Published in final edited form as:

Cold Spring Harb Protoc. ; 2015(1): pdb.prot076547. doi:10.1101/pdb.prot076547.

Protocol 1: Verifying the function and localization of genetically-encoded Ca^{2+} sensors and converting FRET ratios to Ca^{2+} concentrations

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Abstract

Genetically-encoded, ratiometric, fluorescent, Ca^{2+} biosensors can be used in living cells to quantitatively measure free Ca^{2+} concentrations in the cytosol or in organelles. This protocol describes how to perform a calibration of a Ca^{2+} sensor expressed in cultured mammalian cells as images are acquired using a widefield fluorescence microscope. This protocol also explains how to calculate Förster Resonance Energy Transfer (FRET) ratios from acquired images and how to convert FRET ratios to Ca^{2+} concentrations.

Materials

Reagents

ATP stock solution for ER sensor calibrations <R>

Calcium chloride stock solution (1 M) in water

Chelex® 100 resin, sodium form, 50–100 mesh

Chelex®-treated double deionized H_2O <R>

All solutions in this protocol should be prepared using Chelex®-treated water and should be stored in metal-free plastic containers.

Digitonin stock solution <R>

EGTA stock solution <R>

HEPES-buffered Hanks balanced salt solution (HHBSS) with Ca^{2+} <R>

HEPES-buffered Hanks balanced salt solution without Ca^{2+} (Ca^{2+} -free HHBSS) <R>

Ionomycin stock solution <R>.

Mammalian expression plasmid encoding the ratiometric, genetically-encoded Ca^{2+} biosensor of choice

Probes for verifying sensor localization that have minimal bleed-through into the sensor-imaging channels.

Examples include: MitoTracker® Red (Life Technologies) or a mammalian expression plasmid encoding a red FP-tagged localization marker (*e.g.* mCherry-GalT(Qin et al. 2011; Zaal et al. 2011) for Golgi, mCherry-KDEL(Zurek et al. 2011) for endoplasmic reticulum (ER))

Saponin (0.01 to 0.1%)

Thapsigargin stock solution for ER-targeted sensors <R>

Transfection reagent, such as Lipofectamine (Life Technologies) or TransIT-LT1 (Mirus Bio)

Equipment

Epifluorescence microscope system (equipped with a mercury or xenon arc lamp and power supply; excitation and emission filter wheels; Lambda SC Smart Shutter controller; cooled CCD camera; 20X, 40X, 60X and/or 100X plan apochromatic objectives)

Other microscopy systems, such as confocal, FLIM, and spectral imaging, can also be used to monitor FRET, but they are beyond the scope of this protocol.

Excitation (x) and emission (m) filter sets (Chroma) and dichroic mirrors

CFPx and FRETx 430/24, YFPx 495/10, CFPm 470/24, YFPm and FRETm 535/25, CFP and FRET dichroic 450, YFP dichroic 515. Sputter-coated/ET or brightline filters that provide high transmission will give rise to the brightest images.

Glass-bottom imaging dishes

These are commercially available and can be economically constructed using 35 mm polystyrene cell culture dishes, an industrial-strength 0.375 in. hole punch (Roper Whitney), SYLGARD 184 (Dow Corning), an 18-gauge needle and syringe, and 18 mm x 18 mm No. 1 glass coverslips.

Neutral density filters (Chroma)

Software for image acquisition and analysis

Examples include: MetaFluor (Molecular Devices), NIS Elements (Nikon), or MATLAB (Mathworks); ImageJ (NIH) or Excel (Microsoft), for analysis only.

Method

1. Construct and sterilize glass-bottom imaging dishes (skip this step if commercially-made glass-bottom dishes will be used). Punch a hole in the middle of a cell culture dish and place it bottom-up. Use the needle and syringe to judiciously apply pre-mixed SYLGARD 184 around the rim of the punched hole. Place a glass coverslip on top of the glue, and let the glue cure. This procedure is illustrated in Figure 1. Sterilize the dish with 70% ethanol and UV light.

Constructed dishes can be coated with reagents, such as polylysine, that improve the adherence of some cell types.

2. Plate cells on several imaging dishes and transfect them with a plasmid encoding the biosensor of choice. Co-transfect a couple dishes of cells with plasmids encoding subcellularly-targeted red FP, if these will be used to verify sensor localization.

Optimal cell plating density and transfection conditions will differ for each cell type and biosensor. For example, we transfect each imaging dish of HeLa cells with 5 μ l Mirus TransIT-LT1 transfection reagent, and 0.5 μ g plasmid DNA encoding a mitochondria-targeted Ca^{2+} sensor or 1.25 μ g plasmid DNA encoding the same Ca^{2+} sensor targeted to the cytosol. In our experience, it is worthwhile to identify conditions that minimize cell toxicity, promote expression levels detectable by the microscope, and maximize correct localization of biosensors targeted to organelles.

3. Image cells 24–72 hours after transfection, or when sensor expression and cell density are appropriate for the experiment. If a small-molecule fluorescent probe, such as MitoTracker® Red, will be used to verify correct localization of the sensor, add the probe to cells before imaging.
4. Gently remove cell culture media from the imaging dish and replace with 2 mL HHBSS. All solutions are kept at room temperature for the remainder of the experiment. Wash the dish 3–5 times to remove all cell culture media.

Optically-clear salt solutions are preferred for cellular imaging because the autofluorescence of Phenol Red and serum in complete media increases background fluorescence. In addition, components of complete media, such as amino acids and proteins, will bind metal ions and affect sensor calibrations.

5. Place the imaging dish on the microscope stage. Identify and focus on cells.
6. Verify that the sensor is correctly localized to the subcellular compartment. It is unnecessary to have 100% transfection efficiency or

100% correct localization because analysis will be performed on single cells. See Figure 2 for typical localization of targeted sensors in HeLa cells.

Sensor mislocalization could lead to incorrect estimates of Ca^{2+} concentrations. In our experience, the most common mislocalization problems include mitochondrial sensors found in both the mitochondria and the cytosol, nuclear localization signal-tagged sensors found in the cytosol during mitosis, or Golgi (or other secretory pathway) sensors incorrectly localized to the ER. Co-localization of a sensor and an organelle marker can be quantified by running a Pearson's correlation analysis. Another method is to quantify the percentage of cells displaying incorrect localization when optimizing conditions for the best localization (Palmer et al. 2006). Localization can be improved by changing transfection conditions, changing the targeting sequence, or creating a stable cell line.

See Troubleshooting.

7. Set up acquisition parameters. If you have added a fluorescent dye for co-localization, begin this step with a fresh dish of cells and repeat steps 3–5. Acquire one set of images that includes a FRET, a donor, an acceptor, and a brightfield/DIC image. Confirm that the fluorescence intensity of the biosensor is about 2.5–8 times the background intensity and does not saturate the camera. Many software programs controlling image acquisition allow you to select regions of interest and calculate the ratio of two images or regions of interest in during acquisition. This function is helpful because it allows you to observe the sensor response during the experiment.

Limit fluorescent light exposure by closing the shutter unless you are focusing or acquiring images in order to prevent photobleaching of the sensor.

8. Begin the experiment by acquiring a full set of images (CFP, FRET, and YFP) every 20–60 seconds for at least 5 minutes. Make sure that the FRET ratio, defined as the intensity of the biosensor in the FRET image divided by its intensity in the donor image, is stable during this time.

See Troubleshooting.

9. Measure the FRET ratios of the fully unbound sensor (R_{free}). Wash cells 3 times with Ca^{2+} -free HHBSS. Dilute EGTA and ionomycin stock solutions to 2X the final concentration in Ca^{2+} -free HHBSS (final (1X) concentrations: 5 μM ionomycin and 3 mM EGTA), and add 1 mL of the 2X EGTA/ionomycin solution drop-by-drop to 1 mL Ca^{2+} -free HHBSS in the imaging dish. Acquire images every 20–30 seconds until the FRET ratio stabilizes. This protocol should suffice for cytosolic, nuclear, and

mitochondrial-targeted sensors. For an ER-targeted sensor, first treat cells with 1–10 μM thapsigargin to deplete the ER slowly, and after 5–10 min, add ionomycin and EGTA as described above.

The FRET ratio in the cytosol, nucleus, and mitochondria will initially increase after the addition of ionomycin/EGTA due to the permeabilization of the ER and release of Ca^{2+} stores. The FRET ratio of cytosolic sensors, such as D3cpV, will decrease for up to 10 minutes before reaching R_{free} . Higher-affinity sensors may have a slow off rate, and it may take up to 40 minutes for the FRET ratio to reach R_{free} . An alternative is to monitor the FRET ratio for 15–20 minutes, fit the data to an exponential decay, and extrapolate to R_{free} .

10. Wash cells 3–5 times with 2 mL HHBSS. The FRET ratio should increase as Ca^{2+} from the HHBSS enters cells.
11. Measure the FRET ratios of the fully saturated (R_{bound}) Ca^{2+} sensor. Use the same procedure described in Step 9 to add the following reagents to the imaging dish (dilute reagents in HHBSS to a 2X solution, and then add 1 mL of this solution to 1 mL HHBSS in the imaging dish). To saturate a sensor expressed in the cytosol, nucleus, or mitochondria, use 5 μM ionomycin and 10 mM CaCl_2 . To saturate an ER-targeted sensor, first treat cells with a permeabilization agent such as digitonin (5 – 25 μM) or saponin (0.01 to 0.1%) and wait 5–10 min. During this time, there should be no rise in the FRET ratio. Next, add 5–10 mM Ca^{2+} , 1 mM Mg^{2+} , and 1 mM ATP to facilitate activation of SERCA. At this point, the FRET ratio should increase and reach a plateau at R_{bound} .
See Troubleshooting.
12. A typical image analysis workflow is shown in Figure 3. After image acquisition, calculate the average FRET ratio of each cell using the full spectrum unprocessed images (usually stored as .tiff files), not images that have been autoscaled and saved as new images (usually .jpg or .png files). Define regions of interest (ROI) and measure the mean intensity of the same ROI in each set of images (CFP, FRET, and YFP) using image analysis software. Subtract the mean background intensity from each ROI's mean intensity in every image. Calculate the FRET ratio by dividing the ROI's background-subtracted mean intensity in the FRET image by that in the CFP image at each time point. Plot the FRET ratio over time and confirm that the sensor responds to perturbations of $[\text{Ca}^{2+}]$ (Figure 3B). In addition, plot each ROI's mean intensity in the YFP image over time to observe potential photobleaching (Figure 3C).
13. Determine R_{free} , R_{bound} , the dynamic range (DR), and R for each cell. For the majority of sensors, R_{free} corresponds to the minimum FRET ratio (R_{min}) and R_{bound} corresponds to the maximum FRET ratio (R_{max}). DR is

defined as $R_{\max} / R_{\min}$ and R is equal to $R_{\max} - R_{\min}$. Verify that the DR of each cell is comparable to the published DR.

See Troubleshooting.

14. Convert single-cell FRET ratios to fractional saturation (Y , Figure 3D), which is equal to $(R - R_{\text{free}}) / R$. To ensure that the sensor is not perturbing basal $[\text{Ca}^{2+}]$, plot Y as a function of YFP intensity for a range of cells, as shown in Figure 3E.

Figure 4 compares the relative expected resting Y for common cytosolic, mitochondrial, and ER sensors in HeLa cells. Note that the resting Y may be significantly different in different cell types.

15. To convert the FRET ratio into a $[\text{Ca}^{2+}]$, you will need to experimentally determine R , R_{free} , and R_{bound} , and obtain the relevant binding parameters for the sensor you are using. The binding parameters can be obtained from published work (Horikawa et al. 2010; Mank et al. 2008; Nagai et al. 2004; Palmer et al. 2004; Palmer et al. 2006) or can be obtained experimentally (see Protocol 2). Depending on the sensor, binding data may be fit to a one-site (i.e. single apparent K_d value) or two-site (i.e. two apparent K_d values) with or without the empirical Hill coefficient (n), which accounts for cooperativity.

When using published parameters obtained from the literature, it is essential to ensure your data are treated as close to the calibration data used to obtain the binding parameters as possible. Figure 5 presents examples of a sensor titration in terms of $R - R_{\text{free}}$ or Y . The binding parameters for the sensors D3cpV and D1 (Palmer et al. 2004; Palmer et al. 2006), obtained by fitting to $R - R_{\text{free}}$ or Y , are very similar and within the margin of error, as seen in Table 1.

16. If using a sensor with a single K_d , use the expression presented in Figure 5, along with the relevant K_d and n values, and experimentally derived R , R_{free} , and R_{bound} .

The expression presented in Figure 5 is related, but not identical,

to the widely used $[\text{Ca}^{2+}] = \frac{K_d'(R - R_{\min})}{(R_{\max} - R)} \left(\frac{S_{f2}}{S_{b2}} \right)$ derived for small molecule fluorescent indicators. This original expression contains an instrument factor (S_{f2}/S_{b2}) that naturally fell out of the derivation and represents the ratio of the fluorescence intensity of the unbound to bound sensor at wavelength 2 (Grynkiewicz et al. 1985). The instrument factor is absent from all expressions used to convert FRET ratios to $[\text{Ca}^{2+}]$ because the genetically-encoded Ca^{2+} sensors don't adhere to a strict 1:1 complexation, an assumption used to derive the above expression.

17. If using a sensor with two K_d values, first convert your data to Y using the experimentally derived R , R_{free} , and R_{bound} values. Then, use the reported F_1 , K_{d1} , n_1 , F_2 , K_{d2} , n_2 values and an iterative approximation approach to solve the expression outlined in Figure 5 for the $[\text{Ca}^{2+}]$.

One approach for doing this is to use the SOLVER tool in Excel. The iterative approximation involves making an initial guess for the $[\text{Ca}^{2+}]$, then calculating Y , and successively repeating until the calculated Y matches the experimental Y .

Troubleshooting

Problem (Step 6)

The FRET sensor does not co-localize with the appropriate organelle marker, or the organelle morphology is distorted.

Solution

Poor co-localization or distorted organelle morphology can occur if the sensor expression is too high. To minimize this problem, identify cells that have low levels of expression, or sub-clone the biosensor into an inducible expression vector (*e.g.* pRevTRE or pTet-tight from Clontech; or T-Rex from Life Technologies) and optimize expression levels accordingly.

Problem (Step 8)

The FRET ratio decreases or increases during the first 5 min of acquisition, without perturbation of cellular Ca^{2+} .

Solution

Change the acquisition parameters to decrease light exposure. For example, decrease the frequency of image acquisition, decrease the exposure time of each acquisition, or change the neutral density filter to minimize transmitted light. If the FRET ratio does not stabilize after the adjustment of acquisition parameters, change the imaging buffer, select new cells, or start over with a new dish.

Problem (Step 11)

When trying to measure R_{bound} of a cytosolic, nuclear, or mitochondrial sensor, the FRET ratio spikes, then declines before reaching a plateau.

Solution

This phenomenon typically arises because the cell dies before the sensor has an opportunity to be saturated. The solution is to optimize the concentration of ionomycin and calcium and typically involves *lowering* the amount of calcium used in the calibration, because adding Ca^{2+} too quickly can lead to cell death before R_{bound} is reached. Another approach is to use a different method for getting calcium into the cell, such as an alternate ionophore BromoA23187 (final concentration 0.1 to 20 μM) or permeabilization of the plasma

membrane with digitonin (final concentration 5–20 μM) or saponin (final concentration 0.01 to 0.1%).

Problem (Step 11)

When trying to measure R_{bound} of an ER sensor, there is no increase.

Solution

Since the ER contains high amounts of Ca^{2+} under resting conditions, and the D1ER biosensor is close to saturation (it may be fully saturated in some cell types), it can be challenging to measure R_{bound} . For the ER, R_{bound} should always be measured before R_{free} because it is difficult to increase the level of Ca^{2+} in the ER. Note that addition of ionomycin and Ca^{2+} will actually facilitate release of Ca^{2+} from the ER, and after such treatment, it is difficult to attain the original resting ratio before cell death. Thus, to measure R_{bound} , our protocol involves permeabilizing the plasma membrane and adding Ca^{2+} , Mg^{2+} , and ATP to facilitate uptake of Ca^{2+} into the ER by the SERCA pump. This protocol should be optimized for different cell types by changing the concentration, nature, and time of incubation of the permeabilization agent, and the concentration of exogenous Ca^{2+} . As a last resort, if we can't be sure that we can accurately measure R_{bound} , we perform a partial calibration in which we measure R and R_{free} and report the relative changes as R/R_{free} . This allows quantitative comparison of the relative amount of calcium in different cell types or under different environmental conditions, but does not allow for conversion to a calcium concentration (Mccombs et al. 2010; Ravier et al. 2011).

Problem (Step 13)

You observe an unusually low dynamic range ($R_{\text{max}}/R_{\text{min}}$).

Solution

The appropriate chemical perturbations to reach R_{min} or R_{max} will depend on the cell type, the biosensor, and the organelle to which it is targeted. Researchers are advised to try out a series of ionomycin, Ca^{2+} , and EGTA or BAPTA concentrations to obtain the highest and most consistent dynamic range possible. An unusually low dynamic range could result from proteolysis, misfolding, or oxidation of the sensor. It is often valuable to plot the dynamic range as a function of the YFP intensity, for a range of cell intensities, to determine whether the low dynamic range is due to unusually low or high sensor expression. These data are difficult to interpret and may have to be discarded.

Recipes

ATP stock solution for ER sensor calibrations

Prepare a stock solution of ~5 mM in HBSS and adjust pH to 7.0. Aliquot and store them at -20°C .

Chelex®-treated double deionized H₂O

Mix Chelex® 100 with autoclaved water in a large plastic container (~3–4 L) on a stir plate for at least 18 hours. Let the resin settle to the bottom of the container for several hours. Use a bottle-top filter to remove all Chelex® from the water, and store the filtered water in a plastic container.

Digitonin stock solution

Dissolve purified digitonin to a final concentration of 3 mM in 100% DMSO or in warm 100% ethanol and cool to room temperature. Aliquot & store at –20°C.

EGTA stock solution

EGTA = ethylene glycol bis(b-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (FW=380.35 g/mol). Weigh out 190.1 g EGTA and add to 800 mL Chelex®-treated double deionized H₂O. Stir vigorously. Adjust pH to 8.0, first with NaOH pellets, then with 1M NaOH solution. Note that EGTA will not go into solution until pH is ~ 8.0. Adjust final volume to 1 L and dispense into 100 mL aliquots. Sterilize by autoclaving.

HEPES-buffered Hanks balanced salt solution (HHBSS) with Ca²⁺

<u>Reagent</u>	<u>Final concentration</u>
10X HBSS <i>with</i> Ca ²⁺ and Mg ²⁺	1X
HEPES	20 mM
D-glucose	2 g/L

10X HBSS (Life Technologies) does not contain Phenol Red. To make 1 L HHBSS, add 800 mL milliQ water to 100 mL 10X HBSS, 4.76 g HEPES, and 2 g D-glucose. Adjust pH to 7.2 and add water to 1 L. Sterilize using a bottle-top 0.22 micron filter.

HEPES-buffered Hanks balanced salt solution without Ca²⁺ (Ca²⁺-free HHBSS)

<u>Reagent</u>	<u>Final concentration</u>
10X HBSS <i>without</i> Ca ²⁺ or Mg ²⁺	1X
MgCl ₂	0.49 mM
MgSO ₄	0.45 mM
HEPES	20 mM
D-glucose	2 g/L

10X HBSS (Life Technologies) does not contain Phenol Red. To make 1 L HHBSS, add 800 mL milliQ water to 100 mL 10X Ca²⁺-, Mg²⁺-free HBSS, 0.490 mL 1 M MgCl₂, 0.450 mL 1 M MgSO₄, 4.76 g HEPES and 2 g D-glucose. Adjust pH to 7.2 and add water to 1 L. Sterilize using a bottle-top 0.22 micron filter.

Ionomycin stock solution

Ionomycin (free acid, 709 g/mol) usually comes as 1 mg in a sealed ampule. Add 141 μ L 100% DMSO to yield a 10 mM stock solution. Mix well (vortex). Make 5 μ L aliquots and store them in the dark at -20°C . Dilute the 10 mM stock to 1 mM when preparing to use.

Thapsigargin stock solution

Thapsigargin (650.8 g/mol) comes as 1 mg in an amber vial. Add 153 μ L 100% DMSO to yield a 10 mM stock solution. Mix well (vortex). Store in the dark at -20°C . Dilute the 10 mM stock to a 2 mM working solution with 100% DMSO. Note: you can re-freeze any thapsigargin that you do not use.

Acknowledgments

Financial support was provided by the Signaling and Cell Cycle Regulation Training Grant (NIH T32 GM08759) to J.G.P. and by NIH GM084027 and Alfred P. Sloan Fellowship to A.E.P.

References

- Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem*. 1985; 260:3440–3450. [PubMed: 3838314]
- Horikawa K, Yamada Y, Matsuda T, Kobayashi K, Hashimoto M, Matsu-Ura T, Miyawaki A, Michikawa T, Mikoshiba K, Nagai T. Spontaneous network activity visualized by ultrasensitive Ca^{2+} indicators, yellow Cameleon-Nano. *Nat Meth*. 2010; 7:729–732.
- Mank M, Santos AF, Drenth S, Mrcic-Flogel TD, Hofer SB, Stein V, Hendel T, Reiff DF, Levelt C, Borst A, et al. A genetically encoded calcium indicator for chronic in vivo two-photon imaging. *Nat Meth*. 2008; 5:805–811.
- Mccombs JE, Gibson EA, Palmer A. Using a genetically targeted sensor to investigate the role of presenilin-1 in ER Ca^{2+} levels and dynamics. *Mol Biosyst*. 2010; 6:1640–1649. [PubMed: 20379593]
- Nagai T, Sawano A, Park ES, Miyawaki A. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+} *Proc Natl Acad Sci USA*. 2001; 98:3197–3202. [PubMed: 11248055]
- 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*. 2004; 101:10554–10559. [PubMed: 15247428]
- Palmer A, Giacomello M, Kortemme T, Hires SA, Lev-Ram V, Baker D, Tsien RY. Ca^{2+} indicators based on computationally redesigned calmodulin-peptide pairs. *Chemistry & Biology*. 2006; 13:521–530. [PubMed: 16720273]
- Palmer AE, Jin C, Reed JC, Tsien RY. Bcl-2-mediated alterations in endoplasmic reticulum Ca^{2+} analyzed with an improved genetically encoded fluorescent sensor. *Proc Natl Acad Sci USA*. 2004; 101:17404–17409. [PubMed: 15585581]
- Qin Y, Dittmer PJ, Park JG, Jansen KB, Palmer A. Measuring steady-state and dynamic endoplasmic reticulum and Golgi Zn^{2+} with genetically encoded sensors. *Proc Natl Acad Sci USA*. 2011; 108:7351–7356. [PubMed: 21502528]
- Ravier MA, Daro D, Roma LP, Jonas JC, Cheng-Xue R, Schuit FC, Gilon P. Mechanisms of control of the free Ca^{2+} concentration in the endoplasmic reticulum of mouse pancreatic β -cells: interplay with cell metabolism and $[\text{Ca}^{2+}]_c$ and role of SERCA2b and SERCA3. *Diabetes*. 2011; 60:2533–2545. [PubMed: 21885870]
- Zaal KJ, Reid E, Mousavi K, Zhang T, Mehta A, Bugnard E, Sartorelli V, Ralston E. Who needs microtubules? Myogenic reorganization of MTOC, Golgi complex and ER exit sites persists despite lack of normal microtubule tracks. *PLoS ONE*. 2011; 6:e29057. [PubMed: 22216166]
- Zurek N, Sparks L, Voeltz G. Reticulon short hairpin transmembrane domains are used to shape ER tubules. *Traffic*. 2011; 12:28–41. [PubMed: 20955502]

FIGURE 1.

Construction of glass-bottom imaging dishes. Step 1: Use an industrial-strength hole punch to make a 0.375 inch hole in the bottom of a 35 mm cell culture dish, and then place the dish bottom-up. Step 2: Use an 18-gauge needle and syringe to judiciously apply pre-mixed SYLGARD 184 around the hole. Step 3: Place a glass coverslip on top of the glue. Step 4: Gently press down on the glass coverslip to remove air bubbles and to spread out the glue. Let the glue cure at room temperature for 48 hours or for 2–3 hours at 60°C.

FIGURE 2.

Images of HeLa cells transfected with subcellularly targeted Ca^{2+} sensors. (A) Untargeted sensors are found in both the cytosol and nucleus. (B) Sensors with an N-terminal nuclear export signal (MLQLPPLERLTLSDP) are retained in the cytosol. (C) Sensors with an N-terminal nuclear localization sequence (MPKKKKRKVEDASDPM) are retained in the nucleus. (D) Sensors with an N-terminal calreticulin signal sequence (MLLPVLLLGLLGAAAD) and a C-terminal ER-retention sequence (KDEL) localize to the ER (Palmer et al. 2004). Sometimes, a significant fraction of mitochondrial sensors localize to the cytosol (E), but changing the transfection conditions can result in good mitochondrial localization (F) (Palmer et al. 2006).

FIGURE 3.

Image analysis of a cytosolic Ca^{2+} sensor calibration. (A) A background (BG) region of interest (ROI) and an ROI for each cell are defined within the acquired images. The mean intensities of these ROIs in the FRET and CFP images are used to calculate the FRET ratio (R). (B) The R of each cell is plotted over the course of the sensor calibration, and each line in the plot corresponds to R of an ROI in a different cell. Notice the relatively stable R before any perturbations, the increase in R after the addition of thapsigargin (Thapsi), and the initial increase in R after the addition of ionomycin and EGTA (Iono/EGTA). The sensor reaches R_{free} at ~1750 seconds and R_{bound} at ~2000 seconds. (C) The mean intensity of each ROI in the YFP image can be plotted over time to check for photobleaching. The YFP intensity is independent of FRET and dependent on the sensor's concentration and fluorescence. The decrease in YFP intensity of some ROIs could be due to photobleaching or a change in the cell's volume. (D) R values are converted to the fractional saturation (Y) of the sensor using the values determined for R_{free} and R_{bound} . (E) Each cell's Y , before any perturbation, can be plotted against its mean YFP intensity, which is directly proportional to the sensor's intracellular concentration. This plot can reveal whether sensor expression levels do or do not perturb resting $[\text{Ca}^{2+}]$ (open circles and closed circles, respectively). (F) Ca^{2+} concentrations ($[\text{Ca}^{2+}]$) are calculated from the experimentally determined R , R_{free} , and R_{bound} values and the sensor's binding parameters (K_d 's and Hill coefficients). Note that the values approaching R_{bound} are not converted to $[\text{Ca}^{2+}]$ because they approach infinity. (E) Equations for calculating R and Y are presented in Steps 13 and 14 of the protocol.

FIGURE 4.

The fractional saturation of commonly-used Ca^{2+} sensors in the cytosol, mitochondria, and ER of HeLa cells at rest. The figure shows Ca^{2+} binding curves of Pericam, YC-Nano50, YC2.60, YC3.60, TN-XXL, D3cpV, and D1 (Horikawa et al. 2010; Mank et al. 2008; Nagai et al. 2001; Nagai et al. 2004; Palmer et al. 2004; Palmer et al. 2006). D3cpV is 20% saturated in the cytosol or mitochondria, and D1 is 80% saturated in the ER.

FIGURE 5.

Models used to describe sensors binding to Ca^{2+} . A single-site or two-site binding model is used to derive the values for K_d , Hill coefficient (n), F_1 , and F_2 from sensor titration data. Two different methods can be used to fit these data: the $R - R_{\text{free}}$ method or the fractional saturation method, and both result in similar values for the binding parameters (see Table 1). FRET ratios of sensors that are fit to a single-site binding model, such as D3cpV and TN-XXL, can be easily converted to $[Ca^{2+}]$ using the experimentally determined R_{free} and R_{bound} and the reported K_d' and n . If a sensor, such as D1 or YC-Nano50, has two K_d , n , and F values, FRET ratios must be converted to Y , and iterative approximation must be used to determine the corresponding $[Ca^{2+}]$.

TABLE 1

Binding parameters of D3cpV and D1 sensors (Palmer et al. 2004; Palmer et al. 2006).

Sensor	Method	K_d' (μM)	n	R (or F_1 & F_2)
D3cpV	$R-R_{\min}$	0.57	0.85	10
	Fractional saturation	0.64	0.79	...
D1	$R-R_{\min}$	0.59	2.1	0.29
		55	1.7	0.78
	Fractional saturation	0.57	2.2	0.26
		58	1.6	0.74