

LOW AFFINITY RED FLUORESCENT INDICATORS FOR IMAGING Ca^{2+} IN EXCITABLE AND NON-EXCITABLE CELLS

FIELD

[0001] This invention relates generally to low-affinity, fluorescent Ca^{2+} indicators, which may be targeted to the endoplasmic reticulum, the sarcoplasmic reticulum and/or the mitochondria.

BACKGROUND

[0002] In heart cells, the sarcoplasmic reticulum (SR) is responsible for amplification of Ca^{2+} induced Ca^{2+} release (CICR), which enables voltage dependent Ca^{2+} entry triggering myofilament contraction. As contraction is associated with motion of the SR, ratiometric (as opposed to intensimetric) imaging approaches are necessary to correct for movement artefacts.

[0003] Sub-cellular compartments such as the mitochondria, the endoplasmic reticulum (ER), and the SR, have calcium ion (Ca^{2+}) concentrations ranges spanning from low micromolar to high millimolar. In compartments with high Ca^{2+} concentrations, fluorescent indicators which are optimized for the detection of cytoplasmic Ca^{2+} (typically in the 0.1 to 10 μM range) become saturated and unresponsive to physiologically relevant changes in Ca^{2+} concentration. To address this problem, substantial research effort has gone into developing low affinity Ca^{2+} indicators, including genetically-encoded fluorescent proteins (FP). In contrast to synthetic dye-based indicators, FP-based indicators are delivered to the cell as their corresponding DNA coding sequences and can include additional sequences for expression in specific tissues or targeted to specific subcellular compartments.

[0004] Early examples of low affinity indicators include D1ER and D4cpv, which are based on Ca^{2+} -dependent Förster Resonance Energy Transfer (FRET) between cyan and yellow FPs. FRET-based indicators are inherently ratiometric, providing quantitative measurements that are not subject to imaging artefacts due to the movement of organelles or the cell. Indicators engineered from single FPs tend to be intensimetric and often provide larger signal changes. The first single FP-based low affinity Ca^{2+} indicator targeted to the ER was CatchERTM. More recently, a number of low affinity GCaMP-type Ca^{2+} indicators have been discovered and are

composed of circularly permuted (cp) FP fused to calmodulin (CaM) and a peptide that binds to the Ca^{2+} bound form of CaM. These include the CEPIATM, LAR-GECOTM, and ER-GCaMPTM series. Another low affinity single FP-based Ca^{2+} indicator that is emission ratiometric is GEM-CEPIA1erTM, but it requires excitation with high-energy ultraviolet light (≤ 400 nm), which is often associated with increased phototoxicity and autofluorescence.

[0005] It may be desirable to use indicators that can be excited with longer wavelengths (i.e., more red-shifted or > 400 nm) light as they are often associated with decreased phototoxicity and autofluorescence.

[0006] This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY

[0007] In one aspect, the invention may comprise A method of detecting changes in Ca^{2+} levels in a cell, the method comprising:

(a) obtaining a sample comprising cells engineered to express one or more low affinity Ca^{2+} indicator selected from the group consisting of: LAR-GECO1.5, LAR-GECO2, and LAR-GECO3, LAR-GECO4, LAREX-GECO1, LAREX-GECO2, LAREX-GECO3, and LAREX-GECO4, or a polypeptide having a substantially similar amino acid sequence to any one of the foregoing;

(b) exposing the cells to excitation light; and

(c) detecting changes in ER, SR and/or mitochondria Ca^{2+} levels by visualizing or imaging the cells.

[0008] In another aspect, the invention may comprise a low affinity fluorescent Ca^{2+} polypeptide selected from the group consisting of: LAR-GECO1.5, LAR-GECO2, and LAR-GECO3, LAR-GECO4, LAREX-GECO1, LAREX-GECO2, LAREX-GECO3, and LAREX-GECO4, or a polypeptide having a substantially similar amino acid sequence to any one of the

foregoing. In some embodiments, the polypeptide may have the amino acid sequence of one of SEQ ID NOs. 4, 6, 8, 10, 12, 14, 16 or 18.

[0009] In some embodiments, the polypeptide may comprise a mutation selected from the group consisting of: I54A, I330M, and D327N/I330M/D363N. The polypeptide may have a K_d for Ca^{2+} greater than 20 μM , or preferably about 60 μM .

[0010] In another aspect, the invention may comprise a polynucleotide encoding a low affinity fluorescent Ca^{2+} polypeptide of the present invention, or a substantially similar polynucleotide sequence. In some embodiments, the polynucleotide may comprise a nucleic acid sequence selected from the group consisting of:

- (a) SEQ ID NO. 3, 5, 7, 9, 11, 13, 15, or 17;
- (b) a nucleic acid sequence having at least 90% sequence identity to one of SEQ ID NO. 3, 5, 7, 9, 11, 13, 15, or 17, and encoding a fluorescent Ca^{2+} indicator, having a K_d for Ca^{2+} greater than 20 μM , or optionally about 60 μM , but excluding SEQ ID NO. 1;
- (c) a nucleic acid sequence encoding a fluorescent Ca^{2+} indicator comprising an amino acid sequence of SEQ ID No. 4, 6, 8, 10, 12, 14, 16 or 18; and
- (d) a nucleic acid sequence encoding a fluorescent Ca^{2+} greater than 20 μM , or optionally about 60 μM , and having at least 90% sequence identity to an amino acid sequence of SEQ ID No. 4, 6, 8, 10, 12, 14, 16 or 18, but excluding SEQ ID NO. 2.

[0011] In some embodiments, the polynucleotide comprises a mutation which encodes an amino acid mutation selected from the group consisting of: I54A, I330M, and D327N/I330M/D363N.

[0012] In other aspects, the invention may comprise a vector or a host cell comprising a polynucleotide sequence of the present invention. In some embodiments, the host cell is a cardiomyocyte.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Referring to the drawings, several aspects of the present invention are illustrated by way of example, and not by way of limitation, in detail in the figures, wherein:

[0014] Figure 1 shows schematic strategies for engineering of low affinity Ca^{2+} indicators.

[0015] Figure 2 shows an amino acid sequence alignment for LAR-GECO1, LAR-GECO1.5, LAR-GECO2, LAR-GECO3, and LAR-GECO4.

[0016] Figure 3 shows an amino acid sequence alignment of LAREX-GECO1, LAREX-GECO2, LAREX-GECO3, and LAREX-GECO4.

[0017] Figure 4 shows intensimetric and ratiometric red Ca^{2+} indicators with a wide range of affinities to Ca^{2+} .

[0018] Figure 5 shows in vitro characterizations of LAR-GECOs. (A, D, G and J) Excitation and emission spectra of LAR-GECO1.5 (A), LAR-GECO2 (D), LAR-GECO3 (G), and LAR-GECO4 (J). (B, E, H and K) Absorbance and emission spectra of LAR-GECO1.5 (B), LAR-GECO2 (E), LAR-GECO3 (H), and LAR-GECO4 (K) in both the Ca^{2+} -free state (dotted line) and the Ca^{2+} -bound state (solid line). (C, F, I, L) Fluorescence intensity of LAR-GECO1.5 (C), LAR-GECO2 (F), LAR-GECO3 (I), and LAR-GECO4 (L) as a function of pH.

[0019] Figure 6 shows in vitro characterizations of LAREX-GECOs. (A, D, G, and J) Excitation and emission spectra of LAREX-GECO1 (A), LAREX-GECO2 (D), LAREX-GECO3 (G) and LAREX-GECO4 (J). (B, E, H, and K) Absorbance and emission spectra of LAREX-GECO1 (B) and LAREX-GECO2 (E), LAREX-GECO3 (H), and LAREX-GECO4 (K) in both the Ca^{2+} -free state (dotted line) and the Ca^{2+} -bound state (solid line). (C, F, I, and L) Fluorescence intensity of LAREX-GECO1 (C), LAREX-GECO2 (F), LAREX-GECO3 (I), and LAREX-GECO4 (L) as a function of pH.

[0020] Figure 7 shows that ER-LAREX-GECO4 (n=7) expressed in HeLa Cells can detect SR Ca^{2+} dynamics following histamine stimulations. $\Delta R/R = (R_{\text{init}} - R) / R_{\text{init}} * 100\%$, where R is

the ratio of emission intensity with excitation at 470 nm to emission intensity with excitation at 595 nm, R_{init} is the initial ratio. 20 μ M histamine application is indicated by the gray bar.

[0021] Figure 8 shows a comparison of low affinity Ca^{2+} indicators in the immortalized mouse atrial HL1 cell line. **(A)** Expression of ER-LAR-GECO3 and ER-LAR-GECO4 in HL1 cells. Live cell images are pseudocoloured red on the left, fixed images of ER-LAR-GECO3 and ER-LAR-GECO4 taken by confocal microscopy are shown on the right in greyscale. Observation of ER/SR Ca^{2+} change in response to caffeine stimulation with ER-LAR-GECO3 **(B)**, ER-LAR-GECO4 **(C)** and ER-LAREX-GECO4 **(D-F)**. Ratiometric stimulation of ER-LAREX-GECO4 was achieved with laser illumination at 488 nm **(D)** and 594 nm **(E)**. **(F)** $\Delta R/R_0$ trace was calculated from **(D)** and **(E)**. **(G)** Comparison of performance for ER-LAR-GECO4 (n = 21), ER-LAR-GECO3 (n = 14), ER-LAREX-GECO2 (n = 8), ER-LAREX-GECO1 (n = 7), ER-LAREX-GECO4 (n = 14), ER-LAREX-GECO3 (n = 8), R-CEPIAer (n = 15). For intensimetric indicators, $\Delta F_{SR} = (F_{init} - F_{caf}) / F_{init} * 100\%$, where F is the fluorescence intensity, F_{init} is the initial intensity, and F_{caf} is the intensity immediately following caffeine addition. For ratiometric indicators, $\Delta R_{SR} = (R_{init} - R_{caf}) / R_{init} * 100\%$, where R is the ratio of emission intensity with excitation at 488 nm to emission intensity with excitation at 594 nm, R_{init} is the initial ratio and R_{caf} is the ratio immediately following caffeine addition.

[0022] Figure 9 shows a comparative performance of ER-LAR-GECOs and ER-LAREX-GECOs in human embryonic stem cell derived cardiomyocytes (hES-CMs) relative to a G-CEPIAer benchmark. hES-CMs were co-transfected with ER-LAR-GECOs, ER-LAREX-GECOs or R-CEPIAer, together with G-CEPIAer. Representative emission signals (vertical pairs of panels) from each reporter pair, in single cells, were obtained simultaneously through a Dual View system. Some cells (i.e., the R-CEPIA-G-CEPIA pair), underwent spontaneous oscillations that coincided with contraction and relaxation. Inset displays time-lapse of hES-CMs expressing G-CEPIAer and R-CEPIAer from 0.8 to 1 min. Caffeine addition is shown by the grey bar.

[0023] Figure 10 shows observing cytosolic and SR Ca^{2+} in iPSC derived cardiomyocytes (iPSC-CM). Cells were co-transfected with G-GECO and ER-LAREX-GECO3 to visualize their spontaneous activity and response to caffeine stimulation (grey bar). G-GECO was illuminated by a laser at 488 nm. ER-LAREX-GECO3 was excited by laser illumination at 488 nm and 594

nm. Two types of responses were observed. **(A and B)** In one group of cells a large initial response to caffeine application was observed, but coupling of spontaneous SR depletion and subsequent Ca^{2+} oscillations were not apparent. **(C)** A second group of cells demonstrate coupling of spontaneous SR emptying with changes in cytoplasmic Ca^{2+} detectable in iPS-CMs prior to (blue arrow), and following, caffeine application. Intensities in individual emission channels is shown on the left and the processed ratiometric data set is shown on the right.

[0024] Figure 11 shows observation of cytosolic Ca^{2+} and ER/SR Ca^{2+} change in response to caffeine stimulation by G-GECO1 with (A) ER-LAR-GECO4 and (B) ER-LAR-GECO3 in HL 1 cells. The thick grey trace represents the averaged response of the G-GECO1 cytoplasmic emission with the associated left y axis scale bar (F/F_0 (Cyto)). The thick black trace represents the averaged response of the SR targeted red shifted indicator, with the right y axis scale bar (F/F_0 (SR)). Individual cell responses are shown in thin grey traces. Caffeine application is indicated by the grey bar.

[0025] Figure 12 shows characterization of ER/SR store in human embryonic stem cell derived cardiomyocytes (hES-CM) by ratiometric measurement using ER-LAREX-GECO3. ER-LAREX-GECO3 was excited by with laser illumination at 488 nm and 594 nm. Caffeine depletes the SR store and Ca^{2+} refills slowly with small Ca^{2+} oscillations that are more clearly observed in the ratiometric (black, iii) trace.

[0026] Figure 13 shows demonstration of single wavelength excitation for observing cytoplasmic Ca^{2+} (G-GECO) and ER/SR Ca^{2+} (ER-LAREX-GECO4) in hES-CM. (A) Excitation of G-GECO and ER-LAREX-GECO4 by blue light is shown. Image of ER-LAREX-GECO4 was further taken by confocal microscopy (right greyscale image) showing the typically unorganised arrangement of the SR in these cell types. (B) Time-lapse of hES-CM responding to caffeine treatment. A 480 nm LED was used to excite both G-GECO and ER-LAREX-GECO4. Signal is simultaneously observed by a dual view system at 10 Hz. Caffeine application is demonstrated by the grey bar.

[0027] Figure 14 shows that the ER/SR Ca^{2+} dynamics in iPSC-CMs can be monitored by ratiometric measurement using ER-LAREX-GECO3 under electrical pacing. (A) Time-lapse of iPSC-CMs expressing ER-LAREX-GECO3 in response to electrical pacing at 0.5Hz and 1.0Hz.

ER-LAREX-GECO3 was excited by LED illumination at 470 nm (i) and 595 nm (ii) for acquiring ratiometric imaging. Signal is observed at 25Hz. (B) F/F_0 was calculated from (A), where F is the fluorescence intensity, F_0 is the resting intensity. R is ratio of F/F_0 (ex 470) / F/F_0 (ex 595) shown in black line (iii). Cells were paced by C-Pace EP (ION OPTIX), voltage condition was set at 15V. The grey boxes indicate the time slot that cells were stimulated with the electrode.

[0028] Figure 15 shows immunofluorescence characterization of stem cell derived cardiomyocytes showing the typical rudimentary circular rather than elongated appearance with immunofluorescence staining of sarcomeric components Troponin-T, and alpha-actinin to confirm cardiomyocyte identity. Within these mixed populations, a small proportion of cells are binucleate with some areas of apparently more organized SERCA staining potentially indicative of an evolving cellular maturity in contrast to Figure 13A. Scale bar, 10 micron. Zoomed panels taken from the main image as indicated.

[0029] Figure 16 shows that expression of mt-LAREX-GECO4 in HeLa cells for ratiometric observing calcium dynamic in mitochondria. (A) Subcellular distribution of mt-LAREX-GECO4. Scale bar indicates 10 μm . (B) A huge Ca^{2+} influx in mitochondria was detected in response to 20 μM histamine. mt-LAREX-GECO4 was excited by LED illumination at 470nm and 595nm. Histamine application is indicated by the gray bar.

DETAILED DESCRIPTION

[0030] The detailed description set forth below and the appended drawings are intended as a description of various embodiments of the present invention and is not intended to represent the only embodiments contemplated by the inventor. The detailed description includes specific details for the purpose of providing a comprehensive understanding of the present invention, however, the claimed invention may not be limited by such specific details.

[0031] Examples of the present invention may provide a toolbox of novel red shifted low affinity Ca^{2+} indicators with a useful dynamic range and Ca^{2+} affinity, as well as polynucleotide sequences encoding such indicators. The Ca^{2+} indicators described herein may be selectively expressed and retained in organelles by fusing organelle-specific targeting sequences to the

indicator molecule. Thus, these indicators can be targeted to high concentration Ca^{2+} stores, for example the SR in cultured cardiomyocytes or the mitochondria, and can be imaged alone or in combination with other indicators, enabling direct visualization of an important aspect of disease relevant biology that to date has typically been studied indirectly.

[0032] In some embodiments, the invention may comprise intensimetric red fluorescent low affinity Ca^{2+} indicators derived from LAR-GECO1 ($K_d = 24 \mu\text{M}$) [SEQ ID NO. 2]. To engineer intensimetric red fluorescent low affinity Ca^{2+} indicators, the dissociation constant of LAR-GECO1 was tuned by altering the interaction between calmodulin (CaM) and a short peptide from chicken gizzard myosin light chain kinase (RS20) and by modifying CaM's affinity for Ca^{2+} . With reference to Figure 1, different strategies were pursued in the synthesis of the red fluorescent low affinity Ca^{2+} indicators as described herein.

[0033] A first strategy involved modification of the indicator topology by fusing the N-terminus of RS20 to the C-terminus of CaM, while reinstating the original non-circularly permuted (ncp) FP termini (i.e. a “camgaroo” topology, so called because the smaller companion is carried the pouch of the indicator). The structure of circularly permuted (cp) R-GECO1 (PDB ID 4I2Y), which is used here to represent the LAR-GECO1 variant, is shown on the left side of Figure 1A. The red fluorescent protein domain is linked to the Ca^{2+} binding domain comprised of calmodulin (orange cylinders) and RS20 (grey cylinder). Ca^{2+} is represented as purple spheres. On the right side of Figure 1A is a representation of the non-circularly permuted (ncp) LAR-GECO1.5 [SEQ ID NO. 4]. Blue line represents the cp linker or the CaM-RS20 linker for the ncp topology.

[0034] Alternative strategies involved site-specific mutagenesis, for example, alanine-scanning of the CaM-RS20 interface to weaken this interaction, incorporation of mutations at positions outside of the Ca^{2+} binding sites, or incorporation of mutations in the Ca^{2+} -binding sites of CaM. Examples of the second, third and fourth strategies are shown schematically in Figure 1B. On the left, the LAR-GECO1.5 structure is shown with the targeted residues from strategies 2 to 4 highlighted. On the right, primary sequence of RS20 and CaM with targeted residues highlighted as in LAR-GECO1.5 structure.

[0035] Based on strategy 1 shown in Figure 1, LAR-GECO1 was converted to the ncp topology resulting in LAR-GECO1.5, in which CaM and RS20 are connected by a Gly-Gly-Gly-Gly-Ser-

Val-Asp linker, and wherein the FP terminuses are restored. Without restriction to a theory, there may be two possible advantages for this altered topology. The first is that the linker between RS20 and CaM could be engineered to potentially alter the effective K_d . The second is that, due to the direct linkage between RS20 and CaM, they could be less available for interaction with endogenous proteins in the ER or SR.

[0036] LAR-GECO1.5 has a similar Ca^{2+} affinity as LAR-GECO1, while maintaining a fluorescent response to Ca^{2+} of 7.4-fold, indicating that ncp topology does not adversely affect this function. Figure 4 shows normalized fluorescence intensity as a function of free Ca^{2+} concentration in buffer (10 mM MOPS, 100 mM KCl, pH 7.2). LAR-GECO1.5's trace is essentially identical to LAR-GECO1. Consequently, the ncp topology was retained for the design and engineering of low affinity Ca^{2+} indicators.

[0037] Using the LAR-GECO1.5 as a template, strategies 2, 3, and 4 (and/or combinations thereof) were explored to create genetic variants and express them in the context of *Escherichia coli* colonies. Fluorescence imaging of colonies was used to identify brightly fluorescent clones, which were picked, cultured, and tested for their Ca^{2+} response and affinity. This procedure led to the identification of three exemplary indicators with a decreased affinity to Ca^{2+} .

[0038] Among the alanine-scanning constructs, an indicator (designated LAR-GECO2 [SEQ ID NO. 6]) with the Ile54Ala mutation exhibits a Ca^{2+} K_d of 60 μM and a 5.7-fold increase in fluorescence upon binding to Ca^{2+} was discovered. Based on an Ile330Met mutation, an indicator (designated LAR-GECO3 [SEQ ID NO. 8]) with a K_d of 110 μM and a fluorescent response to Ca^{2+} of 7.5-fold was discovered. Based on mutations of Asp327Asn, Ile330Met, and Asp363Asn, an indicator (designated LAR-GECO4 [SEQ ID NO. 10]) with a K_d of 540 μM and a fluorescent response to Ca^{2+} of 13-fold was discovered.

[0039] The low affinities of LAR-GECO2, 3 and 4 are related to the identified mutations, therefore, some embodiments of the invention may include variant polypeptides which vary in other domains, but retain the same or similar functionality and retain one or more of these mutations.

[0040] Genetic fusing of all the indicators to ER targeting and retention sequences and expression in HeLa cells exhibited the expected pattern of ER-localization and bright red fluorescence. Figure 7 shows that ER-LAREX-GECO4 expressed in HeLa cells can detect ER/SR Ca^{2+} dynamics following histamine stimulations.

Table 1: In vitro characterisation of the LAR-GECO series

Protein	Ca^{2+}	λ_{abs} (nm) (ϵ) (mM^{-1} $\cdot\text{cm}^{-1}$)	λ_{em} (nm) (ϕ)	Brightness ¹ ($\text{mM}^{-1}\cdot$ cm^{-1})	pK_a	Intensity change $\pm \text{Ca}^{2+}$	K_d for Ca^{2+} (μM), (Hill coefficient)
LAR-GECO1	-	574 (5.3)	598 (0.13)	0.69	8.6	10x	24 (1.3)
	+	561 (35.8)	589 (0.20)	7.2	5.4/8.8 ²		
LAR-GECO1.5	-	574 (9)	599 (0.19)	1.7	9.3	7.4x	24 (1.1)
	+	561 (47)	587 (0.27)	12	6.0/9.0 ²		
LAR-GECO2	-	574 (5.0)	598 (0.13)	0.65	8.9	5.7x	60 (1.2)
	+	561 (19.7)	589 (0.19)	3.7	6.4/9.0 ²		
LAR-GECO3	-	574 (5.5)	598 (0.11)	0.61	9.4	7.5x	110 (1.1)
	+	561 (23.2)	589 (0.20)	4.6	5.9/8.8 ²		
LAR-GECO4	-	574 (5.3)	598 (0.10)	0.53	9.1	13x	540 (1.2)
	+	561 (35.2)	589 (0.19)	6.7	6.5/8.8 ²		

¹Brightness is defined as the product of ϵ and ϕ .
²In the Ca^{2+} -bound state, all LAR-GECOs show biphasic pH dependence.

[0041] Thus, as summarised in Table 1, LAR-GECO2, -3 and -4 are red fluorescent Ca^{2+} indicators that are intensimetric and have lower affinities than their parental indicator LAR-GECO1.

[0042] In another aspect, the invention comprises ratiometric low affinity red GECOs. In some embodiments, these indicators have ratiometric properties, which can reduce sensitivity to movement, improve quantitative measurement and enable single wavelength excitation with two-colour imaging strategies. Thus, in some embodiments, the present invention comprises at least four new ratiometric low affinity red GECOs with affinities to Ca^{2+} ranging from 146 μM to 1023 μM , described here as LAREX-GECOs.

[0043] These novel new indicators were derived from REX-GECO1, a previously reported excitation ratiometric red Ca^{2+} indicator, which was engineered into the ncp topology. Then the same mutations used to engineer LAR-GECO3 and -4 above were then introduced, to produce new indicators LAREX-GECO1 [SEQ ID NO. 12] and LAREX-GECO2 [SEQ ID NO. 14]. Figure 4, panel B, shows normalized excitation ratio as a function of free Ca^{2+} concentration in buffer (10 mM MOPS, 100 mM KCl, pH 7.2). Excitation ratio = 480 nm / 580 nm excitation fluorescence intensity ratio. K_d is dissociation constant of Ca^{2+} . Relative to REX-GECO1 (K_d of 240 nM), the novel indicators, designated as LAREX-GECO1 and LAREX-GECO2, provide substantially lower Ca^{2+} affinities of 146 μM and 1023 μM , respectively.

[0044] In other embodiments, further LAREX-GECOs derivatives were produced, wherein the CaM portion of REX-GECO1 was replaced with the CaM portion of R-CEPIA1er, a previously reported intensimetric low affinity red Ca^{2+} indicator. The resulting new indicator, designated as LAREX-GECO3 [SEQ ID NO. 16], exhibits a Ca^{2+} K_d of 564 μM and a dynamic range of 23-fold. Converting LAREX-GECO3 protein to the ncp topology resulted in another new indicator, designated as LAREX-GECO4 [SEQ ID NO. 18] with a similar K_d of 593 μM and a dynamic range of 18-fold.

[0045] The characterisation of LAREX-GECOs is summarised in table 2.

Table 2: Summary of Ratiometric Indicators

Protein	Ca ²⁺	λ_{abs} (nm) (ϵ) (mM ⁻¹ cm ⁻¹)	λ_{em} (ϕ)	Brightness ¹ (mM ⁻¹ • cm ⁻¹)	pK _a ³	Ratio change ² ± Ca ²⁺	K _d for Ca ²⁺ (μ M), (Hill coefficient)
LAREX- GECO1	-	578 (39)	605 (0.14)	5.5	6.1	4.5x	146 (0.93)
	+	467 (29)	586 (0.14)	4.0			
LAREX- GECO2	-	578 (36)	605 (0.13)	4.7	5.7	23x	1023 (0.8)
	+	471 (32)	586 (0.20)	6.4			
LAREX- GECO3	-	579 (39)	605 (0.08)	3.1	6.5	23x	564 (1.7)
	+	474 (32)	587 (0.17)	5.4			
LAREX- GECO4	-	578 (33)	605 (0.08)	2.6	6.2	18x	593 (1.6)
	+	471 (31)	587 (0.17)	5.3			

¹Brightness is defined as the product of ϵ and ϕ .
²Defined as the change of the excitation ratio (450 nm / 580 nm).
³pK_a is the pH at which the dynamic range is 50% of maximum.

[0046] Table 3 provides a summary of the calcium affinity of the indicators. Characterization of these indicators is described below.

Table 3. Summary of Ca²⁺ indicators

Name	K _d (μ M)	Topology
LAR-GECO1	24	cp
LAR-GECO1.5	24	ncp
LAR-GECO2	60	ncp
LAR-GECO3	110	ncp
LAR-GECO4	540	ncp
LAREX-GECO1	146	ncp
LAREX-GECO2	1023	ncp
LAREX-GECO3	564	cp
LAREX-GECO4	593	ncp

Observing Ca²⁺ dynamics in heart muscle cells

[0047] In heart muscle cells, called cardiomyocytes, contraction and relaxation requires cyclical release and reuptake of Ca²⁺, which consequently is a critical regulator of contraction. Typically, cytoplasmic concentrations change from a diastolic range (~0.1 μ M free Ca²⁺) to a systolic range one order of magnitude higher (~1 μ M free Ca²⁺). As intracellular Ca²⁺ buffering is significant, ~100 μ M total Ca²⁺ is required to effect this change. Most of the required Ca²⁺ comes from the SR, which comprises only a fraction of the cell volume, and therefore contains Ca²⁺ concentrations much higher than the cytoplasm. As a result, observation of Ca²⁺ dynamics in the SR is difficult due to lack of low affinity Ca²⁺ indicators. For this reason, indirect measurements of cytoplasmic Ca²⁺ in response to caffeine induced SR emptying in the presence or absence of various chemical inhibitors is typically used.

[0048] The low affinity Ca²⁺ dye Fluo-5N ($K_d = 97 \mu$ M) has been used to visualize SR Ca²⁺ changes in isolated permeabilized adult ventricular myocytes but specific SR loading without cytoplasmic contamination may be difficult to achieve and as an intensimetric indicator, it may be susceptible to motion artefact. Stem cell derived cardiomyocytes lack the typical spatial T-tubule/SR architecture seen in ventricular myocytes and erroneous cytoplasmic signals therefore cannot be identified based on positional information.

[0049] In one embodiment, the indicators of the present invention may mitigate these challenges and provide physiological beat-to-beat changes in SR Ca²⁺, which can be directly visualised in a cell culture; and stem cell derived cardiomyocytes.

[0050] *Physiological changes in SR Ca²⁺ visualised in a cell culture*

[0051] A large variety of models are used in cardiovascular research. In one aspect of the present invention a cell culture of stable immortalized cell lines, known as the HL1 cell line, derived from mouse atrial cardiomyocytes is used as a model.

[0052] With reference to Figure 8 (panels A, B and C) and Figure 11, ER-LAR-GECO3 and ER-LAR-GECO4 were evaluated with the simultaneous expression of cytoplasmic G-GECO1 in the HL1 cell line. In response to 10 mM caffeine addition, a rise in the cytosolic Ca²⁺ signal can be accompanied by a decrease in the ER/SR Ca²⁺ signal.

[0053] With reference to Figure 8, panels D, E and F, ratiometric imaging of ER-LAREX-GECO4 was achieved by dividing the emission intensity with excitation at 488 nm with the emission intensity at 594 nm excitation.

[0054] With reference to Figure 8, panel G, a comparison of the intensimetric or ratiometric responses of the various indicators of the present invention upon caffeine stimulation (ΔF_{SR} or ΔR_{SR}) in the HL1 cell line show that ER-LAREX-GECO4 and ER-LAREX-GECO3 have the largest signal changes (-72.9 +/- 15.2% and -76.0 +/- 16.1% change, respectively). The present invention also provides in vitro characterization demonstrating ER-LAREX-GECO4 (dynamic range 18 $\times$, K_d = 593 μ M) and ER-LAREX-GECO3 (dynamic range 23 $\times$, K_d = 564 μ M) having large dynamic ranges and optimal K_d values for detection of cyclical diastolic (~1000 to 1500 μ M) to systolic (~300 to 600 μ M) Ca^{2+} changes in the cardiomyocyte of the SR.

Physiological changes in SR Ca^{2+} visualised in stem cells

[0055] In another aspect, the indicators described herein may provide visualization of changes in SR Ca^{2+} levels, such as in cardiomyocytes derived from human embryonic stem cells (hES) or human induced pluripotent stem cells (hiPSCs). Such stem cells can be a model of inherited heart disease or in vitro drug toxicity and drug screening platforms.

[0056] With reference to Figure 9, a green low affinity indicator G-CEPIAer (reporting a dynamic range 4.7 $\times$, K_d = 672 μ M) was used as an internal standard to minimize the impact of cell phenotype variability and immaturity. The indicators described herein were compared to green low affinity indicator G-CEPIAer, in stem-cell derived cardiomyocytes. The present invention may permit visualization of physiological beat to beat SR emptying in addition to provoked SR Ca^{2+} depletion in response to caffeine application.

[0057] From the intensity traces, the response (ΔF_{SR}) of the red indicators, which could be divided by the paired ΔF_{SR} for G-CEPIAer producing a comparative $R_{red/green}$ ratio in the same cell, (ΔF_{SR} from red channel/ ΔF_{SR} from G-CEPIAer). ER-LAREX-GECO3 ($R_{red/green}$ = 1.03 +/- 0.08) it appears equivalent to the G-CEPIAer. Both ER-LAREX-GECO3 and ER-LAREX-GECO4 ($R_{red/green}$ = 0.71 +/- 0.02) appear to perform better than R-CEPIAer ($R_{red/green}$ = 0.60 +/- 0.06) in this system, which is consistent with results obtained in HL1 cultured cell line and the in

vitro data. Isolated comparisons between cells, for example using the G-CEPIAer traces alone, can reveal significant heterogeneity in individual responses, which could be a weakness of current in vitro stem cell derived cardiomyocyte models.

[0058] Ratiometric LAREX-GECO3 and LAREX-GECO4 indicators may offer advantages in the in vitro systems can be further characterized in stem cell models.

[0059] An advantage of ratiometric, relative to some intensimetric indicators, is that they self-correct for cell movement. This is a particular problem for caffeine stimulation methods, as emptying of the SR can provoke larger movements than the regular oscillatory contraction and relaxation of the cultured cardiomyocyte. This ratiometric imaging provides observation of spontaneous beat-to-beat Ca^{2+} release and reuptake. With reference to Figure 12, following a caffeine application to deplete the SR Ca^{2+} concentrations, oscillations during Ca^{2+} reuptake to SR can be easily detected.

[0060] In another aspect, changes in beat-to-beat Ca^{2+} concentrations in iPSC-CMs under electrical pacing can also be detected by ER-LAREX-GECO3, as shown in Figure 14.

[0061] Since ratiometric indicators have a Ca^{2+} dependent excitation in the blue-green light spectrum, as shown in Figure 6, which appears to capture most of the information of SR emptying and refilling as shown in Figure 12, embodiments of the present invention may include single wavelength two-colour imaging using G-GECO1 and ER-LAREX-GECO4 in stem-cell derived cardiomyocytes, as shown in Figure 13. This avoids the need to switch illumination sources and is therefore a strategy for high frame rate imaging or prolonged observation that can be desirable in some circumstances.

[0062] With reference to Figure 9, since physiological SR Ca^{2+} depletion may not be detected in all cells expressing G-CEPIA, even though they were all visibly contracting, the present invention may permit the ratiometric measurement of SR Ca^{2+} release with cytosolic Ca^{2+} observation using the co-expression of G-GECO and ER-LAREX-GECO3 in iPSC cardiomyocytes, as shown in Figure 10.

[0063] With reference to Figure 10 panel B, although some cells appear to have initial coupling between the initial caffeine provoked SR Ca^{2+} depletion and cytoplasmic Ca^{2+} accumulation, it

may be seen that subsequent oscillations are not linked. However with reference to Figure 10 panel C, other cells from the same stem cell differentiation have shown coupling of spontaneous cytosolic Ca^{2+} transients with Ca^{2+} fluctuation in the adjacent SR, indicative of physiological Ca^{2+} release from the SR store contributing to cytosolic Ca^{2+} before caffeine treatment. Following caffeine application these cells show a correlation between the amplitude of cytoplasmic Ca^{2+} transient recovery and the gradual restoration of SR Ca^{2+} content and durable coupling of cytoplasmic and SR signals during subsequent oscillations.

[0064] It is possible this cell autonomous behavior, which is likely not identifiable using cytoplasmic Ca^{2+} traces alone, reflects the distinct stages of *in vitro* maturity. In support of this, a small proportion of stem-cell derived cardiomyocytes appear to develop a higher order structure to components such as SERCATM, which may be implicated in the excitation and contraction coupling was observed as shown in Figure 13.

Observing Ca^{2+} dynamics in the mitochondria

[0065] It is known that calcium signaling plays an important role in regulating mitochondrial function. Mitochondrial calcium (Ca^{2+}) overload is one of the pro-apoptotic ways to induce the swelling of mitochondria. Thus, real-time monitoring Ca^{2+} dynamics in prediction of cellular states or response to different stimulation would be of interest. However, like ER/SR, mitochondria also contain high concentrations of Ca^{2+} , and therefore there are relatively few variants optimized for use to study calcium signaling in mitochondria. The low affinity indicators of the present invention may provide a solution. Figure 16 shows that expression of mt-LAREX-GECO4 in HeLa cells for ratiometric observing calcium dynamic in mitochondria. (A) Subcellular distribution of mt-LAREX-GECO4. Scale bar indicates 10 μm . (B) A huge Ca^{2+} influx in mitochondria was detected in response to 20 μM histamine. mt- LAREX-GECO4 was excited by LED illumination at 470nm and 595nm. Histamine application is indicated by the gray bar.

Polypeptide and Nucleotide Sequences

[0066] Aspects of the invention include the fluorescent polypeptides described herein, having the amino acid sequences indicated, or a substantially similar amino acid sequence. A substantially

similar amino acid sequence will have at least some level of sequence identity, with the same or similar function. It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying polypeptides, wherein such polypeptides have the same or similar function or activity. Percent identities of 90% or greater (ie. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) may be useful.

[0067] In examples of the present invention, polypeptides will have the same or similar function if they are similarly fluorescent and have a low-affinity for Ca^{2+} , with a K_d of greater than 20 μM , and more preferably greater than about 60 μM . However, it will be understood that the progenitor fluorescent polypeptides LAR-GECO1 and REX-GECO1 are not included as having substantially similar sequences, nor are any nucleic acid sequences which encode for the progenitor fluorescent polypeptides.

[0068] As used herein, "nucleic acid" means a polynucleotide and includes single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases. Nucleic acids may also include fragments and modified nucleotides. Thus, the terms "polynucleotide", "nucleic acid sequence", "nucleotide sequence" or "nucleic acid fragment" are used interchangeably and is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deosycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deosythymidylate, "R" for purines (A or G), "Y" for pyrimidiens (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

[0069] The terms "homology", "homologous", "substantially similar" and "corresponding substantially" are used interchangeably herein. They refer to nucleic acid fragments wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences.

[0070] The invention may also comprise a nucleic acid sequence encoding a polypeptide having an amino acid sequence described herein, or a substantially similar amino acid sequence, as well as substantially similar nucleic acid sequences. Substantially similar nucleic acid sequences may have 90% or greater sequence identity (ie. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%).

[0071] "Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to the nucleic acid bases or amino acid residues in two sequences that are the same when aligned for maximum correspondence over a specified comparison window. Thus, "percentage of sequence identity" refers to the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the results by 100 to yield the percentage of sequence identity. These identities can be determined by those skilled in the art, including the use of any of the programs described herein.

[0072] Sequence alignments and percent identity or similarity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, Wis.). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

[0073] The "Clustal V method of alignment" corresponds to the alignment method labeled Clustal V (described by Higgins and Sharp, CABIOS. 5:151-153 (1989); Higgins, D. G. et al. (1992) Comput. Appl. Biosci. 8:189-191) and found in the MegAlign™ program of the

LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, Wis.). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

[0074] "BLASTN method of alignment" is an algorithm provided by the National Center for Biotechnology Information (NCBI) to compare nucleotide sequences using default parameters.

[0075] Moreover, the skilled artisan recognizes that substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize (under moderately stringent conditions, e.g., 0.5xSSC, 0.1% SDS, 60° C.) with the sequences exemplified herein, or to any portion of the nucleotide sequences disclosed herein and which are functionally equivalent to any of the nucleic acid sequences disclosed herein. Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions.

[0076] The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, or 85%, 90% or 95% sequence identity, up to and including 100% sequence identity (i.e., fully complementary) with each other.

[0077] The term "stringent conditions" or "stringent hybridization conditions" includes reference to conditions under which a probe will selectively hybridize to its target sequence. Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can

be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optionally less than 500 nucleotides in length.

[0078] Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C., and a wash in 1x to 2xSSC (20xSSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.5x to 1xSSC at 55 to 60° C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37° C, and a wash in 0.1xSSC at 60 to 65° C.

[0079] Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth et al., Anal. Biochem. 138:267-284 (1984): $T_m = 81.5^\circ \text{C} + 16.6 (\log M) + 0.41 (\% \text{ GC}) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1° C for each 1% of mismatching; thus, T_m , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with > 90% identity are sought, the T_m can be decreased 10° C. Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4° C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or

wash at 6, 7, 8, 9, or 10° C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20° C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45° C. (aqueous solution) or 32° C. (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). Hybridization and/or wash conditions can be applied for at least 10, 30, 60, 90, 120, or 240 minutes.

EXAMPLES

[0080] Embodiments of the present invention are described with reference to the following Examples. These Examples are provided for the purpose of illustration only.

[0081] Example 1A: Engineering of LAR-GECOs

[0082] LAR-GECO1 in pBAD/His B vectorTM (Life Technologies) was used as the initial template to assemble LAR-GECO1.5 (strategy 1 – Figure 1). The development of LAR-GECO1 is described in Wu et al. *Red fluorescent genetically encoded Ca²⁺ indicators for use in mitochondria and endoplasmic reticulum*, Biochem J. 2014 Nov 15;464(1):13-22, the entire contents of which are incorporated herein by reference, where permitted.

[0083] The N-terminus of RS20 and the C-terminus of CaM in LAR-GECO1 were connected by amino acid sequence (GGGGSVD), while the original ncp FP termini were reinstated by overlap extension polymerase chain reactions (PCR). To explore strategies 2, 3, and 4, which led to the development of LAR-GECO2, 3, and 4, point mutations listed in Table 4 were introduced to LAR-GECO1.5 using Quikchange Lightning Site-Directed Mutagenesis KitTM (Agilent)

following manufacturer's instructions. Oligonucleotides containing specific mutations were designed in the aid of Agilent online mutagenesis primer design program.

Table 4: Summary of mutations introduced to engineer the LAR indicator series

Strategy	Mutation	Comments
Strategy 2 Alanine-scanning through RS20-CaM interface	R41A	
	R42A	
	K43A	
	W44A	
	N45A	
	K46A	
	G48A	
	H49A	
	W51A	
	R52A	
	I54A	Designated as LAR-GECO2
	R56A	
	L57A	
	E314A	
	L321A	
	F322A	
	M375A	
	E387A	
	M412A	
	E417A	
	M448A	
Strategy 3 CaM mutations from O- GECO1 and R-GECO1.2	I330M	Designated as LAR-GECO3
	K397N	Previously reported (<i>Wu et al., 2013</i>)
Strategy 4 Mutations in the EF-hands of CaM	T329D/T365 D/D367N	Previously reported (<i>Sun et al., 2013</i>)
	T365D/D367 N	Previously reported (<i>Sun et al., 2013</i>)

T329D/T331 D/T365D	Previously reported (<i>Sun et al., 2013</i>)
T363D	Previously reported (<i>Sun et al., 2013</i>)
T329D/T331 D	Previously reported (<i>Sun et al., 2013</i>)
D363N/D367 N	Previously reported (<i>Sun et al., 2013</i>)
D327N/I330 M	Previously reported (<i>Sun et al., 2013; Wu et al., 2013</i>)
D327N/I330 M/D363N	Designated as LAR-GECO4
E334A	Previously reported (<i>Sun et al., 2013</i>)
T365D	Previously reported (<i>Sun et al., 2013</i>)

Example 1B: Engineering of LAREX-GECOs

[0084] To engineer LAREX-GECO1 and 2, REX-GECO1 in pBAD/His B vector (Life Technologies) was first turned into the ncp topology by overlap extension PCR as described above. Point mutations from LAR-GECO3 and 4 were then introduced to this ncp version of REX-GECO1 using Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent) as described above to make LAREX-GECO1 and 2 respectively. To construct LAREX-GECO3, the CaM domain of REX-GECO1 was replaced by the CaM domain of R-CEPIA1er via overlap extension PCR. pCMV R-CEPIA1erTM was a gift from Masamitsu IinoTM (Addgene plasmid #58216). LAREX-GECO4 was constructed by changing the topology of LAREX-GECO3 to ncp as described above. The sequence of all the LAR-GECO and LAREX-GECO constructs was verified by sequencing.

[0085] To test the Ca²⁺ affinity of all the LAR-GECO and LAREX-GECO variants, each variant in pBAD/His B vector (Life Technologies) was electroporated into *E. coli* strain DH10BTM (Invitrogen). *E. coli* containing these variants were then cultured on 10 cm LB-agar Petri dishes supplemented with 400 µg/mL ampicillin (Sigma) and 0.02% (wt/vol) L-arabinose (Alfa Aesar) at 37 °C overnight. These Petri dishes were then placed at room temperature for 24 h before imaging. During imaging, an image was captured for each Petri dish by using excitation filter of

542/27 nm (for LAR-GECO variants), or both 438/24nm and 542/27 nm (for LAREX-GECO variants) to illuminate *E. coli* colonies and emission filter of 609/57 nm. A single *E. coli* colony emitting red fluorescence of each variant was then picked and cultured in 4 mL liquid LB with 100 µg/mL ampicillin and 0.02% (wt/vol) L-arabinose at 37 °C overnight. Proteins were then extracted from the liquid LB culture by B-PER™ (Pierce) following manufacturer's instructions. The extracted protein solution of each variant was then subjected to Ca²⁺ titration. In the Ca²⁺ titration, extracted protein solutions were added into Ca²⁺ buffers with different free Ca²⁺ concentrations. Ca²⁺/HEDTA, and Ca²⁺/NTA buffers were prepared by mixing Ca²⁺-saturated and Ca²⁺-free buffers (30 mM MOPS, 100 mM KCl, 10 mM chelating reagent, pH 7.2, either with or without 10 mM Ca²⁺) to achieve the buffer Ca²⁺ concentrations from 0 mM to 1.3 mM. Fluorescence spectra of each variant in different Ca²⁺ concentrations were recorded by using a Safire2™ fluorescence microplate reader (Tecan). These fluorescence intensities were then plotted against Ca²⁺ concentrations and fitted by Hill equation to calculate the dissociation constant to Ca²⁺ of each variant.

[0086] **Example 2: In vitro characterization**

[0087] For detailed characterization of LAR-GECOs, proteins were expressed and purified as described in Wu J, Liu L, Matsuda T, Zhao Y, Rebane A, Drobizhev M, et al. *Improved orange and red Ca²⁺ indicators and photophysical considerations for optogenetic applications*. ACS Chem Neurosci. 2013;4: 963–972 (Wu et al. 2013). Spectral measurements were performed in solutions containing 10 mM EGTA or 10 mM CaNTA, 30 mM MOPS, 100 mM KCl, pH 7.2. For determination of fluorescence quantum yield of LAR-GECOs and LAREX-GECOs, mCherry and LSS-mKate2 were used as standards. Procedures for measurement of fluorescence quantum yield, extinction coefficient, pK_a, K_d for Ca²⁺ have been described in Wu et al. 2013. For Ca²⁺ titration, purified proteins were added into Ca²⁺/HEDTA, and Ca²⁺/NTA buffers, and fluorescence measurements were performed as described above.

[0088] With reference to Figure 5, in vitro characterization of LAR-GECO1.5, LAR-GECO2, LAR-GECO3, and LAR-GECO4 shows that all four ncp Ca²⁺ indicators share substantially identical spectral properties with their progenitor, LAR-GECO1. In addition, these new LAR-GECOs exhibit a similar monophasic dependence on pH in the Ca²⁺ free state. Upon binding to

Ca²⁺, this dependence on pH switches from monophasic to biphasic, which is very similar to LAR-GECO1's pH dependence.

[0089] With reference to Figure 6, the new LAREX-GECOs share very similar spectral properties with their progenitor, REX-GECO1. Furthermore, these LAREX-GECOs display a similar pH dependence profile with REX-GECO1, with the largest Ca²⁺-dependent change in ratio occurring between pH 7 to 9.

[0090] **Example 3: Plasmids for mammalian cell imaging**

[0091] The ER targeted GECO genes were generated using primers containing ER targeting sequence (MLLPVPLLLGLLGAAAD [SEQ ID NO. 19]) and ER retention signal sequence (KDEL). The PCR products were subjected to digestion with the BamHITM and EcoRITM restriction enzymes (Thermo). The digested DNA fragments were ligated with a modified pcDNA3 plasmid that had previously been digested with the same two enzymes. Plasmid were purified with the GeneJET miniprep kitTM (Thermo) and then sequenced to verify the inserted genes.

[0092] **Example 4: Cell culture conditions and transfection**

[0093] To culture the HL1 cell line, flasks were pre-coated with gelatin/fibronectin at 37 °C overnight. Cells were cultured in supplemented Claycomb MediumTM (Claycomb Medium with 10% fetal bovine serum (Sigma Aldrich 12103C (Batch 8A0177)), 1 U/ml penicillin/streptomycin, 0.1 mM norepinephrine and 2 mM L-glutamine) and split 1:3 when they reached confluency. Cells were transfected using transfection reagent, Lipofectamine 2000 (Invitrogen), for 48 hours before acquiring images.

[0094] The OxF2 human embryonic stem cell line was cultured on mouse embryonic fibroblasts (MEF) in ES medium containing DMEM/F12TM (Invitrogen), 20% Knockout Serum ReplacerTM (KSR, Invitrogen), 1 mM glutamine, 1% non-essential amino acids, 125 µM mercaptoethanol, 0.625% penicillin/streptomycin and 4 ng/ml basic Fibroblast Growth Factor (bFGF) (Peprotech). One week before differentiation, ES colonies were manually cut and placed on GeltrexTM (Gibco) coated six-well plates in mTeSR1 mediumTM (Stemcell).

[0095] Human iPSC-derived cardiomyocytes (Human iPSC Cardiomyocytes - Male | ax2505™) were bought from Axol Bioscience. The cells were plated in two wells of 6-well plate and cultured for eight days in Axol's Cardiomyocyte Maintenance Medium™ to 80-90% confluency. Cells then were replated on Fibronectin/Gelatin (0.5% / 0.1%) coated glass bottom dishes, and were transfected using transfection reagent, Lipofectamine 2000 (Invitrogen). Tyrode's buffer was used for final observation.

[0096] HeLa cells were cultured in homemade 35-mm glass-bottom dishes in Dulbecco's modified Eagle medium (Sigma–Aldrich) containing 10% fetal bovine serum (Invitrogen). Cells were transfected with CMV-mito-LAREX-GE04, ER-LAREX-GE03 and ER-LAREX-GE04 using a transfection reagent of Lipofectamine 2000 (Invitrogen).

[0097] **Example 5: Cardiomyocyte differentiation from human pluripotent stem cells**

[0098] This protocol is based on method reported in Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, et al. *Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin signaling under fully defined conditions*. Nat Protoc. 2013;8: 162–175. ES cell colonies were dissociated into single cells using accutase and put into 6-well plates coated with Geltrex at 0.5×10^6 cells per well, in mTeSR1 with added rock inhibitor, Y27632 (10 μ M). On day 3, at 80 - 90% confluence, medium was changed to RPMI/B27 (B27 supplement without insulin – Gibco) containing 12 μ M GSK-3 inhibitor, CHIR 99021Tocris™). After 24 hours, medium was changed to remove CHIR. 48 hours later, half the medium (1 ml) from each well was aspirated and replaced with fresh RPMI/B27 containing a final concentration of 5 μ M wnt inhibitor, IWP 2™ (Tocris). 48 hours later the IWP was removed and after a further 48 hours the medium was changed to RPMI + B27™ with insulin (Gibco). Cultures were maintained in this medium, which was changed twice weekly. Cells then were replated on Fibronectin/Gelatin (0.5% / 0.1%) coated glass bottom dishes, and were transfected using transfection reagent, Lipofectamine 2000 (Invitrogen).

[0099] **Example 6: Immunostaining for characterization of hES derived cardiomyocytes**

[00100] Primary antibodies were mouse monoclonal anti-actinin (Sigma no. A7811) rabbit polyclonal anti-troponin I (abcam, ab47003) and mouse monoclonal anti-SERCA2 ATPase™

(ABR no MA3-910). Secondary antibodies were Fab fragment anti-mouse 488 and anti-rabbit 568TM (Molecular Probes). The procedure was as follows: 4% paraformaldehyde fixation (10 min room temperature), 0.1% Triton x-100 in Tris-buffered saline (TBST) to permeabilize and wash, 2% BSA with 0.001% sodium azide in TBST for blocking (1 hr room temperature), primary antibodies at 1:200 (2 hr room temperature), 3× wash with TBST (5 mins per wash), secondary antibodies 1:1000 (1 hr room temperature), 3× wash with TBST (5 mins per wash), dry the coverslip and mount in VectorshieldTM (Vector Laboratories). Fluorescence imaging was done with a Leica SP5 confocal microscope using a 63× oil lens with 488 nm and 543 nm excitation.

[00101] **Example 7: Live cell imaging conditions**

[00102] For non-ratiometric imaging, an inverted microscope (IX81TM, Olympus) equipped with a 60× objective lens (NA 1.42TM, Olympus) and a multiwavelength LED light source (OptoLEDTM, CARIN) was used. Blue (470 nm) and green (550 nm) excitation were used to illuminate G-GECO or G-CEPIA and LAR-GECOs, respectively. The GFP filter set (DS/FF02-485/20-25, T495lpxr dichroic mirror, and ET525/50 emission filter) was used to observe G-GECO signal in HL1 cells. The RFP filter set (DS/FF01-560/25-25, T565lpxr dichroic mirror, and ET620/60 emission filter) was used to observe signal of LAR-GECO3 and LAR-GECO4 in HL1 cells. A quad-band filter set including a quad-band bandpass filter (DS/FF01-387/485/559/649-25, Semrock), dichroic quad-edge beamsplitter (DS/FF410/504/582/669-Di01-25x36TM, Semrock) and a quad-band bandpass emission filter (DS/FF01-440/521/607/700-25TM, Semrock) was used to simultaneously observe G-CEPIA and LAR-GECOs or G-GECO and LAR-GECOs in ES-CMs. Fluorescence signals were recorded through Dual-View system (DC2TM, Photometrics) with green (520/30 nm) and red (630/50 nm) channels to EM-CCD cameras (ImagEMTM, Hamamatsu) controlled by software (CellRTM, Olympus).

[00103] For ratiometric imaging of HL1 cells, ES-CMs and iPS-CMs by LAREX-GECOs, an inverted confocal microscope ZEISS LSM710TM, equipped with 63× 1.40 NA oil objective and multi-argon ion laser was used. In HL1 cells, images of red fluorescence and far red signals of LAREX-GECOs were detected at 560-710 nm, and 630-720 nm wavelength range,

respectively, using 488 nm excitation and 594 nm excitation. For simultaneous ratiometric ER and cytoplasmic Ca²⁺ transients in iPS-CMs, green, red and far red signals were detected at 492-540 nm, 630-728 nm, and 630-728 nm wavelength range, respectively, using 488 nm excitation and 594 nm excitation.

[00104] For ratiometric imaging in HeLa cells (Figures 7 and 16) and iPSC-CMs (Figure 14), An inverted microscope (D1, Zeiss) equipped with a 63× objective lens (NA 1.4, Zeiss) and a multiwavelength LED light source (pE-4000, CoolLED) was used. Blue (470 nm) and orange (595 nm) excitation were used to illuminate LAREX-GECOs for ratiometric excitation. The RFP filter set (T590lpxr dichroic mirror, and ET 590lp emission filter) was used to imaging of LAREXs. Fluorescence signals were recorded using a CMOS camera (ORCA-Flash4.0LT, HAMAMATSU) controlled by a software (HC Image).

[00105] Example 8: Construction of CMV-mito-LAREX-GECO4 vector

[00106] LAREX-GECO4 were subcloned from pcDNA3-LAREX-GECO4 (without ER targeting and retention sequence) as follow: PCR primers with a 5' BamHI linker (MT-BamHI-LAREX_GECO4-F) and a 3' HindIII linker (MT-HindIII-LAREX-GECO4-R) were used to amplify LAREX-GECO4 that do not containing ER targeting (MLLPVPLLLGLLGAAAD [SEQ ID NO. 19]) and retention sequences (KDEL) from pcDNA3-LAREX-GECO4 plasmid and ligated with BamHI, HindIII-digested CMV-mito-LAR-GECO1.2 (Addgene #61245) to replace LAR-GECO1.2 fragment. A start codon (ATG) were added to replace ER targeting sequences and a stop codon (TAA) were added in place of retention sequences.

[00107] Oligonucleotides used in the cloning steps are, MT-BamHI-LAREX_GECO4-F:5'- GATCGGATCCAACCATGGTGAGCAAGGGCGAGGAGGAT-3' [SEQ ID NO. 20] and MT-HindIII-LAREX_GECO4-R:5'-GATCAAGCTTTTACTTGTACAGCTCGTCCATGCC-3' [SEQ ID NO. 21].

Sequence Listing

[00108] The Sequence Listing associated with this application is filed in electronic format via e-PCT and hereby incorporated by reference into the specification in its entirety. The name of

the text file containing the Sequence Listing is 55326-272-Mar1-2019.txt. The size of the text file is 48 KB and the text file was created on March 1, 2019.

Interpretation

[00109] The description of the present invention has been presented for purposes of illustration and description, but it is not intended to be exhaustive or limited to the invention in the form disclosed. Many modifications and variations will be apparent to those of ordinary skill in the art without departing from the scope and spirit of the invention. Embodiments were chosen and described in order to best explain the principles of the invention and the practical application, and to enable others of ordinary skill in the art to understand the invention for various embodiments with various modifications as are suited to the particular use contemplated. To the extent that the following description is of a specific embodiment or a particular use of the invention, it is intended to be illustrative only, and not limiting of the claimed invention.

[00110] The corresponding structures, materials, acts, and equivalents of all means or steps plus function elements in the claims appended to this specification are intended to include any structure, material, or act for performing the function in combination with other claimed elements as specifically claimed.

[00111] References in the specification to "one embodiment", "an embodiment", etc., indicate that the embodiment described may include a particular aspect, feature, structure, or characteristic, but not every embodiment necessarily includes that aspect, feature, structure, or characteristic. Moreover, such phrases may, but do not necessarily, refer to the same embodiment referred to in other portions of the specification. Further, when a particular aspect, feature, structure, or characteristic is described in connection with an embodiment, it is within the knowledge of one skilled in the art to combine, affect or connect such aspect, feature, structure, or characteristic with other embodiments, whether or not such connection or combination is explicitly described. In other words, any element or feature may be combined with any other element or feature in different embodiments, unless there is an obvious or inherent incompatibility between the two, or it is specifically excluded.

[00112] It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for the use of exclusive terminology, such as "solely," "only," and the like, in connection with the recitation of claim elements or use of a "negative" limitation. The terms "preferably," "preferred," "prefer," "optionally," "may," and similar terms are used to indicate that an item, condition or step being referred to is an optional (not required) feature of the invention.

[00113] The singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise. The term "and/or" means any one of the items, any combination of the items, or all of the items with which this term is associated.

[00114] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges recited herein also encompass any and all possible sub-ranges and combinations of sub-ranges thereof, as well as the individual values making up the range, particularly integer values. A recited range (e.g., weight percents or carbon groups) includes each specific value, integer, decimal, or identity within the range. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, or tenths. As a non-limiting example, any range discussed herein can be readily broken down into a lower third, middle third and upper third, etc.

[00115] As will also be understood by one skilled in the art, all ranges described herein, and all language such as "up to", "at least", "greater than", "less than", "more than", "or more", and the like, include the number(s) recited and such terms refer to ranges that can be subsequently broken down into sub-ranges as discussed above.

CLAIMS

1. A method of detecting changes in Ca^{2+} levels in a cell, the method comprising:
 - a. obtaining a sample comprising cells engineered to express one or more low affinity Ca^{2+} indicator selected from the group consisting of: SEQ ID Nos 12, 14, 16 and 18;
 - b. exposing the cells to excitation light; and
 - c. detecting changes in endoplasmic reticulum (ER), sarcoplasmic reticulum (SR) and/or mitochondria Ca^{2+} levels by visualizing or imaging the cells.
2. The method in claim 1, wherein the sample comprises a cell culture or stem cells.
3. The method of claim 2 wherein the sample comprises a cell culture of stable immortalized cell line.
4. The method of claim 3 wherein the cell culture comprises a HL1 cell line.
5. The method in claim 1, wherein the indicator is ratiometric.
6. The method in claim 1, wherein the indicator is intensimetric.
7. The method in any one of claims 1-6, wherein the indicator is used in combination with another fluorescent indicator.
8. The method of claim 7 which uses a single wavelength two-colour imaging method.
9. The method of claim 7 wherein the other fluorescent indicator is a cytoplasmic calcium indicator.
10. The method of any one of claims 1-9, wherein the indicator is targeted to an organelle with an organelle-specific targeting sequence.
11. A low affinity fluorescent Ca^{2+} polypeptide selected from the group consisting of: SEQ ID Nos 12, 14, 16 and 18.
12. The polypeptide of claim 11 further comprising an organelle-specific targeting sequence.
13. The polypeptide of claim 12 comprising the targeting sequence of SEQ ID NO. 19.
14. The polypeptide of claim 11 having a K_d for Ca^{2+} greater than 60 μM .

15. A polynucleotide encoding a polypeptide of one of claims 11 -16.
16. The polynucleotide of claim 17 comprising a nucleic acid sequence selected from the group consisting of
SEQ ID NO. 11, 13, 15, or 17
 - a. .
17. The polynucleotide of claim 15 or 16, further comprising a sequence which encodes an organelle-specific targeting sequence.
18. The polynucleotide of claim 17 wherein the organelle-specific targeting sequence encodes SEQ ID NO. 19.
19. A vector comprising the polynucleotide of one of claims 15-18.
20. A host cell comprising the polynucleotide sequence of one of claims 15-18, or a vector of claim 19.
21. The host cell of claim 20 which is a cardiomyocyte.
22. The host cell of claim 20 which co-expresses another fluorescent calcium indicator.