Measuring calcium signaling using genetically targetable fluorescent indicators

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Genetically encoded Ca^{2+} indicators allow researchers to quantitatively measure Ca^{2+} dynamics in a variety of experimental systems. This protocol summarizes the indicators that are available, and highlights those that are most appropriate for a number of experimental conditions, such as measuring Ca^{2+} in specific organelles and localizations in mammalian tissue-culture cells. The protocol itself focuses on the use of a cameleon, which is a fluorescence resonance-energy transfer (FRET)-based indicator comprising two fluorescent proteins and two Ca^{2+}-responsive elements (a variant of calmodulin (CaM) and a CaM-binding peptide). This protocol details how to set up and conduct a Ca^{2+}-imaging experiment, accomplish offline data processing (such as background correction) and convert the observed FRET ratio changes to Ca^{2+} concentrations. Additionally, we highlight some of the challenges in observing organellar Ca^{2+} and the alternative strategies researchers can employ for effectively calibrating the genetically encoded Ca^{2+} indicators in these locations. Setting up and conducting an initial calibration of the microscope system is estimated to take ~1 week, assuming that all the component parts are readily available. Cell culture and transfection is estimated to take ~3 d (from the time of plating cells on imaging dishes). An experiment and calibration will probably take a few hours. Finally, the offline data workup can take ~1 d depending on the extent of analysis.

INTRODUCTION

These days, researchers who aim to quantitatively monitor Ca^{2+} dynamics in living cells have a plethora of indicators from which to choose. Many of the available sensors have some redeeming qualities, as well as potential shortcomings, making it difficult to determine which indicator is best for a given application. This overview is meant to help the researcher decide how to choose the most appropriate genetically encoded Ca^{2+} sensor, as well as how to use it for quantitative Ca^{2+} imaging.

After many years, contributions from numerous researchers and multiple rounds of design, small-molecule Ca^{2+} indicators (such as fura-2, indo-1, fluo-3 and Oregon green- 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)) still exhibit greater dynamic range (percent change in ratio or intensity) than the best genetically encoded sensors. Genetically encoded Ca^{2+} indicators are generally defined as optical sensors that are produced by translation of a nucleic-acid sequence, and comprise at least one light-emitting protein and Ca^{2+}-responsive element, such that Ca^{2+} binding changes their optical properties. The primary advantages of small-molecule indicators over genetically encoded ones are increased sensitivity, rapid response kinetics (although a number of genetically encoded sensors now exhibit these) and lack of need for gene transfer. The disadvantages of small-molecule indicators include the following: the localization of dyes cannot be tightly controlled, so they cannot be targeted to different organelles; the dyes cannot be targeted to a subset of cells within an intact tissue or organism; loading via membrane-permanent esters is often difficult or impossible in nonvertebrate organisms; and, finally, the dyes can slowly leak out of a cell during a long-time-course experiment (hours to days). For these reasons, the genetically encoded Ca^{2+} sensors are preferred for many applications, including measuring Ca^{2+} dynamics (i) in biochemically defined subcellular locations, (ii) in a micro-domain or nano-domain in the immediate vicinity of a given protein, (iii) in cell-specific or tissue-specific expression in transgenic or virally transduced organisms, (iv) in conjunction with other transfected genes so that only the co-transfected subpopulation is monitored, (v) in nonvertebrate but genetically tractable organisms, (vi) in extended time-lapse experiments where dyes might slowly leak out of a cell and (vii) in experiments where the concentration of the indicator is controlled by an inducible promoter.

Genetically encoded Ca^{2+} indicators can be categorized into the following three classes: (i) bioluminescent reporters based on photoproteins, such as aequorin[1,2]; (ii) constructs, such as cameleons, in which Ca^{2+}-responsive elements alter the efficiency of fluorescence resonance-energy transfer (FRET) between two fluorescent proteins[3–11]; and (iii) probes (camgaroos[12,13], G-CaMP[14,15] and pericams[16]) in which Ca^{2+}-responsive elements, including calmodulin (CaM), are inserted into a single fluorescent protein to modulate the protonation state of its chromophore. Aequorin-based probes generate light by chemical reactions of modest turnover rate, so their signal is generally too weak for single-cell Ca^{2+} imaging. These probes will therefore not be considered further in this protocol. **Table 1** summarizes the most widely used fluorescent sensors in each class, as well as the Ca^{2+}-responsive elements, apparent affinities (K_d') for Ca^{2+} and relevant references.

To date, these genetically encoded Ca^{2+} indicators have been targeted to various organelles, including the nucleus[3], endoplasmic reticulum (ER)[3,8,11], mitochondria[10,17,18], Golgi[13] and plasma membrane[7,9,10,19] of individual cells. In addition, indicators have been expressed in a number of transgenic organisms, including plants[20], nematodes[21], flies[9,22–24], zebrafish[25] and mice[26,27].

When choosing the best genetically encoded Ca^{2+} indicator, it is important to remember that 'one size does not fit all' and, therefore, the researcher should evaluate a number of factors. First and foremost, researchers should consider the equipment available. Ratiometric indicators are more quantitative and easily interpreted,

TABLE 1 | Summary of the properties of commonly used genetically encoded Ca^{2+} indicators.

Name	Version	Ca^{2+}-responsive elements	K_d' for Ca^{2+}	Strategy for Ca^{2+} sensing	Specific comments	References
Cameleon	YC2.1	CaM, M13p	0.8, 2 μM	FRET between ECFP and EYFP	Obsolete; replaced by D3 or D3cpv (for greater dynamic range)	3
	YC3.3	CaM E104Q, M13p	1.5 μM	FRET between ECFP and citrine	Obsolete; replaced by D3 or D3cpv (for greater dynamic range)	3,13
	YC4.3	CaM E31Q, M13p	0.8, 700 μM	FRET between ECFP and citrine		3,13
	YC6.1	cpCaM, CKKp	0.11 μM	FRET between ECFP and EYFP		6
	YC3.6	CaM E104Q, M13p	0.25 μM	FRET between ECFP and cp-Venus		19
	Split YC7.3er	CaM E31D, M13p	130 μM	FRET between ECFP and citrine	Intermolecular FRET between ECFP-CaM and M13-YFP	11
	D1	mCaM, mM13p	0.8, 60 μM	FRET between ECFP and citrine	Does not bind wt CaM, fast kinetics	8
	D2cpv[a]	mCaM, m-smMLCKp	0.03, 3 μM	FRET between ECFP and cp-Venus	Does not bind wt CaM	10
	D3cpv[a]	mCaM, m-smMLCKp	0.6 μM	FRET between ECFP and cp-Venus	Does not bind wt CaM	10
	D4cpv[a]	mCaM, m-smMLCKp	64 μM	FRET between ECFP and cp-Venus	Reduced affinity for wt CaM	10
TroponinC	Tn-L15	csm troponinC	29 μM	ECFP and citrine	Mg^{2+} sensitive	7
biosensor	TN-XL	m-csm troponinC	2.5 μM	ECFP and cp-citrine	Mg^{2+} insensitive, fast kinetics	9
Camgaroo		CaM	7 μM	CaM inserted into cpYFP or cp-citrine	If a single fluorescent protein indicator must be used, it would be preferable to use G-CaMP1.6 or pericam	12,13
G-CaMP		CaM, M13p	0.235 μM	CaM and M13p inserted into cpGFP	Dim, pH sensitive, slow maturation	14
	1.6 E140K	mCaM, M13p	0.16 μM	CaM E140K and M13p inserted into cpGFP	40× as bright as G-CaMP; non-ratiometric but fast kinetics	15
Pericam		cpCaM, M13p	0.2 μM, 0.7 μM, 1.7 μM[b]	CaM inserted into cpEYFP	Can be ratiometric	16

[a]Variants of D2, D3 and D4 that contain citrine instead of cp-Venus are also available (details can be found in ref. 10). [b]Different pericam versions have different Ca^{2+} affinities. CKK, CaM kinase kinase; cp, circularly permuted (p indicates peptide); csm, chicken skeletal muscle; m, mutant (M13 is the peptide from skeletal muscle myosin light-chain kinase); smMLCK, smooth-muscle myosin light-chain kinase; p, peptide; wt, wild type.

and less subject to artifacts and pH interference; however, these advantages generally require a microscope with motorized filter wheels or two emission channels so that ratio images can be rapidly collected. Second, it is sensible to pick the indicator with the greatest dynamic range so that small Ca^{2+} rises will be easily detectable over the noise. As originally shown by Miyawaki and co-workers[19], and later corroborated by two other groups[9,10], sensors that incorporate a circularly permuted (cp) version of the acceptor fluorescent protein (citrine or Venus) generally exhibit greater ratiometric sensitivity and expanded dynamic ranges. However, we observed that sensors with cp-Venus did not express well in the ER (A.E.P. and R.Y.T., unpublished observations); therefore, it is possible that cp variants might not always express well in every location in a cell. A third important consideration is to choose a sensor with a K_d' that is appropriate for the anticipated Ca^{2+} concentration in the region of interest (ROI). Fourth, the original cameleons suffered from interference by endogenous CaM, leading to loss of Ca^{2+} sensitivity or even inversion of the response. Such interference has now been greatly reduced or eliminated by engineering complementary mutations into the CaM and its binding peptide[8,10]. Other CaM-based indicators, such as G-CaMPs and pericams, might also benefit from similar mutations. Fifth and finally, for some applications, such as measurements of rapid neuronal Ca^{2+} dynamics, the most important criterion might be to choose a sensor with the fastest response kinetics. **Table 2** summarizes the preferred genetically encoded Ca^{2+} indicators for a number of experimental applications, such as localizing the sensor to different subcellular domains.

The protocol outlined below summarizes how to use a cameleon FRET-based indicator to monitor intracellular Ca^{2+} in mammalian tissue-culture cells. Note that a similar protocol should apply for all the genetically encoded indicators, and the specifics for using single fluorescent protein Ca^{2+} indicators have been detailed by others[15–17]. The current protocol takes the researcher through the steps of cell transfection, experimental set-up, running an actual experiment, *in situ* calibration of the sensor and offline data processing.

TABLE 2 | Recommended sensors for different applications.

Experimental requirement	Indicator of choice	Reasons sensor was chosen over others
Cytoplasm	D3cpv, TN-XL, YC3.6	K_d', dynamic range
ER	D1ER	K_d', fast response kinetics
Mitochondria	4mtD2cpv, 4mtD3cpv	K_d', dynamic range, improved targeting
Plasma membrane	LynD3cpv, TN-XL	Demonstrated to work in PM of neurons and therefore impervious to binding by wt CaM
Fast processes	TN-XL, D1, G-CaMP	Rapid response kinetics[a]

[a]It should be noted that the response kinetics of D2, D3 and D4 have not yet been measured.

MATERIALS

REAGENTS

- Mammalian expression plasmid containing the genetically encoded indicator
- HEPES-buffered Hanks balanced salt solution (HHBSS; 10× HBSS stock available from Invitrogen/Gibco)
- Ca^{2+}-free HHBSS (10× Ca^{2+}/Mg^{2+}-free HBSS stock available from Invitrogen/Gibco)
- Ionomycin, free acid (Calbiochem, cat. no. 407950)
- $CaCl_2$ 1 M stock solution
- EGTA 1 M stock solution, pH 7.4
- Ca^{2+}-perturbing agents
- FuGene 6 (Roche Diagnostics)
- OPTI-MEM (Invitrogen/Gibco)

EQUIPMENT

- Axiovert 200M microscope (Zeiss); equivalent microscope systems from other manufacturers (e.g., Olympus or Nikon) might be available
- Xenon arc lamp (XBO75) and power supply (both from Zeiss)
- 10-position excitation filter wheel with Smart Shutter, 10-position emission filter wheel, Lambda 10-2 controller or Lambda 10-3 controller (all from Sutter Instruments)
- Photometrics CoolSNAP CCD camera or Cascade 512B CCD camera (Roper Scientific Inc.); comparable cameras are available from Hamamatsu
- Filter sets (e.g., CFPx 425/20, YFPx 495/10, CFPm 480/40, YFPm 525/20, CFP and FRET dichroic 450, and YFP dichroic 515) and neutral density filters (all from Chroma Technology Corp.)
- MetaFluor (Universal Imaging), TillVision (Till Photonics), Slidebook (SciTech), ImageJ with the Ratio Plus plugin (National Institutes of Health (NIH) freeware; see http://rsb.info.nih.gov/ij/) or equivalent software
- 3.5 cm dishes (MatTek Corporation or Corning)
- Industrial-strength hole punch (Roper Whitney)
- Sylgard 182 (Dow Corning Corporation)
- Glass coverslips (Fisher Scientific)
- Maxiprep kit (Qiagen or Sigma)
- Excel (Microsoft) or equivalent data-analysis program
- Perfusion system (e.g., AutoMate Scientific, Bioscience Tools or Braintree Scientific; optional)

REAGENT SETUP

Ionomycin, free acid Prepare a 1-mM stock solution in 100% DMSO. Aliquot and store at – 20 °C.

HHBSS 20 mM HEPES, 1× HBSS, 2 g l^{-1} D-glucose (pH 7.4). HBSS is diluted from a 10× stock (Invitrogen/Gibco).

Ca^{2+}-free HHBSS 20 mM HEPES, 1× Ca^{2+}-free/Mg^{2+}-free HBSS, 2 g l^{-1} D-glucose, 490 µl of 1 M $MgCl_2$, 450 µl of 1 M $MgSO_4$ (pH 7.4) to yield a 1-l solution. Ca^{2+}-free/Mg^{2+}-free HBSS is diluted from a 10× stock (Invitrogen/Gibco).

EGTA 1 M stock solution EGTA is insoluble at low pH; therefore, two equivalents of base (NaOH or KOH) must be added for it to go into solution.

Plasmid DNA Prepare a maxiprep of the plasmid containing the genetically encoded indicator.

Autofluorescence Different cells might have different degrees of autofluorescence; therefore, the autofluorescence of the cell lines to be used in the experiment must be determined. To assess this, simply place untransfected cells in an imaging dish and determine the I_{DD}, I_{AA} and I_{DA}, where I_{DA} is the intensity of fluorescence upon donor excitation in the acceptor-emission channel and the other variables follow the same nomenclature (i.e., the direct cyan fluorescent protein (CFP), direct yellow fluorescent protein (YFP) and FRET intensities). This will help to provide an indication of the signal intensity needed to be above this background level in later experiments.

EQUIPMENT SETUP

Inverted fluorescent microscope with accessories for rapid ratio imaging We use a Zeiss Axiovert 200M microscope outfitted with a motorized dichroic turret, and 10-position excitation and emission filter wheels, controlled by a Lambda 10-2 or Lambda 10-3 controller. Equivalent microscope systems from other manufacturers might be available. Alternatives to a motorized dichroic turret (a feature of the Zeiss Axiovert 200M) include the following: (i) use of a dual-pass dichroic mirror, which would selectively allow two (or more) wavelengths to pass; and (ii) beamsplitters, which allow the simultaneous collection of two (dual-view; Optical Insights) or four (quad view; Optical Insights) emission wavelengths. The microscope should be equipped with a high-sensitivity CCD camera, such as CoolSNAP or Cascade 512B. A xenon arc lamp (XBO) is preferable to a mercury arc lamp (HBO), because the light intensity is more uniform over the visible spectrum. **Figure 1** shows the overall layout of our experimental set-up.

Excitation filters (x), dichroic mirrors and emission filters (m) For sensors based on FRET between CFP and a variant of YFP (e.g., enhanced yellow fluorescent protein (EYFP), citrine, Venus, cp-citrine or cp-Venus), recommended filter sets include CFPx 425/20, YFPx 495/10, CFPm 480/40, YFPm 525/20, CFP and FRET dichroic 450, and YFP dichroic 515 (ref. 28). Newer sputter-coated (ET) filter sets that have increased transmission (93–97%) are preferable because they result in better signal to noise and the filters can be manufactured with a smaller bandwidth to minimize potential cross-talk between fluorescence channels. Such filters are available from Chroma Technology Corporation as well as Semrock.

Software package to interface with microscope, filter changer and camera While not absolutely required, it is preferable to use a software package that allows online ratioing, so that the progress of the experiment can be monitored in real time. MetaFluor, TillVision and Slidebook fulfill all of these criteria, because the entire experiment can be run from the computer, and online ratio images as well as time-course graphs of ratios can be plotted in real time.

Imaging dishes Imaging dishes should have a glass coverslip bottom. The thickness of the coverslip should be matched with the appropriate objective. Dishes (3.5 cm) can be purchased or can be more economically prepared by the

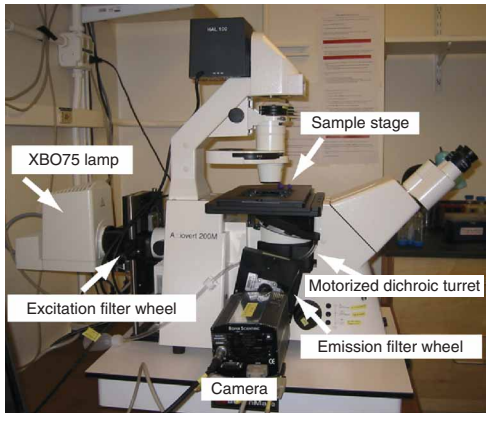

Figure 1 | Layout of the experimental set-up.

investigator by punching a hole in a standard 3.5 cm cell-culture dish with an industrial punch, affixing a glass coverslip with a non-toxic bondable material (such as Sylgard 182), and sterilizing the dishes before use with ethanol and UV light.

Measure crosstalk or bleedthrough between the donor (CFP) and acceptor (YFP variant) fluorescence channels This is accomplished by transfecting a plasmid containing only CFP and measuring I_{DA}/I_{DD} and I_{AA}/I_{DD}. Likewise, transfect cells with a plasmid containing only YFP and measure I_{DA}/I_{AA} and I_{DD}/I_{AA}. The crosstalk can be removed offline upon completion of an experiment through the process of linear spectral unmixing[29,30]. See Step 15 for instructions on how to do this. Methods for quantifying FRET of the calcium indicators, such as external calibration, acceptor photobleaching and donor lifetime, have been discussed elsewhere[31].

Optimize parameters for signal strength and illumination levels In setting up an experiment, it is important to strike a balance between signal strength (fluorescence intensity) and illumination light levels. The user must adjust the intensity of the light and the duration of excitation exposure to obtain satisfactory signal to noise without inducing unnecessary photobleaching. Modulate the illumination light by placing neutral density filters in the light path and altering the excitation exposure time. These parameters must be optimized for each microscope system because the sensitivity depends on the light source, microscope optics and camera sensitivity. We typically use a 1.3 or 1.0 neutral density filter (i.e., 5 or 10% transmitted light), and excitation exposure times between 300 and 1,000 ms. Researchers should also consider the rate of data acquisition. If images will be acquired every few seconds (or even more frequently) it is best to use less illumination light, whereas if images will be collected less frequently it is possible to use more light. We generally try to ensure that our least intense signal is at least a few thousand counts above background for a 16-bit camera or a few hundred counts above background for a 12-bit camera.

Choose the experimental parameters For genetically encoded calcium indicators based on FRET between CFP and a variant of YFP, at least two images should be collected at each time point: a direct CFP image (I_{DD}, CFP excitation CFP emission, the CFP channel) and a FRET image (I_{DA}, CFP excitation YFP emission, the FRET channel). **Figure 2a** depicts the CFP and FRET images for a mitochondrially-targeted cameleon. If the microscope is equipped with a motorized dichroic turret, and time permits, it might also be desirable to collect the direct YFP image (I_{AA}, YFP excitation YFP emission, the YFP channel) to monitor photobleaching. If online ratioing is available, divide the FRET image by the CFP image to yield the FRET ratio (**Fig. 2b**). A higher ratio is indicative of higher Ca^{2+}.

PROCEDURE

Cell transfection

1| Plate cells on imaging dishes. Cells are typically plated so they will reach 40–60% confluency within 24 h. Because cells grow at different rates, individual researchers will have to optimize plating densities for each cell line.

■ **PAUSE POINT** After plating cells, wait at least 24 h.

2| When cells have reached 40–60% confluency, transfect them with the chosen genetically encoded Ca^{2+} indicator. While a number of transfection reagents are available, we typically use FuGene 6 in OPTI-MEM and transfect 1 μg maxiprepped plasmid DNA according to the manufacturer's instructions. Researchers are encouraged to choose the transfection reagent and optimized protocol appropriate for their particular cell line. Alternatively, create a stable cell line using standard protocols, such as antibiotic selection with G418 or fluorescence-activated cell sorting (FACS; e.g., see www.protocol-online.org/prot/Cell_Biology/ and ref. 32). This might be preferable if transfection reagents alter the cellular phenotype.

■ **PAUSE POINT** After transfecting cells, wait ~48 h.

3| Image cells after 36–48 h. While fluorescence is often visible after 24 h, best results are typically obtained after 48 h.

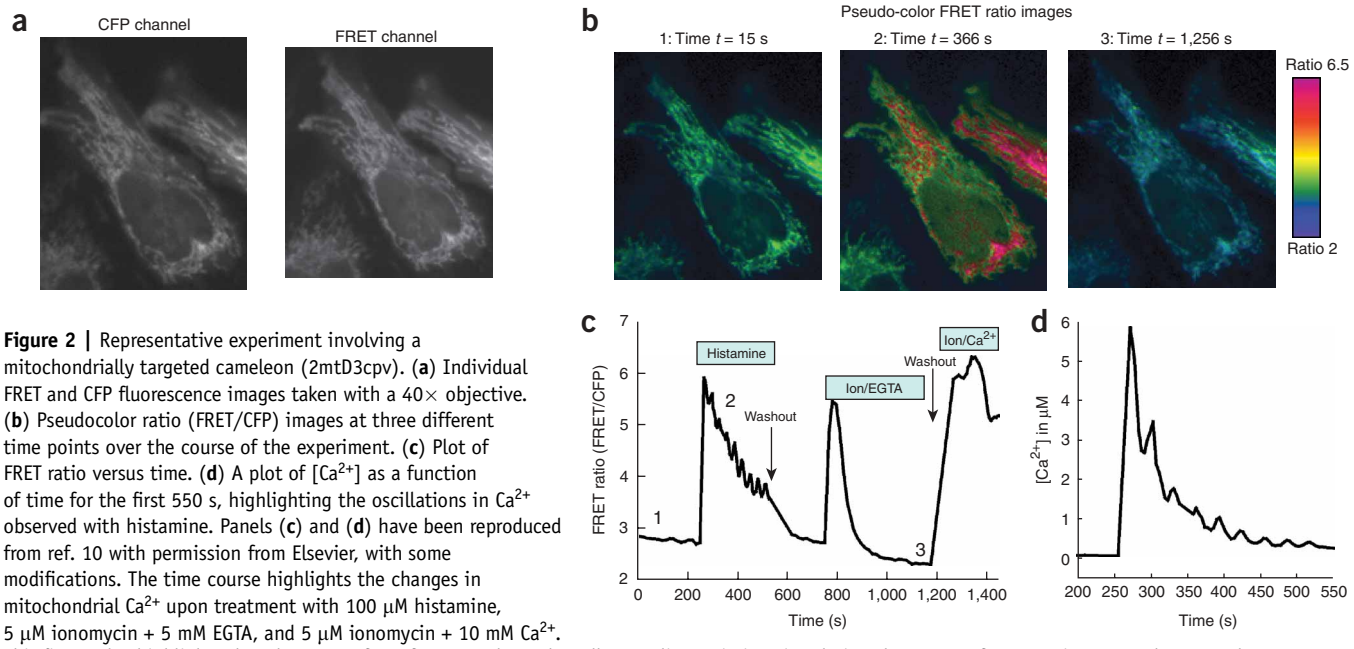

Figure 2 | Representative experiment involving a mitochondrially targeted cameleon (2mtD3cpv). (**a**) Individual FRET and CFP fluorescence images taken with a 40× objective. (**b**) Pseudocolor ratio (FRET/CFP) images at three different time points over the course of the experiment. (**c**) Plot of FRET ratio versus time. (**d**) A plot of $[Ca^{2+}]$ as a function of time for the first 550 s, highlighting the oscillations in Ca^{2+} observed with histamine. Panels (**c**) and (**d**) have been reproduced from ref. 10 with permission from Elsevier, with some modifications. The time course highlights the changes in mitochondrial Ca^{2+} upon treatment with 100 μM histamine, 5 μM ionomycin + 5 mM EGTA, and 5 μM ionomycin + 10 mM Ca^{2+}. This figure also highlights the advantage of a software package that allows online ratio imaging during the course of an experiment, as the researcher can visually monitor changes in Ca^{2+}.

Ca^{2+}-imaging experiment

4| Remove cells from incubator and rinse twice with HHBSS. After rinsing, add 1 ml HHBSS to the 3.5-cm imaging dish.
▲ CRITICAL STEP Wash the cells carefully so that they are not dislodged.

5| Use brightfield illumination to focus on cells, and fluorescence illumination to choose a field of view. It is desirable to have at least two to four transfected cells in the field of view. If using a stable cell line expressing the indicator, all the cells in the field of view will be fluorescent. In choosing which cells to image, it is advisable not to choose the brightest or the dimmest, but rather those with medium fluorescence. As with any imaging protocol, the fluorescence localization should be confirmed and cells should appear healthy. Investigators should also confirm that the desired Ca^{2+} phenotype is observed and is unperturbed by expressing the genetically encoded indicator. If there are concerns over Ca^{2+} buffering or other effects of indicator overexpression, it is often useful to compare the responses of cells of different brightness.

6| To measure the resting Ca^{2+}, start the data acquisition and collect images every ~20 s for a few hundred seconds. It is important to make sure that the starting ratio and microscope focus are stable over this period.

7| Add agents that perturb or activate Ca^{2+} pathways. Prepare 1 ml agent in HHBSS at 2× the final desired cellular concentration (**Table 3**). Add the 1 ml 2× stock and mix rapidly two or three times with the pipetman without touching the microscope stage or imaging dish.
▲ CRITICAL STEP The biggest challenge in working manually is to add the agent without changing the focus. It should be possible to add the agent and mix in a few seconds in between data acquisition. Alternatively, the agents can be added using a perfusion system.

8| Following observation of the Ca^{2+} response, wash the agent out. Pause the data acquisition, remove the imaging media and rinse three times with HHBSS. Resume data acquisition and ensure that the ratios return to the resting starting levels

TABLE 3 | Common Ca^{2+}-perturbing agents.

Agent	Final [cellular]	[Stock solution]	Anticipated effect
Activate GPCR, hydrolysis of PIP_2 to IP_3 and DAG, IP_3-mediated release of ER Ca^{2+} from IP_3R			
ATP	10–100 μM	5 mM in HHBSS, pH 7.4	$[Ca^{2+}]_{ER}$ ↓ transiently $[Ca^{2+}]_{cyt}$ ↑ transiently $[Ca^{2+}]_{mito}$ ↑ transiently Oscillations can be observed in all three locations
UTP	10–100 μM	5 mM in HHBSS, pH 7.4	
Histamine	10–100 μM	5 mM in HHBSS, pH 7.4	
Carbachol	10–100 μM	5 mM in HHBSS, pH 7.4	
Agents to perturb RyR			
Ryanodine	1–10 μM	100 mM in methanol or DMSO	Inhibits Ca^{2+} release from RyR
Caffeine	1–40 mM		Release of ER Ca^{2+} through RyR
RTK activators of PLCγ			
PDGF	1–20 ng ml^{-1}		$[Ca^{2+}]_{ER}$ ↓ transiently $[Ca^{2+}]_{cyt}$ ↑ transiently Oscillations might be observed
Modulators of neuronal NMDAR, AMPAR and VGCC			
Glutamate	100 μM	5–10 mM in HHBSS, pH 7.4	$[Ca^{2+}]_{cyt}$ ↑ transiently $[Ca^{2+}]_{mito}$ ↑ transiently
KCl	50 mM	Isotonic solution in HHBSS or Ringers buffer	
Electrical stimulation			
SERCA inhibitors			
Thapsigargin	0.2–4 μM	2–10 mM in DMSO	Irreversible $[Ca^{2+}]_{ER}$ ↓ Transient $[Ca^{2+}]_{cyt}$ ↑ Transient $[Ca^{2+}]_{mito}$ ↑
BHQ	10–100 μM	5–10 mM in DMSO	Reversible $[Ca^{2+}]_{ER}$ ↓ Transient $[Ca^{2+}]_{cyt}$ ↑ Transient $[Ca^{2+}]_{mito}$ ↑
Agents to calibrate Ca^{2+} indicators			
Ionomycin[a]/Ca^{2+}	5–10 μM/5–20 mM	1 mM in DMSO/1 M in aqueous solution	Saturation of Ca^{2+} indicator (R_{max}), ultimately cell death
Ionomycin[a]/EGTA[b]	5–10 μM/3–5 mM	1 mM in DMSO/1 M in aqueous solution	Chelation of cellular Ca^{2+} (R_{min})

[a]Bromo-A23187 is preferred to ionomycin in some cell lines. [b]The Ca^{2+}-specific chelator BAPTA can be used in place of EGTA. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; BHQ, 1,4-dihydroxy-2, 5-di-tert-butylbenzene; DAG, diacylglycerol; GPCR, G-protein coupled receptor; IP_3, inositol (1,4,5) trisphosphate; IP_3R, inositol (1,4,5) trisphosphate receptor; NMDAR, *N*-methyl D-aspartate receptor; PIP_2, phosphoinositide (4,5) bisphosphate; PLCγ, phospholipase Cγ; RTK, receptor tyrosine kinase; RyR, ryanodine receptor; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; VGCC, voltage-gated Ca^{2+} channel.

(i.e., before perturbation). **Figure 2c** depicts a typical time course highlighting the ratio changes over time upon addition of different Ca^{2+}-perturbing agents.

9| To calibrate the indicator, measure the minimum ratio under Ca^{2+}-free conditions (R_{min}) and the maximum ratio under conditions of saturating Ca^{2+} (R_{max}) at the end of each experiment. A calibration is required in order to convert the observed FRET ratios to calcium concentrations. Because the calibration can often take around 15–20 min, we typically slow the data acquisition (e.g., to 20–30 s) to avoid unnecessary photobleaching over the course of the calibration. To determine the R_{min}, remove the HHBSS and add 1 ml Ca^{2+}-free HHBSS. Add 1 ml of a 2× solution containing EGTA and ionomycin in Ca^{2+}-free HBSS, where the final cellular concentration of ionomycin is 5 μM and the final EGTA concentration is 3 mM. Addition of ionomycin and EGTA will cause an initial increase in cytoplasmic Ca^{2+} as it is released from the ER store. In order to accurately determine the R_{min}, wait until there is no further change in the ratio before moving on to the R_{max}; it might take >10 min for the ratio to stop changing.

▲ **CRITICAL STEP** It is important to use Ca^{2+}-free HHBSS rather than adding extra EGTA to chelate the 1.3 mM Ca^{2+} in standard HHBSS, because EGTA releases 2 H^+ when it binds Ca^{2+}, and therefore addition of EGTA to HHBSS will cause the pH to drop.

▲ **CRITICAL STEP** To ensure that the R_{min} value obtained with ionomycin and EGTA represents an accurate measurement, treat cells with BAPTA-AM, which is a Ca^{2+}-specific chelator that acts much faster than EGTA, in EGTA-containing HHBSS. This is particularly important for high-affinity indicators that might require a lengthy incubation with EGTA to reach the R_{min}.

10| To determine the R_{max}, pause the data acquisition and remove the media containing ionomycin and EGTA. Wash three times with HHBSS and then leave 1 ml HHBSS in the dish. Resume data acquisition. The FRET ratio will increase as Ca^{2+} (from the HBSS) enters the cell. To saturate the Ca^{2+}, add 1 ml of a 2× stock solution containing ionomycin and $CaCl_2$, where the final cellular concentration of ionomycin is 5 μM and the final Ca^{2+} concentration is 10 mM. The concentration of Ca^{2+} and ionomycin might need to be optimized so that probe saturation is reached before the cells begin to die.

▲ **CRITICAL STEP** The treatment with ionomycin and high Ca^{2+} is likely to lead to cell death, and must be done at the end of the experiment. Cell death is usually obvious and involves blebbing, a change in cell size and, for organellar (ER and mitochondria) localized probes, a punctuate appearance of the fluorescence.

▲ **CRITICAL STEP** Ionomycin and high Ca^{2+} can induce changes in organelle and cytoplasmic pH; this is not a significant problem with the cameleons, but is a larger problem with the circular-permuted single fluorescent protein indicators (camgaroos, pericams and G-CaMP). To accommodate this, an alternative approach to obtain the R_{max} is to permeabilize the cells with digitonin and add defined concentrations of Ca^{2+} to the extracellular medium. This strategy was used effectively by Fillipin *et al.*[17], and a variation for determining the R_{max} for ER-targeted indicators is presented in **Box 1**.

■ **PAUSE POINT** After conducting the experiment and calibration, the offline data workup can be done at any time.

Offline data workup

11| Select ROIs. Each experiment should generate a FRET and CFP (and potentially YFP) image at each time point. The software program should allow the researcher to choose ROIs on individual cells as well as for the background. It is preferable to choose an untransfected cell as the background to allow the cellular autofluorescence to be adequately subtracted. However, if this is not possible, a region in the field of view that does not contain any cells (i.e., a bare area of the coverslip) can be used as a substitute. Most programs will average the fluorescence intensity within this region. An alternative is to sum all the photons collected within the ROI. If the fluorescence intensity is summed rather than averaged, it is imperative that the background ROI is the same size as the cellular ROI. Summing the intensity might be preferable when monitoring plasma membrane-targeted or mitochondrially-targeted indicators. The software program should allow data (i.e., the averaged or summed fluorescence intensity for each channel and each ROI) to be exported in an ASCII or delimited format for import into a data-processing program, such as Excel.

12| Background correction and plotting time courses of ROI. It is often desirable to revisit a given experiment and choose an ROI based on the observed cellular responses, such as identifying regions of spatial heterogeneity or highlighting cell-to-cell variability. The experiment can be reanalyzed using acquisition software, such as MetaFluor, or can be processed independently using ImageJ with the Ratio Plus plugin. The goal is to present the background-corrected ratio data according to the following equation:

$$\mathrm{RATIO} = (\mathrm{FRET}_{\mathrm{Int}} - \mathrm{FRET}_{\mathrm{BKGND}})/(\mathrm{CFP}_{\mathrm{Int}} - \mathrm{CFP}_{\mathrm{BKGND}}).$$

Here, $FRET_{Int}$ and CFP_{Int} represent the intensity of the FRET and CFP channels in the ROI, and $FRET_{BKGND}$ and CFP_{BKGND} represent the intensity of FRET and CFP in the background ROI. As depicted in **Figure 2**, the background-corrected ratio can be represented as an image or as a time course (ratio versus time). To create a background-corrected ratio image, a program such as MetaFluor or ImageJ must be used; to simply calculate the background-corrected ratio of a ROI as a function of time, a data-processing program (such as Excel) is sufficient.

BOX 1 | CALIBRATION OF CAMELEON SENSORS WITHIN ORGANELLES.

Determining an accurate R_{max} value in subcellular organelles poses a challenge, because high concentrations of Ca^{2+} are toxic to the cell and the researcher must ensure that the localized probe is saturated before the cell starts to die. Underestimating the R_{max} will lead to an overestimate of the Ca^{2+} concentration. One potential control that can help determine whether a localized probe is saturated is to compare the total dynamic range of the localized probe to a cytoplasmically localized probe in the same cell type and on the same microscope system. However, it should be noted that some localized probes have been shown to have reduced dynamic ranges[17].

Calibration of R_{max} for an indicator targeted to the ER

For HeLa and MCF7 cell lines, ionomycin and high Ca^{2+} were insufficient for saturating the probe in the ER before cell death. However, this might vary with cell type, so researchers are encouraged to try different protocols. An alternative strategy for determining the R_{max} in the ER is to permeabilize the plasma membrane with digitonin and to flood the cell with extra Ca^{2+}, allowing the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) to pump Ca^{2+} into the ER. The specific protocol is as follows.

1. Treat cells with 25 μM digitonin and wait ~5 min for the plasma membrane to permeabilize. During this time there should be essentially no change to ER Ca^{2+} levels.

▲ CRITICAL STEP If ER Ca^{2+} levels begin to rise during this time, the plasma membrane likely permeabilized earlier than expected and the rise is due to the fact that there is an excess of Ca^{2+} in HHBSS (1.3 mM versus 100 nM Ca^{2+} in the cytoplasm); therefore, even in the absence of adding extra Ca^{2+}, permeabilization will increase cytoplasmic, and hence ER, Ca^{2+}. However, we find this is typically not enough to saturate the probe, so it is preferable to add a solution containing extra Ca^{2+}.

2. After waiting ~5 min from the addition of digitonin, add 5–10 mM Ca^{2+}, 1 mM ATP and 1 mM Mg^{2+}. The extra Mg^{2+} and ATP are included because they are required for the SERCA pump activity. At this point there should be an immediate rise in ER Ca^{2+}, which eventually reaches a plateau before it drops (as the cell dies). This plateau should be the R_{max}.

▲ CRITICAL STEP If there is not an immediate rise after adding the high Ca^{2+} solution, it is likely that the membrane has not yet permeabilized, and researchers are advised to wait an additional ~5 min or optimize the digitonin concentration.

Considerations for calibration of the R_{max} for a mitochondrially targeted indicator

Fillipin *et al.*[17] have developed an effective protocol for passive Ca^{2+} loading in digitonin-permeabilized cells. Alternatively, the strategies used in Step 10 can be employed to increase mitochondrial Ca^{2+}. One challenge of mitochondrially targeted indicators is that data are often collected at 100× rather than 40× to observe mitochondrial details with better resolution. This increased magnification requires illuminating for longer (1,000 ms versus 500 ms) to obtain images of comparable brightness. Therefore, photobleaching of the mitochondrial cameleons might be more prominent. Researchers are encouraged to optimize illumination intensity and acquisition times to minimize photobleaching and/or to collect the direct YFP image so that photobleaching can be corrected.

13| Convert FRET ratios to Ca^{2+} concentration. Use the R_{min} and R_{max} values obtained in each experiment to convert the cell data to the % FRET ratio of maximum (%Δ*R*). Briefly, in Excel, calculate the following:

$$(R - R_{min})/(R_{max} - R_{min}) \times 100.$$

Here, *R* is the background-corrected ratio (calculated in Step 12) at a given time. Finally, use the *in vitro* parameters and relevant equation (**Table 4**) to determine the Ca^{2+} concentration at each time point. Note that for cameleons fitted with a one-site saturation model (D3cpv and D4cpv), the *in vitro* equation can be rearranged so that $[Ca^{2+}]$ can be easily calculated. For cameleons fitted with a two-site saturation model (D1 and D2cpv), the equation cannot be as easily rearranged to isolate Ca^{2+}; therefore, it is necessary to use an algorithm that will input the relevant parameters and calculate Ca^{2+}. This is readily done using the SOLVER tool in Excel, which allows the researcher to 'guess' an initial value for $[Ca^{2+}]$ and then iteratively vary this value until the calculated %Δ*R* matches the experimentally determined %Δ*R* at each time point.

TABLE 4 | *In vitro* calibration parameters for a number of cameleon sensors.

Cameleon	*In vitro* equation[a]	Parameters obtained from *in vitro* fit[b]
D1	$\%\Delta R=\{R_{max1}[Ca^{2+}]^{n1}/(K'_{d1}{}^{n1}+[Ca^{2+}]^{n1})\}+\{R_{max2}[Ca^{2+}]^{n2}/(K'_{d2}{}^{n2}+[Ca^{2+}]^{n2})\}$	K'_{d1} (0.58), K'_{d2} (56.46), R_{max1} (28), R_{max2} (72), n_1 (1.18), n_2 (1.67)
D2cpv	$\%\Delta R=\{R_{max1}[Ca^{2+}]^{n1}/(K'_{d1}{}^{n1}+[Ca^{2+}]^{n1})\}+\{R_{max2}[Ca^{2+}]^{n2}/(K'_{d2}{}^{n2}+[Ca^{2+}]^{n2})\}$	K'_{d1} (0.097), K'_{d2} (7.67), R_{max1} (65.9), R_{max2} (33.53), n_1 (1.34), n_2 (0.77)
D3cpv	$\%\Delta R=R_{max}[Ca^{2+}]^{n}/(K'_{d}{}^{n}+[Ca^{2+}]^{n})$; $[Ca^{2+}]=\{K'_{d}{}^{n}\times(R-R_{min})/(R_{max}-R)\}^{1/n}$	K'_{d} (0.76), R_{max} (105.3), n (0.74)
D4cpv	$\%\Delta R=R_{max}[Ca^{2+}]^{n}/(K'_{d}{}^{n}+[Ca^{2+}]^{n})$; $[Ca^{2+}]=\{K'_{d}{}^{n}\times(R-R_{min})/(R_{max}-R)\}^{1/n}$	K'_{d} (49.68), R_{max} (98.04), n (1.35)

[a]Where $\%\Delta R=(R-R_{min})/(R_{max}-R_{min})$ and $\Delta R_{max}=R_{max}-R_{min}$. [b]$K'_d$ reported in μM and for a one-site Hill model, the apparent dissociation constant (K'_d) is related to the true Ca^{2+} dissociation constant K_d by the equation $K'_d=K_d(S_{f2}/S_{b2})^{1/n}$, where (S_{f2}/S_{b2}) is the ratio of emission intensities of the Ca^{2+}-free cameleon to Ca^{2+}-bound cameleon, measured over the denominator wavelength passband, (i.e., the FRET-excited YFP emission band; see ref. 33). For cameleons incorporating cp-Venus, (S_{f2}/S_{b2}) is ~0.47.

▲ CRITICAL STEP There is always a possibility that the *in situ* (i.e., within cells) K_d values differ from the *in vitro* values; hence, all conversions to $[Ca^{2+}]$ should note the exact parameters used. We generally favor reporting time traces of both FRET ratio changes and $[Ca^{2+}]$ (e.g., using left and right vertical axes for the two calibrations).

14| Correction of photobleaching. Photobleaching leads to a steady decrease in the FRET ratio over time, because variants of YFP are less photostable than CFP. To correct for photobleaching, multiply the intensity of the FRET channel (I_{DA}) by a correction factor. This correction factor is calculated by dividing the intensity of direct YFP at the start of the exp (I_{AA}^0) by the YFP intensity at a given time point (I_{AA}^t).

15| Subtract crosstalk. The process of subtracting the bleedthrough from one channel into another is called spectral unmixing and has been detailed by others[29,30]. Briefly, determine the coefficients of crosstalk as described in the EQUIPMENT SETUP ($a = I_{DA}/I_{AA}$, $b = I_{DD}/I_{AA}$, $c = I_{DA}/I_{DD}$, $d = I_{AA}/I_{DD}$). Each channel can be corrected for bleedthrough by subtracting the intensity of other channels multiplied by their bleedthrough coefficients; for example, the intensity of the CFP signal will be given by $I_{DD} - b \times I_{AA}$ and the intensity of the FRET signal will be given by $I_{DA} - a \times I_{AA} - c \times I_{DD}$.

● TIMING

Equipment and reagent setup: 1 week.
Step 1: 1 d.
Steps 2 and 3: 2 d.
Steps 4–8: 1–2 h.
Steps 9 and 10: 0.5–1 h.
Steps 11–15: 2–24 h.

ANTICIPATED RESULTS

A typical experiment is presented in **Figure 2**. The individual fluorescent channels (FRET and CFP) showed a strong signal above background. A plot of the FRET ratio and $[Ca^{2+}]$ as a function of time (**Fig. 2c, 2d**) showed the expected increase in FRET ratio or Ca^{2+} upon addition of histamine to the cells. Washout of histamine caused the FRET ratio to return to baseline. Treatment with ionomycin/EGTA caused a transient increase in ratio change and Ca^{2+} due to release of Ca^{2+} from the ER, followed by a slow decline. The R_{min} value was below the resting/starting level. Treatment with Ca^{2+} and ionomycin increased the ratio and Ca^{2+} to a level higher than that observed for histamine.

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1. Rizzuto, R., Brini, M. & Pozzan, T. Targeting recombinant aequorin to specific intracellular organelles. *Methods Cell Biol.* **40**, 339–358 (1994).
2. Robert, V., Pinton, P., Tosello, V., Rizzuto, R. & Pozzan, T. Recombinant aequorin as tool for monitoring calcium concentration in subcellular compartments. *Methods Enzymol.* **327**, 440–456 (2000).
3. Miyawaki, A. *et al.* Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**, 882–887 (1997).
4. Romoser, V.A., Hinkle, P.M. & Persechini, A. Detection in living cells of Ca^{2+}-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. *J. Biol. Chem.* **272**, 13270–13274 (1997).
5. Miyawaki, A., Griesbeck, O., Heim, R. & Tsien, R.Y. Dynamic and quantitative Ca^{2+} measurements using improved cameleons. *Proc. Natl. Acad. Sci. USA* **96**, 2135–2140 (1999).
6. Truong, K. *et al.* FRET-based *in vivo* Ca^{2+} imaging by a new calmodulin-GFP type molecule. *Nat. Struct. Biol.* **8**, 1069–1073 (2001).
7. Heim, N. & Griesbeck, O. Genetically encoded indicators of cellular calcium dynamics based on troponin C and green fluorescent protein. *J. Biol. Chem.* **279**, 14280–14286 (2004).
8. Palmer, A.E., Jin, C., Reed, J.C. & Tsien, R.Y. Bcl-2-mediated alterations in endoplasmic reticulum Ca^{2+} analyzed with an improved genetically encoded fluorescent sensor. *Proc. Natl. Acad. Sci. USA* **101**, 17404–17409 (2004).
9. Mank, M. *et al.* A FRET-based calcium biosensor with fast signal kinetics and high fluorescence change. *Biophys. J.* **90**, 1790–1796 (2006).
10. Palmer, A.E. *et al.* Ca^{2+} indicators based on computationally-redesigned calmodulin–peptide pairs. *Chem. Biol.* **13**, 521–530 (2006).
11. Ishii, K., Hirose, K. & Iino, M. Ca^{2+} shuttling between endoplasmic reticulum and mitochondria underlying Ca^{2+} oscillations. *EMBO Rep.* **7**, 390–396 (2006).
12. Baird, G.S., Zacharias, D.A. & Tsien, R.Y. Circular permutation and receptor insertion within green fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **96**, 11241–11246 (1999).
13. Griesbeck, O., Baird, G.S., Campbell, R.E., Zacharias, D.A. & Tsien, R.Y. Reducing the environmental sensitivity of yellow fluorescent protein. *J. Biol. Chem.* **276**, 29188–29194 (2001).
14. Nakai, J., Ohkura, M. & Imoto, K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nat. Biotech.* **19**, 137–141 (2001).
15. Ohkura, M., Matsuzaki, M., Kasai, H., Imoto, K. & Nakai, J. Genetically encoded bright Ca^{2+} probe applicable for dynamic Ca^{2+} imaging of dendritic spines. *Anal. Chem.* **77**, 5861–5869 (2005).
16. Nagai, T., Sawano, A., Park, E.S. & Miyawaki, A. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. *Proc. Natl. Acad. Sci. USA* **98**, 3197–3202 (2001).
17. Filippin, L., Magalhaes, P.J., Benedetto, G.D., Colella, M. & Pozzan, T. Stable interactions between mitochondria and endoplasmic reticulum allow rapid accumulation of calcium in a subpopulation of mitochondria. *J. Biol. Chem.* **278**, 39224–39234 (2003).
18. Filippin, L. *et al.* Improved strategies for the delivery of GFP-based Ca^{2+} sensors into the mitochondrial matrix. *Cell Calcium* **37**, 129–136 (2005).
19. Nagai, T., Yamada, S., Tominaga, T., Ichikawa, M. & Miyawaki, A. Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **101**, 10554–10559 (2004).

20. Iwano, M. *et al.* Ca^{2+} dynamics in a pollen grain and papilla cell during pollination of *Arabidopsis*. *Plant Physiol.* **136**, 3562–3571 (2004).
21. Kerr, R. *et al.* Optical imaging of calcium in neurons and pharyngeal muscle of *C. elegans*. *Neuron* **26**, 583–594 (2000).
22. Reiff, D.F., Thiel, P.R. & Schuster, C.M. Differential regulation of active zone density during long-term strengthening of *Drosophila* neuromuscular junctions. *J. Neurosci.* **22**, 9399–9409 (2002).
23. Fiala, A. *et al.* Genetically expressed cameleon in *Drosophila melanogaster* is used to visualize olfactory information in projection neurons. *Curr. Biol.* **12**, 1877–1884 (2002).
24. Reiff, D.F. *et al. In vivo* performance of genetically encoded indicators of neural activity in flies. *J. Neurosci.* **25**, 4766–4778 (2005).
25. Higashijima, S., Masino, M.A., Mandel, G. & Fetcho, J.R. Imaging neuronal activity during zebrafish behavior with a genetically encoded calcium indicator. *J. Neurophysiology* **90**, 3986–3997 (2003).
26. Hasan, M.T. *et al.* Functional fluorescent Ca^{2+} indicator proteins in transgenic mice under TET control. *PLoS Biol.* **2**, 763–775 (2004).
27. Ji, G. *et al.* Ca^{2+}-sensing transgenic mice: postsynaptic signaling in smooth muscle. *J. Biol. Chem.* **279**, 21461–21468 (2004).
28. Shaner, N.C., Steinbach, P.A. & Tsien, R.Y. A guide to choosing fluorescent proteins. *Nat. Methods* **2**, 905–909 (2005).
29. Gordon, G.W., Berry, G., Liang, X.H., Levine, B. & Herman, B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys. J.* **74**, 2702–2713 (1998).
30. Zal, T. & Gascoigne, N.R. Photobleaching-corrected FRET efficiency imaging of live cells. *Biophys. J.* **86**, 3923–3939 (2004).
31. Miyawaki, A. & Tsien, R.Y. Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol.* **327**, 472–436 (2000).
32. Sambrook, J. & Russel, D.W. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 2001).
33. Adams, S.R., Bacskai, B.J., Taylor, S.S. & Tsien, R.Y. in *Fluorescent Probes for Biological Activity of Living Cells — A Practical Guide* (eds. Matson, W.T. & Relf, G.) 133–149 (Academic Press, New York, 1993).