myocytes;(^{117}) adenoviral subcellular expression in adult rat myocytes;(^{117}) numerous applications in which aequorin is not expressed but injected, and it therefore cannot be regarded as a genetically encoded indicator</td>
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<td>cameroon</td>
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<td></td>
<td>1.6 / 2.5</td>
<td>mitochondrial(^{36}) utilizing the blue fluorescent protein (BFP)</td>
<td>mitochondrial expression in neonatal rat myocytes(^{118})</td>
<td></td>
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<tr>
<td>YC2.1</td>
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<td></td>
<td>0.1 / 2.10</td>
<td>caireticolin leading sequence and the retention signal KDEL (SR);(^{109}) nuclear (nucleus)(^{109})</td>
<td>different (K_d) reported by others (0.63(^{37}))  lack of fluorescence lifetime change (donor) with (Ca^{2+}) change</td>
<td>SR-expression in neonatal myocytes;(^{109}) neonatal myocytes(^{119})</td>
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<tr>
<td>YC3.1</td>
<td>ex: 416 and/or 494 em: 515</td>
<td></td>
<td>1.5 / 2.10</td>
<td>PTX-receptor;(^{110}) nuclear(^{110})</td>
<td>different (K_d) reported by others (0.63(^{37}))  lack of fluorescence lifetime change (donor) with (Ca^{2+}) change</td>
<td>SR-expression in neonatal myocytes;(^{109}) neonatal myocytes(^{119})</td>
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<td>YC3.6</td>
<td>ex: 495 and/or 490 em: 514</td>
<td></td>
<td>0.2 / 6.60</td>
<td>subunit VIII of cytochrome c oxidasen (mitochondria)(^{126})</td>
<td>decreasing intensity with increasing (Ca^{2+}) concentration</td>
<td>adenoviral expression in adult rat myocytes(^{118})</td>
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<tr>
<td>D1</td>
<td>ex: 495 em: 516</td>
<td></td>
<td>0.2 / 2.60</td>
<td>mitochondrial(^{36}) utilizing the blue fluorescent protein (BFP)</td>
<td>mitochondrial expression in neonatal cardiac myocytes(^{112})</td>
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<td>ratio-</td>
<td>ex: 416 and/or 494 em: 515</td>
<td></td>
<td>1.7 / 10.0</td>
<td>FKBP;(^{110}) mitochondrial, nuclear(^{36})</td>
<td>quantitative read-out only with double (consecutive) excitation</td>
<td>mitochondrial in neonatal myocytes;(^{107,113}) adenoviral mitochondrial in adult mice(^{111})</td>
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<td>metric</td>
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<td>0.7 / 8.50</td>
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<td>flash</td>
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<td>0.2 / 6.60</td>
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<tr>
<td>gecos</td>
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<td></td>
<td>1.5 / 10.0</td>
<td>FKBP;(^{110}) mitochondrial, nuclear(^{36})</td>
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<td>mitochondrial in neonatal myocytes;(^{107,113}) adenoviral mitochondrial in adult mice(^{111})</td>
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<td>GCaMP1</td>
<td>no exitation</td>
<td></td>
<td>0.23 / 3.50</td>
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<td>GCaMP2</td>
<td>ex: (400)/490 em: 510</td>
<td></td>
<td>0.15 / 4.00</td>
<td>MARCKS, aktin;(^{115}) adenylif cyclase;(^{116}) bax;(^{117}) synaptophysin;(^{118}) connexin43;(^{119}) PMCA4;(^{120}) subunit VIII of cytochrome c oxidasen (mitochondria)(^{126})</td>
<td>different (K_d) reported by others (0.44(^{37}) and 0.84(^{37}))</td>
<td>transgenic mouse(^{118}) functional engraftment of transplanted embryonic myocytes;(^{118}) fused to the Ca(^{2+}) pump (PMCA4) in rat neonatal and adult myocytes;(^{118}) mitochondrial in neonatal myocytes</td>
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<td>GCaMP3</td>
<td>ex: (400)/490 em: 510</td>
<td></td>
<td>0.66 / 12.0</td>
<td>synaptophysin;(^{118}) lymphocyte-specific protein tyrosine kinase (membrane)(^{126})</td>
<td>superb signal to noise ratio</td>
<td>human embryonic stem cell-derived cardiomyocytes;(^{118,120}) adenoviral expression in adult rat myocytes (this paper)</td>
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<tr>
<td>GCaMP6/</td>
<td>ex: 435/515 em: 440/535</td>
<td></td>
<td>0.63 / 4.00</td>
<td>triadin1, junctin1;(^{118})</td>
<td>superb signal to noise ratio</td>
<td>adenoviral expression of a triadin1 or junctin1 fused GCaMP6 in adult rat myocytes(^{118})</td>
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<td>TN-L15</td>
<td>ex: 435/515 em: 440/535</td>
<td></td>
<td>1.2 / 1.40</td>
<td>tropolin C based; therefore, no unwanted interaction with calmodulin</td>
<td>tropolin C based; therefore, no unwanted interaction with calmodulin</td>
<td>herpesvirus expression in adult canine myocytes(^{118})</td>
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<td></td>
<td>2.5 / 4.00</td>
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<td>mitochondrial(^{36}) utilizing the blue fluorescent protein (BFP)</td>
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<td>TN-XXL</td>
<td>ex: 435/515 em: 440/535</td>
<td></td>
<td>0.8 / 2.30</td>
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<td>mitochondrial(^{36}) utilizing the blue fluorescent protein (BFP)</td>
<td>adenoviral expression in adult rat myocytes (this paper)</td>
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<td>GECOs</td>
<td>ex: 370/570 em: 446/600</td>
<td></td>
<td>0.16-0.48 / 8-111</td>
<td>PKC(^{31})</td>
<td>entire group of new sensors</td>
<td>will be used by authors in several cardiac applications</td>
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Figure 2. Overview of genetically encoded Ca\(^{2+}\) indicators that have been expressed in cardiac myocytes. The 2 excitation values of Förster resonance energy transfer (FRET)-based probes refer to the measurement mode for the apparent FRET efficiency (compare Equations 1–4 and Figure 6A and 6B). The targeted versions represent a selection of popular or innovative fusion proteins, which were not necessarily applied in cardiomyocytes. The expression in cardiac cells is listed in the rightmost column, providing examples going beyond the discussion in the main text. BFP indicates blue fluorescent protein; ER, endoplasmic reticulum; em, emission; ex, excitation; FKBP, FK506 binding protein; GECOs, genetically encoded Ca\(^{2+}\) indicators for optical imaging; MARKCS, myristoylated alanine-rich C-kinase substrate; PKC, protein kinase C; PMCA4, plasma membrane Ca\(^{2+}\) ATPase isof orm 4; PTX, cation-permeable ion channel that opens in response to the binding of extracellular adenosine 5'-triphosphate; and SR, sarcoplasmic reticulum. The images with the scheme of principle are adapted from Pérez Koldenkova and Nagai\(^{26}\) with the permission of the publisher (Elsevier, 2013).
particular, the cell type. Therefore, for cardiomyocytes, we compared a range of GECI (GCaMP3, ratiometric pericam, inverse pericam, YC3.6, TN-XL, and TN-XXL) with the small molecule dye Fura-2. A primary limiting factor in recording intracellular Ca^{2+} transients with GECI is the signal:noise ratio of the recorded signals. We evaluated this ratio after the adenoviral transduction of the abovementioned sensors. Figure 3A shows representative cell images (left) and typical traces of electrically induced Ca^{2+} transients (right) in cardiac myocytes expressing the GECI for 48 hours. Although the probes used in the upper 4 rows relied on calmodulin as the Ca^{2+}-sensing domain (GCaMP3, ratiometric pericam, inverse pericam, and YC3.6),\textsuperscript{15,56,63} TN-XL and TN-XXL both used part of the Ca^{2+}-sensing domain of troponin-C.\textsuperscript{64,65} Interestingly, for unknown reasons, we were unable to measure TN-XXL-dependent fluorescence changes in cardiac myocytes, despite the sufficient expression of the probe and visible stimulation-dependent contractions of the cells (compare Figure 3A). This result was puzzling given that the viral entry vector for TN-XXL, when expressed in human embryonic kidney cells, was able to display appropriate fluorescence changes in response to ATP stimulation (data not shown). These findings emphasize the care with which GECI should be tested, particularly in cellular environments. In addition to the 6 genetically encoded indicators depicted in Figure 3, an adenovirus was also designed for flash pericam.\textsuperscript{15} Virus production for flash pericam worked well, and the virus could be transduced in HeLa cells, in which it enabled the monitoring of cellular Ca^{2+} transients. Interestingly, in cardiac myocytes, functional expression failed, and we observed large fluorescent aggregates instead. We concluded that the protein did not properly mature. The 5 GECI depicted in the upper rows of Figure 3 permitted the recording of cellular Ca^{2+} transients, albeit with substantially different properties. To quantify the different behaviors of the probes, we analyzed their signal:noise ratios (Figure 3C), the upstroke duration of the apparent Ca^{2+} transients reported by the probes (Figure 3D), and their corresponding time constants characterizing the falling phase of the Ca^{2+} transient (Figure 3Db). For all parameters, we also included the data obtained for the small molecule dye Fura-2 (blue bars in all 3 panels). With respect to the signal:noise ratio, the GCaMP3 sensor was the only probe that could compete with Fura-2 (Figure 3C). All other genetically encoded probes revealed a lower signal:noise ratio. When analyzing the upstroke duration of the resulting signals, we realized that in comparison with the values from Fura-2, nearly all GECI displayed substantially increased durations, likely because of intramolecular steric changes in response to Ca^{2+} binding preceding the changes in the fluorescence properties. Such extended upstrokes occurred independently of the Ca^{2+}-sensing domain. Interestingly, the upstroke duration was largely independent of the actual mechanism generating the fluorescence changes. Although GCaMP3 and the ratiometric and inverse pericams are based on CPFPs and the fluorescence changes were produced by sterically changing the relationship between the 2 halves of the fluorescence protein,\textsuperscript{66} both YC3.6 and TN-XL are based on FRET between the CFP and YFP variants.\textsuperscript{50,64} Although it seems that the internal changes in the molecular arrangement might be slower in fluorescence protein-based Ca^{2+} sensors, the situation was much less straightforward when we concentrated on the decay phase of the signal transient (Figure 3D). Although Fura-2, GCaMP3, inverse pericam, and YC3.6 all displayed comparable decay time constants (Figure 3Db), ratiometric pericam and TN-XL exhibited significantly slower and faster decay phases, respectively. These differences were independent of both the sensing Ca^{2+} domain and the mechanism of fluorescence generation. Although the reasons for the slow decay of the ratiometric pericam are unclear, there are more obvious reasons why the signal transient reported by TN-XL was fast. In contrast to the other GECI, in which the sensing domain is derived from calmodulin, the Ca^{2+}-sensing domain of TN-XL originates from troponin-C and is characterized by a relatively high apparent \( K_d \) for Ca^{2+} of 2.5 \( \mu \)mol/L and an unusually high Hill coefficient of 1.7.\textsuperscript{64} Therefore, TN-XL preferentially detected the peak of the cellular Ca^{2+} transients, effectively narrowing the apparent time course and reducing the signal:noise ratio.

As any other Ca^{2+} indicator, GECIs contribute to the cytosolic Ca^{2+} buffer capacity. To quantify this, we measured the sensor concentration of YC3.6 expressed in ventricular cardiac myocytes and found values between 0.4 and 3.8 \( \mu \)mol/L with an average below 2 \( \mu \)mol/L, as depicted in Figure 3E. When compared with the physiological cytosolic Ca^{2+} buffer capacity of cardiac myocytes of \( \leq 100 \) \( \mu \)mol/L,\textsuperscript{67} the additional Ca^{2+} buffer capacity introduced by the GECI was rather small and at least an order of magnitude smaller than the concentrations used for small molecular dyes.\textsuperscript{68,69}

In additional experiments, we analyzed GCaMP2 and Fura-2 using ventricular myocytes from a transgenic mouse line.\textsuperscript{18} This comparison was motivated by (1) the spectral properties of GCaMP2 and Fura-2, which enabled the simultaneous measurement of both indicators in a single cell; (2) the similar \( K_d \) values of the indicators for Ca^{2+} \( \equiv 230 \) nmol/L\textsuperscript{14,69}; and (3) the expression in a transgenic animal to represent the best case scenario in terms of the expression rate and stability. Figure 4Aa depicts typical normalized data from a ventricular myocyte expressing GCaMP2 that was also loaded with Fura-2 and electrically stimulated by extracellular field electrodes (denoted by the arrowhead in Figure 4Aa). Although the measurements were performed at 35°C, there were differences in the kinetics of the recorded signal in both the upstroke (82.5 ms [Fura-2] versus 220 ms [GCaMP2]; \( P<0.0001; 4 \) mice; >50 cells) and decay time constant (268 ms [Fura-2] versus 641 ms [GCaMP2]; \( P<0.0001; 4 \) mice; 46 cells). These time constants display the apparent properties of the same Ca^{2+} transient simultaneously recorded with 2 sensors and should not be confused with the time constant of the sensors. Nevertheless, these apparent time constants correspond well with the published properties of these 2 sensors. GCaMP2 was reported to have a \( \tau_{\text{on}} \) of \( \approx 15 \) ms and a \( \tau_{\text{off}} \) in the range of 75 ms,\textsuperscript{18} whereas \( \tau_{\text{small}} \) for Fura-2 was below 2 ms.\textsuperscript{70} We complemented these measurements with calculations based on a simulated real Ca^{2+} transient in Figure 4Ab, confirming our notion. In contrast to small molecular indicators, in GECI, the \( K_d \) is less influential for the upstroke and decay time constants, except if in combination with the dynamic range, the Ca^{2+} concentration to measure is outside the sensors coverage as outlined above for TN-XL.
Figure 3. Continued
Figure 3. Comparison of genetically encoded Ca\(^{2+}\) indicators expressed in adult ventricular myocytes with Fura-2-loaded cells. 

A. Examples of Ca\(^{2+}\) transients measured with 5 different genetically encoded Ca\(^{2+}\) indicators and Fura-2 under otherwise identical experimental conditions (left; distribution of the fluorescence in myocytes 2 days after transduction; right, typical train of electrically evoked global Ca\(^{2+}\) transients). Although the sensor TN-XXL was expressed in cardiac myocytes, it did not report Ca\(^{2+}\) changes. Adult rat ventricular myocytes were isolated as previously described\(^{156}\) and maintained in optimized culture conditions\(^{25}\) for 3 days. Measurements were performed with a video-imaging set-up as previously described\(^{155}\) at 34°C. The multiplicity of infection (MOI) was in the range of 4×10\(^4\) to 7×10\(^5\) pfu per 1500 cells and determined as depicted in B. Example of determination of adenoviral dose for the ratiometric pericam. The MOI marked with the red circle was used for virus transduction. C. Statistical analysis of the signal:noise (S/N) ratio. D. Relationship between the Ca\(^{2+}\) transient amplitudes recorded at a 0.2-Hz stimulation frequency divided by the amplitude values obtained at a 3-Hz stimulation frequency with Fura-2 (y axis) and GCaMP2 (x axis). B–D. The red lines represent exponential best fits. This figure presents primary experimental results. RFU indicates relative fluorescence units.

To compare the properties of the apparent Ca\(^{2+}\) transients reported by Fura-2 and GCaMP2 further, we plotted the amplitudes of the simultaneously recorded Ca\(^{2+}\) signals measured with these 2 probes against each other (Figure 4B) and observed a good correlation between these 2 parameters (P<0.0001; 4 mice; 123 cells). Interestingly, we noticed a population of myocytes that clearly displayed a Fura-2 transient but lacked any detectable GCaMP2 fluorescence changes. To determine whether these cells represented a population of cells displaying only minute GCaMP2 expression, we plotted the resting Fura-2 ratio against GCaMP2 fluorescence (Figure 4C), which revealed that although there was a broad range of GCaMP2 fluorescence, the number of cells with low expression was negligible. Because both sensors, Fura-2 and GCaMP2, readily detected modulations of the Ca\(^{2+}\) transient amplitude at 2 different stimulation frequencies (Figure 4D), we concluded that efficient Ca\(^{2+}\) binding occurred for both sensors. The significant population of cells that displayed GCaMP2 fluorescence without changes in the transients (compare Figure 4B and 4C) might be because of the altered Ca\(^{2+}\)-binding kinetics of the Ca\(^{2+}\)-sensing domain of GCaMP2 in the cytosolic environment of the myocyte (eg, because of modulated cooperativity between the EF hands in the Ca\(^{2+}\)-sensing domain of GCaMP2). In addition, we may speculate that this is because of incorrect protein maturation of the Ca\(^{2+}\) sensor domain or the linkers independent of the fluorescent proteins.

Ca\(^{2+}\) Indicators Based on CPFPs

GEIs based on CPFP represent 1 of 2 engineering strategies for converting fluorescent proteins into active physiological Ca\(^{2+}\) sensors (see FRET-Based Ca\(^{2+}\) Indicators section of this article). The structural basis for Ca\(^{2+}\) sensing was elucidated for GCaMP2\(^{71}\) and can be described as a switch from a protonated to a deprotonated fluorophore\(^{71}\) by restricting solvent access to the fluorophore and stabilizing it in an ionized form.\(^{72}\)
This stabilization is supported by extensive contacts between the condensed calmodulin–M13 structure and the fluorophore moieties.71

Apart from the design approach, the application of CPFPs has a significant effect on the recording strategy. CPFPs are often used as equivalents to single-excitation, single-emission small molecule dye Ca2+ probes. However, many of the CPFPs can also be used in a ratiometric excitation mode (eg, ratio-metric pericam). Ca2+ sensitivity is introduced into the CPFPs by the molecular insertion of calmodulin or calmodulin–M13 domains. Examples of such Ca2+ sensors include the families of pericams and GCaMPs. In comparison with previous versions, GCaMP2 displays improved folding at 37°C and larger response amplitudes when compared, for example, with FRET-based Ca2+ sensors.14 Despite the relative ease of recording when using the single-excitation single-emission mode, these Ca2+ indicators are associated with many shortcomings because the measured intensity changes can alternatively be a result of sample movement, inhomogeneous Ca2+ sensor distribution, or changing autofluorescence.73,74 In addition to such complications, pH-sensitivity, irreversible or reversible photoconversion, and rather complex Ca2+/intensity relationships can contribute to falsified interpretations of their intensity changes. An example of reversible photoconversion is shown in Figure 5, which depicts Ca2+ transients immediately after the start of illumination. Although the time course of the fluorescence strongly resembles that of irreversible photobleaching (Figure 5A), this process was reversible because the original starting fluorescence recovered after longer dark periods (Figure 5C and 5D). Such behavior is incompatible with the process of photobleaching. Instead, photoconversion at the molecular level induced by cis-trans isomerization in the actual chromophore75 is reversible and may explain these observations. Figure 5A illustrates such behavior for the Ca2+ sensor GCaMP3. The entire family of CPFPs is prone to such photoconversion processes, but photobleaching can be mathematically accounted for, as illustrated in Figure 5B. Despite the shortcomings of CPFP-based Ca2+ sensors, the most recent iteration of these probes, that is, GCaMP3-6, offers the best signal-to-noise ratios76 (Figure 4C).

**FRET-Based Ca2+ Indicators**

An alternative to single fluorophore-based Ca2+ indicators is the use of ratiometric Ca2+ sensors based on FRET between 2 different mutants of GFP, similar to the first set of GECI.12,13 Both approaches use Ca2+ sensing by calmodulin in combination with the Ca2+-dependent interaction of calmodulin and the calmodulin-binding peptide M13 from the myosin light chain kinase. In cameleons,13 calmodulin–M13 is sandwiched between CFP and YFP. In these probes, Ca2+ binding to calmodulin is translated into alterations of the steric arrangement between CFP and YFP by a 2-fold mechanism. First, Ca2+ binding to calmodulin itself leads to significant rearrangement of the EF hands in the molecule. Such steric changes within the molecule result in changes in the CFP–YFP interaction. Nevertheless, the major intramolecular FRET originates from the process of intramolecular interaction of Ca2+-calmodulin with its binding partner M13. In this process, Ca2+-calmodulin almost wraps around the binding partner, and the entire Ca2+-sensing domain complex drastically changes its arrangement, resulting in a substantial alteration of the CFP–YFP interaction, eventually changing the energy transfer between these 2 fluorescent proteins (hence, subsequent changes in the FRET signal). FRET changes can be observed as changes in CFP and YFP in opposite directions, a prerequisite for ratiometric fluorescence.

**Figure 5. Reversible photoconversion of GCaMP3.** **A**, Decrease in baseline fluorescence intensity during recording of a train of electrically evoked Ca2+ transients with GCaMP3. **B**, Correction of photoconversion based on the exponential fit of data at the diastolic Ca2+ concentration. The measured trace was deconvolved by the fitted background data, resulting in a trace that is virtually unaffected by photoconversion. **C**, Reversible photoconversion of GCaMP3 expressed in rat cardiomyocytes. **Left**, Photoconversion in individual myocytes; **right**, the same cell (color code) after dark resting periods of a given length. **D**, On the basis of results from **C**, we calculated a recovery curve for fluorescence. The multiplicity of infection for all GCaMP3 transductions was 7×105 pfu/1500 cells. This figure presents primary experimental results.
measurements. However, particularly for quantitative results, there are several aspects that must be considered before, during, and after FRET measurements. A popular but over-simplified approach is to measure and to calculate the fluorescence ratio between the spectral emission channels of the donor and acceptor after donor excitation. For most sensors, the emission spectra of the donor and the acceptor overlap. Furthermore, the emission spectra of the donor and the acceptor also overlap in the emission channel of the acceptor. Such cross talk between the excitation and emission channels limits the usability of simple donor/acceptor fluorescence ratios as a direct read-out. Such ratios are not independent of uneven bleaching and contraction artifacts, which in beating myocytes are particularly advantageous properties of ratiometric measurements.\(^ {31}\) Quantitative FRET signals rely on 2 major determinants: (1) the FRET efficiency of the sensor itself, which is determined by the distance between the donor and the acceptor and the angle of their dipoles to one another and (2) the number of molecules that interact. For FRET-based genetically encoded Ca\(^ {2+}\) sensors, a particular molecule is expected to be in 1 of 2 conformations, either Ca\(^ {2+}\) free or Ca\(^ {2+}\) occupied. Therefore, the number of molecules binding Ca\(^ {2+}\) dominates the extent of the FRET signal. The measurement of the fluorescence lifetime of the donor is one of the best approaches for quantifying FRET signals.\(^ {77}\) To quantify subsequent FRET measurements, such lifetime recordings can be used for the initial calibration of a Ca\(^ {2+}\) probe.\(^ {78}\) These measurements must be performed under experimental conditions identical to the previous experiments and require fluorescence lifetime imaging technology, which is inherently slow.\(^ {79}\) Alternatively, various algorithms to circumvent fluorescence lifetime imaging measurements have been reported\(^ {80-82}\) and incorporated into software by microscopy manufacturers. However, the most comprehensive method to quantify intramolecular FRET in biosensors that allows a relatively straightforward implementation is the calculation of the apparent FRET efficiency:

\[
E_{app} = E_f \frac{\epsilon_D}{\epsilon_A}
\]

where \(E\) is the FRET efficiency, \(f_D\) is the fraction of the FRET sensor fluorescent entities (ie, 0.5 for a 1:1 expression of donor and acceptor), and \(\epsilon_D\) and \(\epsilon_A\) are the extinction coefficients for the donor and acceptor at the donor excitation wavelength, respectively.\(^ {83}\) \(f_D\), \(\epsilon_D\), and \(\epsilon_A\) can be regarded as constants within a given biosensor, but their actual values do not need to be known to perform the calculation. To determine the apparent FRET efficiency for yellow cameleon, the following formula is used:

\[
E_{app}^{YC} = E_f \frac{\epsilon_D}{\epsilon_A} \frac{F^{YC}_{CFP,YFP} - \alpha F^{YC}_{CFP,YFP} + \beta F^{YC}_{CFP,CFP}}{F^{YC}_{CFP,YFP}}
\]

where \(F^{YC}\) is the fluorescence signal of yellow cameleon, \(CFP_{a}\) and \(YFP_{r}\) are the applied excitation wavelengths, and \(CFP_{a}\) and \(YFP_{r}\) are the recorded emission channels. \(\alpha\) and \(\beta\) are constants that must be individually determined for the donor and acceptor under the same experimental conditions as the FRET sensor. They can be calculated as follows:

\[
\alpha = \frac{F^{CFP}_{CFP,YFP_a}}{F^{CFP}_{CFP,YFP_r}} \frac{F^{CFP}_{CFP,CFP}}{F^{CFP}_{CFP,CFP_a}}
\]

and

\[
\beta = \frac{F^{CFP}_{CFP,YFP_a}}{F^{CFP}_{CFP,YFP_r}} \frac{F^{CFP}_{CFP,CFP}}{F^{CFP}_{CFP,CFP_a}}
\]

This method relies on the individual availability of the fluorescent proteins used in the FRET sensor. The experiment itself requires an alternating excitation at the donor and acceptor excitation wavelengths concomitant with the detection of 2 emission channels for the donor and the acceptor fluorescence. The superiority of determining the FRET efficiency over simple CFP/YFP ratio recordings is highlighted in Figure 6, which illustrates FRET measurements from an YC3.6-expressing rat ventricular myocyte. In comparison with the blue trace (CFP/YFP ratio at the CFP excitation wavelength), which is convoluted by slow-moving artifacts of the beating myocyte, the trace showing the FRET efficiency (Figure 6Ab) calculated from the same raw data displays a constant baseline with a constant amplitude typical for steady-state Ca\(^ {2+}\) transients. Furthermore, this calculation facilitates the comparison of cells under different experimental settings, such as expression level, as depicted in Figure 6B.

Although the apparent FRET efficiency requires more complex measurements and lowers the temporal resolution, it is an optimized procedure that produces quantitative data from FRET-based GECI. This approach is particularly advantageous because it does not assume any molecular properties or constants, such as the molecular quantum efficiency, that need to be acquired via complicated procedures or extracted from published data. With such an approach, we evaluated the maximal recording frequency that could be attained. We were able to achieve an image recording speed of 100 frames per second with a monochromator-driven video-system as outlined in Figure 6C. However, light-emitting diode–based systems in combination with sCMOS (scientific complementary metal oxide semiconductor) cameras can be expected to yield acquisition rates of several hundred Hertz.\(^ {84}\)

**Biocompatibility of Genetically Encoded Ca\(^ {2+}\) Sensors**

In general, the biocompatibility of genetically encoded biosensors is an advantage instead of small molecule dyes because the sensors are naturally produced by the cells themselves, even when the probe is expressed ubiquitously.\(^ {85}\) However, the preservation of the in vitro properties of fluorescent proteins when used as Ca\(^ {2+}\) probes in vivo do not necessarily apply to their Ca\(^ {2+}\)-sensing domains. Although their basic functional properties are retained when expressed in living cells,\(^ {86}\) the dynamic range of many probes (eg, camgaroo-2, GCaMP2, and inverse pericam) is significantly reduced in the cytosol.\(^ {87,88}\) This phenomenon has also been reported for many small molecule dyes, such as Flu-3, for which even the subcellular localization alters the dynamic range (ie, nucleoplasmic versus cytoplasmic environment).\(^ {89}\) Furthermore, protein interactions have been reported that influence their properties, such as Ca\(^ {2+}\) responsiveness and...
intracellular mobility. Such observations for genetically encoded Ca2+ probes suggest that the Ca2+-sensing domains of the molecules might be either modified by the host cell or altered through protein–protein interactions in the cytosol. Because the vast majority of genetically encoded Ca2+ sensors today use calmodulin (or parts of it) as the Ca2+ sensor, putative calmodulin–protein interactions might in fact contribute to such cytosolic alterations of the probe properties. There are numerous intracellular interaction partners for calmodulin, which include the apocalmodulin and Ca2+-occupied forms. In both states, calmodulin can in fact serve as both receiver and donor for protein–protein interactions. Apart from changes in the dynamic range and Ca2+ affinity of the probe properties, simple expression of the Ca2+ sensor itself has been reported to interfere directly and indirectly with the physiology of the host organ or organism or may be a target for upstream signaling and protein modifications, such as kinase-dependent phosphorylation.

Apart from the calmodulin domain of the sensors, the intramolecular calmodulin-binding domain M13 has also been reported to interact with endogenous calmodulin. However, this interaction has been abrogated in a new generation of Ca2+ sensors in which the intramolecular calmodulin–M13 interaction has been remodeled so that endogenous calmodulin and calmodulin cannot bind. Thus, it is likely that in the near future, genetically encoded Ca2+ sensors will be designed to display fewer unwanted intracellular interactions.

A different approach is to avoid using the ubiquitous calmodulin as a Ca2+-sensing domain and to make use of the skeletal and cardiac Ca2+-binding protein troponin-C. To transfer Ca2+ binding into fluorescent changes, genetically encoded Ca2+ probes based on troponin-C use the same approach as calmodulin-based sensors (ie, FRET between CFP and YFP [or their variants]). In addition to minimized intracellular interference, these indicators also display rapid on and off kinetics because the Ca2+-sensing domain (troponin-C) originates from a fast Ca2+ read-out system (ie, the contractile machinery of skeletal and cardiac muscle). Early versions of these Ca2+ probes used troponin-C from chicken skeletal muscle (TN-L15) and human cardiac muscle (TN-hum troponin-C) and displayed favorable Ca2+ affinities of 1.2 and 0.47 μmol/L, respectively. Further molecular evolution yielded molecules with brighter fluorescence, better protein folding (CerTN-L15; Kd=1.2 μmol/L), and reduced Ca2+ affinities (TN-XL; Kd=2.5 μmol/L). Because troponin-C exhibits significant Mg2+ binding, its Mg2+ affinity was substantially higher than Ca2+ affinity.

Figure 6. Comparison of Ca2+ transients calculated as the cyan fluorescent protein (CFP)/yellow fluorescent protein (YFP) ratio and apparent Förster resonance energy transfer (FRET) efficiency. Aa, Adult cardiac myocytes expressing YC3.6. The cells were electrically paced, and the resulting CFP and YFP fluorescence were compared after CFP excitation and displayed as the CFP/YFP ratio. Ab, The same Ca2+ transients as in Aa but displayed as the apparent FRET ratio calculated as outlined in the text (Equations 2-4). B, Cardiac myocytes expressing YC3.6 were electrically stimulated and analyzed. Two representative myocytes displaying substantially different signal transients when calculating simple CFP/YFP fluorescence ratios are shown (dotted lines). Using the same raw data, we calculated the apparent FRET efficiency; the resulting signal transients (solid lines) confirm the expected similar behavior of the 2 myocytes. These results were representative for 20 cells from 4 different preparations. Ca, High-speed recordings of YC3.6 expressing cardiomyocytes. Ca, Image series of a Ca2+ upstroke at an imaging recording frequency of 100 Hz. Each pair of consecutive images was used to calculate the apparent FRET ratio. B, Example trace of consecutive Ca2+ transients of a single cell recorded at 100 Hz. The multiplicity of infection for all YC3.6 transductions was 4×104 pfu/1500 cells. This figure presents primary experimental results. D, Indicates the FRET efficiency; and f0, fraction of the FRET sensor fluorescent entities.
reduced in more advanced versions of these Ca²⁺ probes, such as TN-XL. The latter probe displays the fastest kinetics observed for genetically encoded Ca²⁺ sensors when expressed in Drosophila motor neurons.⁶⁴ When tethered to individual Ca²⁺ channels, this probe sensed local Ca²⁺ at the intracellular mouth of the channel.⁶⁵

Figure 7. In vivo Ca²⁺ imaging using genetically encoded sensors. A–H, Transgenic zebrafish embryos expressing GCaMP1 underwent Ca²⁺ imaging experiments. In animals expressing the dco/c226 mutant (dco−/−), disarrayed cardiac impulse propagation was evident. A, Brightfield micrographs of 48 hours post fertilization (hpf) wild-type (WT) and dco−/− embryos. The black arrow indicates pericardial edema. B, Schematic diagram illustrating the modular structure of Cx46. A connexon consists of 4 transmembrane domains (M1–M4). The M3 domain is the major pore-lining domain. The red asterisk marks the site of the s226 mutation. C–E, Optical maps of Ca²⁺ transient propagation during a single cardiac cycle, represented as isochromes every 20 ms in 60 hpf embryos carrying the Tg(cmlc2:gsCAlmp)s878 reporter and (C) WT at the dco locus (WT), (D) dco/c226 homozygous mutant (dco), or (E) WT with dco/c226 mutant cardiomyocytes carrying the Tg(cmlc2:dsRed)s879 reporter in the ventricle (dco→WT). The black arrow shows the normal direction of ventricular cardiac conduction. The numbers indicate the temporal sequence of Ca²⁺ transients. Cardiac conduction across the WT ventricle proceeds uniformly from the atrioventricular (AV) canal to the outflow tract (OT), whereas cardiac conduction travels aberrantly through the dco/c226 mutant ventricle. Transplanted dco/c226 mutant cardiomyocytes disrupt the organized AV to OT conduction in the WT ventricle. The white asterisk marks the location of transplanted dco mutant cardiomyocytes in the WT ventricle, as illustrated in G and H. F and H, Fluorescent micrographs of the dco→WT mosaic heart imaged in E. F, Green fluorescence shows the WT host ventricle and (G) donor-derived dco/c226 mutant cardiomyocytes expressing Tg(cmlc2:dsRed)s879. H, An overlay of green and red fluorescence images reveals that the donor-derived dco/c226 mutant cardiomyocytes are located at the outer curvature of the ventricle near the AV canal, where cardiac conduction is disrupted in the WT host ventricle (E). I–L, Ca²⁺ fluorescence in the heart from a transgenic mouse line expressing GCaMP2. I, Time sequence of fluorescence images during a single cardiac cycle in an anesthetized and ventilated mouse. Images at peak atrial and ventricular systole are shown from a series obtained at 33 Hz. The heart is illuminated obliquely from the left side of the camera, highlighting the right atrium (RA) and ventricle (RV); each series was separately scaled to 0 to 255 (left atrium [LA]). K, Left, Atrial fluorescence transients recorded in the experiment shown in J; right, transients recorded from the same heart after direct application of 10 μmol/L isoproterenol (Iso). The heart is illuminated obliquely from the left side of the camera, highlighting the right atrium (RA) and ventricle (RV); each series was separately scaled to 0 to 225 (left atrium [LA]). L, In vivo images from a phenotypically normal heart after transgene induction by dox removal. The images show diastole and peak atrial (A) and ventricular (V) systole. Continuous ventricular fluorescence from the full experiment is shown below. The color scale in I applies throughout. Reproduced from Tallini et al ¹⁸ and Chi et al, ⁹⁷ copyright 2006 and 2010, respectively.
Application of GECI in the Heart

During the past 12 years, numerous investigations of GECI have been reported in the heart or in isolated cardiac myocytes. The numerous indicators used in these studies are summarized in Figure 2. Many of these studies confirm the advantageous properties of GECI when compared with small molecule dyes; the results are listed and discussed below.

Cardiac Conduction System and Its Role in the Developing Heart

A series of publications used the transgenic zebrafish line Tg(cmlc2:gcAMP)778 with cardiac-targeted expression of GCaMP1 to reveal novel insights into the development of the heart and the conduction system (Figure 7A–7H).17,97,98 In addition to cell-specific expression, these investigators also took advantage of novel imaging approaches, such as in vivo selective plane imaging.99 These studies identified 4 distinct stages in the development of cardiac conduction that could be linked to anatomic changes in the developing heart. When combining Ca2+ imaging with novel genetic approaches, the authors highlighted a specific homeobox transcription factor, tcf2, that seemed to be critical for the development of conductance between the atrium and the ventricle.17 Furthermore, circulation fluid forces were identified as an essential epigenetic factor for embryonic cardiogenesis.100,101

Ca2+ Communication Within the Cardiac Endothelium in the Mammalian Heart

Two mouse lines expressing cardiac GCaMP2 were recently introduced.18,102 Global cardiac expression was achieved by cloning the Ca2+ sensor downstream of a weakened α myosin heavy chain promoter. Additional incorporation of a tet-off system enabled temporary control of expression, which minimized problems such as cardiac abnormalities, which often occur when calmodulin-based sensors are expressed in the heart over an extended time period (see Biocompatibility section of this article). The results displaying cardiac conduction are illustrated in Figure 7I–7L. Another series of elegant experiments from the same group used targeted GCaMP2 expression under the control of the connexin-40 promoter to limit the expression of the sensor to arterial and arteriolar endothelial cells, Purkinje cells of the cardiac conductance system, and atrial myocytes.102 The authors concluded that in arterioles, in vivo responses to acetylcholine consisted of 2 phases: (1) a rapidly conducted vasodilation initiated by a local rise in endothelial Ca2+ but independent of endothelial cell Ca2+ signaling at remote sites and (2) a slower complementary dilation associated with a Ca2+ wave that propagates along the endothelium.

Functional Engraftment of Transplanted Cardiac Myocytes

To evaluate the functional and electric integration of transplanted cells in infarcted hearts, GECI can be used to read out the coupling to the native myocardium in vivo, as performed with GCaMP2 in transplanted embryonic cardiac myocytes.103 In this particular study, embryonic cardiomyocytes were isolated from the abovementioned transgenic GCaMP2 mouse line.18 This functional assay can be used as a proof-of-principle test but is not appropriate for routine observations. However, recently, a similar approach used human embryonic stem cell–derived cardiomyocytes expressing GCaMP3 to show their electric integration in a Guinea pig chronic infarct model.104,105

Organelle-Specific Ca2+ Signaling in Cardiac Myocytes

On the basis of number of publications, we found that organelle-specific expression of genetically encoded Ca2+ sensors (mitochondria, sarcoplasmic reticulum, and nucleus) is the most popular application of GECI in cardiomyocytes.106–115 Although mitochondrial Ca2+ measurements were possible through the use of small molecule dyes116 before the introduction of GECI, spatial discrimination from cytosolic Ca2+ or the removal of cytosolic Ca2+ indicators (eg, using a patch pipette) is cumbersome.117 Therefore, numerous studies have used mitochondria-targeted Ca2+ sensors106,107,110,111,113–115 to analyze beat-to-beat oscillations in neonatal cardiomyocytes,107 to investigate metabolic problems,110,111,113 or to measure spatiotemporal Ca2+ gradients during excitation–contraction couplings.115

In the sarcoplasmic reticulum, the application of GECI to follow luminal Ca2+ transients has been surprisingly limited.106,112 However, these indicators have greatly contributed to the elucidation of the function of the A-kinase–anchoring protein in Ca2+ reuptake into the sarcoplasmic reticulum.

Ca2+-Signals in the Nanodomains of Cardiac Myocytes

Although a classical application of GECI, the question of Ca2+ concentrations in nanodomains near Ca2+ transport proteins or in the fuzzy space has only recently been addressed after the introduction of a novel series of CPFPs, the GCaMP6s.76 Based on the fastest version of these indicators (GCaMP6f), C-terminal fusion proteins of tradin1 and junctin, GCaMP6f-T and GCaMP6f-J, respectively, allowed Ca2+ measurements exclusively in the dyadic cleft.118 The recorded signals were termed Ca2+ nanosparks because their calculated volume was ≤50× smaller than that of Ca2+ sparks. Although the focus of that article118 was methodology, the peak intensity (FIF) of the nanotransients was 7.8-fold higher because of global Ca2+ elevation.

In another study, the Ca2+--indicator (GCaMP2) was directly fused to the Ca2+-transporting protein, isoform 4 of the plasma membrane Ca2+/calmodulin-dependent ATPase.119 As a proof of principle for this approach, an isoform-specific inhibitor of plasma membrane Ca2+ ATPase isoform 4 was identified (aurintricarboxylic acid).119

These 2 recent publications show the potential of fusion protein-targeted GECI, which will foster our understanding of the molecular regulation of Ca2+ in the heart.

Pharmaceutical Screening Based on Cardiac Myocytes

In pharmaceutical screening applications, the use of GECI has putative advantages instead of small molecule dyes. These advantages are related to the permanent presence of the functional indicator in the correct cellular compartment, which allows the comprehensive measurement of continuous (chronic) applications of compounds of interest during its course of effect. In addition, the screening procedure can be streamlined because cell staining is not required. Although pharmaceutical
or cardiovascular safety screens based on genetically encoded Ca\textsuperscript{2+} sensors expressed in cardiomyocytes have never been published, the enabling technology is well described.\textsuperscript{120,121} A robust isolation procedure\textsuperscript{122} and greatly improved cell culture\textsuperscript{23} enable the use of adult cardiomyocytes, which is a prerequisite that seems to be compulsory, particularly in consideration of the different gene expression patterns of embryonic, neonatal, and adult cardiac myocytes.\textsuperscript{31,123} The major limitation is the requirement for electrodes in multiwell plates, which can be expensive; current designs are limited to 24-well plates.\textsuperscript{84,121} However, the counterparts of GECI and genetically encoded optical manipulators, such as channel rhodopsins, have been successfully used in the heart.\textsuperscript{124} These molecules could provide an alternative means for the electric stimulation of isolated adult cardiac myocytes without electrodes and could thus enable the use of 384-well plates. The excitation wavelength for the channel-rhodopsin requires Ca\textsuperscript{2+} indicators with a bathochromic shift of the excitation, as was recently introduced.\textsuperscript{125–127}

### Recommendation and Outlook

Given the advantages and limitations discussed above, there are numerous applications for which GECI could prove advantageous, particularly in light of the latest developments of GECI with increased sensitivity.\textsuperscript{76} However, the selection of the appropriate GECI for a particular application remains challenging. In any case, the intrinsic properties of GECIs have to match the Ca\textsuperscript{2+} dynamics in cardiac myocytes or their subcellular localization to obtain the best results. On the basis of the discussion and additional data presented in this review, we have developed potential guidelines for selecting an appropriate sensor based on general sensor characteristics (Figure 8). As mentioned above, there are additional important considerations that have been widely neglected, such as the specific $K_e$ for Ca\textsuperscript{2+}, the dynamic range and kinetics in vivo, the availability or ability of fusion proteins for specific targeting, the potential cross talk with other ions, or the pH value within the cell. Such considerations unfortunately render this decision process more complex. Similar considerations have also been discussed repeatedly for small molecule dyes but remain neglected.

The development of GECI is far from being at its end point. Future research will lead to increased fluorescence yield, enlarged dynamic ranges, and faster sensors. The latter requirement seems to be essential to enable Ca\textsuperscript{2+} upstroke-based analysis methods of fast confocal recordings, as recently described.\textsuperscript{128} Furthermore, biocompatibility must be improved, particularly by reducing cross talk with endogenous signaling pathways. There is also potential for broad expansion of the spectral range of GECI. The optical properties of nearly all sensors reside in a narrow spectral band determined by the incorporated fluorescence protein, namely, CFP, GFP, YFP, and their many successors. Interesting exceptions are the original cameleon2, which used blue fluorescent protein\textsuperscript{13} and a series of novel single-emission GECI,\textsuperscript{125,126} which displayed a bathochromic spectral shift. There is a particular demand for indicators with spectral properties shifted into the red or far-red spectral band. These wavelengths reduce the contribution of autofluorescence and enable better light penetration into the tissue, which is particularly important for in vivo imaging approaches. Furthermore, such indicators would also enable new types of measurements, such as quantitative Ca\textsuperscript{2+} measurements in red blood cells,\textsuperscript{129} and would enable combinations with other genetically encoded biosensors, such as membrane potential sensors\textsuperscript{22,130} or phosphorylation sensors.\textsuperscript{131,132}
Beyond the future developments of GECI, their potential field of application is far from being fully exploited. Therefore, it is the responsibility of researchers in the field of cardiovascular sciences to bridge the gap between molecular biological analysis, using the tools provided by genetically encoded Ca\textsuperscript{2+} sensors and cardiovascular studies involving optical imaging of the heart in vivo and in isolated cardiomyocytes.

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Disclosures

None.

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Lars Kaestner, Anke Scholz, Qinghai Tian, Sandra Ruppenthal, Wiebke Tabellion, Kathrina Wiesen, Hugo A. Katus, Oliver J. Müller, Michael I. Kotlikoff and Peter Lipp

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