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Measuring the *in situs* K_d of a genetically-encoded Ca^{2+} sensor

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Abstract

The use of genetically-encoded Ca^{2+} sensors (GECIs) for long-term monitoring of intracellular Ca^{2+} has become increasingly common in the last decade. Emission-ratiometric GECIs, such as those in the yellowameleon family, are capable of making quantitative measurements, meaning that their fluorescence signals can be converted to free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_{\text{free}}$). This conversion is only as accurate as the sensor's apparent dissociation constant for Ca^{2+} (K_d'), which depends on temperature, pH, and salt concentration. This protocol describes a method for performing a titration, in living cells (*in situ*), of cytosolic, nuclear, or mitochondrial sensors. An excellent example of calibration of an ER-targeted sensor is presented elsewhere (Rudolf et al. 2006).

Materials

Reagents

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.

EGTA stock solution <R>

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

HEPES-buffered Hanks balanced salt solution without Ca^{2+} or Mg^{2+} ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HHBSS) <R>

Ionomycin stock solution <R>

Mammalian cells of interest and associated cell culture media

Mammalian expression plasmid encoding the ratiometric GECI of choice

Metal chelate buffer system <R>

Permeabilizing agent (~100X) stock solution: digitonin (1 mM), saponin (100 µg/mL), or alamethicin (5 mg/mL)

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 objective (depends on application). Other microscopy systems, such as confocal and FLIM and spectral imaging, can also be used to monitor FRET, but they will not be discussed in this protocol.

An environment chamber, fit to the microscope stage, to control temperature, humidity, %CO₂, and %O₂.

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 × 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), KaleidaGraph (Synergy Software), GraphPad (GraphPad software), 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) according to the procedure illustrated in Protocol 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. Select the appropriate metal-chelate buffer(s) for the desired free Ca²⁺ concentration ([Ca²⁺]_{free}). For a sensor titration, about 10 (linearly) evenly-spaced [Ca²⁺]_{free} should be

chosen within the range of ~10-fold above and below the expected K_d' . Metal chelate buffers consist of a chelator (*e.g.* EDTA), the ion to be buffered, in this case Ca^{2+} , and sometimes a competing ion (*e.g.* Mg^{2+} or Sr^{2+}). The addition of a competing ion effectively increases the chelator's buffering range, and so all other divalent metals must be eliminated from the *single* metal chelate buffers that are described in this protocol. The final concentration of the chelator must be much greater than $[\text{Ca}^{2+}]_{\text{free}}$, just as in a pH buffer, where the concentration of the weak acid/conjugate base is much greater than the H_3O^+ concentration. In addition, the *total* $[\text{Ca}^{2+}]$ is orders of magnitude greater than $[\text{Ca}^{2+}]_{\text{free}}$. Refer to Table 1 for the buffering range of some metal-chelate buffer systems.

See Troubleshooting

3. Prepare two 100X stock solutions for each buffer: (1) 1 M Ca^{2+} and 1 M chelator, and (2) 1 M chelator *only*. The “pH jump method” (Tsien and Pozzan 1989) should be used to prepare stock solution (1) because it is extremely important to make a 1:1 solution of metal:chelator.

The pH titration method (Tsien and Pozzan 1989) can be used make solutions of polyprotic acid chelators (EDTA, EGTA, HEDTA) and metal ions, so that the concentrations of each are within 0.5% of each other. For example, when Ca^{2+} binds EGTA, two protons are released, causing a drop in the pH of the solution. Therefore, the change in pH upon Ca^{2+} addition (pH/Ca^{2+}) decreases when there are equal concentrations of Ca^{2+} and EGTA. Please refer to the detailed practical description of this method in the recipes section.

4. Mix the two stock solutions in appropriate proportions (from 1:9 to 9:1) to buffer at specific $[\text{Ca}^{2+}]_{\text{free}}$ (see Tables 2-4 and (Tsien and Pozzan 1989)).

5. Image cells 24-72 hours after transfection or when sensor expression and cell density are appropriate for the experiment.

6. Gently remove the cell culture media from the imaging dish and replace it with 2 mL HHBSS. All solutions and cells should be kept at the desired temperature, humidity, % CO_2 , and % O_2 . Historically, this experiment has been performed in ambient air and at room temperature. 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.

7. Place the imaging dish on the microscope stage. Identify and focus on cells.

8. Set up acquisition parameters. 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.

9. 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

10. Measure the FRET ratio 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 sensors.

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 can take longer 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} .

11. Measure the FRET ratio of the sensor in cells as they are permeabilized in the presence of a metal chelate buffer solution that precisely defines the $[\text{Ca}^{2+}]_{\text{free}}$. First, make a 2X metal chelate buffer/permeabilizing solution in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HHBSS. Gently wash the cells 3-5 times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HHBSS, and work efficiently because cells are more delicate at this point. Pipet 1 mL $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HHBSS into the imaging dish and carefully add 1 mL of the 2X metal chelate buffer/permeabilizing solution. Immediately begin acquiring images every 15-20 seconds until the FRET ratio stabilizes at R_{final} .

Typical permeabilization agents include digitonin (final working concentration 5 to 25 μM), saponin (final working concentration 0.1%), or alamethicin (final working concentration 50 $\mu\text{g}/\text{mL}$). It is necessary to identify the most effective agent and appropriate working concentration empirically for each cell line. In our experience, an *in situ* titration of a mitochondrial (matrix) sensor is most successful using alamethicin because it effectively permeabilizes the mitochondrial membranes to ions and small molecules while retaining the sensor in the matrix. Excellent additional resources for parameters to optimize for *in situ* calibrations include a paper by Thomas et al. that details *in situ* calibrations for a range of small molecule Ca^{2+} indicators (Thomas et al. 2000), and another method for mitochondrial sensor calibration (Filippin et al. 2003).

See Troubleshooting

12. A typical image analysis workflow is shown in Figure 1. 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 each 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 determine R_{free} and R_{final} for each cell.

13. Repeat the imaging experiment several times to determine R_{free} and R_{final} at several different defined $[\text{Ca}^{2+}]_{\text{free}}$.

See Troubleshooting

14. Plot $R_{\text{final}} - R_{\text{free}}$ vs. the defined $[\text{Ca}^{2+}]_{\text{free}}$ in each imaging experiment, and fit data to several binding expressions, illustrated in Figure 2, using the least squares method (Goodrich and Kugel 2007). To determine the most appropriate binding expression, first consult the binding model used to fit *in vitro* data, if it's available. The binding model should not change between *in vitro* and *in situ*. Second, as a general principle, use the simplest expression that yields the best fit (i.e. R^2 value closest to 1) to determine the K_d , and the Hill coefficient (n). Third, visually evaluate the fits as expressions with more parameters (i.e. two-site models with or without Hill coefficients) often yield better fits as judged by the R^2 value, but are sometimes physically unrealistic as shown in Figure 2 (negative $R_{\text{final}} - R_{\text{free}}$ or $n = 27$). The best fit to the data presented in Figure 2 is the one-site Hill expression.

See Troubleshooting**Troubleshooting****Problem (Step 2)**

The desired $[\text{Ca}^{2+}]_{\text{free}}$ falls outside the range any of the metal-chelate buffers in Table 2.

Solution

If the desired $[\text{Ca}^{2+}]_{\text{free}}$ is $> 50 \mu\text{M}$, the solution does not need to be buffered because it is unlikely that the sensor or other Ca^{2+} -binding components would perturb these higher $[\text{Ca}^{2+}]$. Mixed metal chelate buffer systems, which contain a fixed amount of a competing metal ion (e.g. Mg^{2+} or Sr^{2+}), broaden the range of $[\text{Ca}^{2+}]_{\text{free}}$ buffers. Examples of mixed metal chelate buffer systems are presented in several publications (Dittmer et al. 2009; Qin et al. 2011; Tsien and Pozzan 1989; Vinkenborg et al. 2009).

Problem (Step 2)

The imaging buffer is at a pH other than 7.2 or 7.4.

Solution

The pH affects the protonation state of chelators like EDTA, EGTA, and HEDTA, and as a result affects the K_d' and the buffering range of the metal-chelate solution. Detailed explanations of how to re-calculate free Ca^{2+} concentrations at a different pH are available (Tsien and Pozzan 1989; Kao et al. 2010).

Problem (Step 9)

The FRET ratio is unstable before any 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. 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)

The FRET ratio does not change after membrane permeabilization, or the FRET ratio spikes after membrane permeabilization instead of stabilizing at R_{final} .

Solution

Change the permeabilization conditions so that upon permeabilization, there is a gradual increase in FRET ratio that reaches a plateau (see Figure 1). The permeabilizing reagent, its concentration, and the time of incubation should be optimized for each cell type.

Problem (Step 13)

R_{free} varies from experiment to experiment.

Solution

Some variation in R_{free} is expected due to cellular heterogeneity. The difference between R before any perturbation and R_{free} should be more consistent.

Problem (Step 14)

None of the binding expressions appear to fit the titration data.

Solution

Check the experimental conditions of your experiments. Environmental factors, such as temperature and pH, can significantly affect binding. Repeat cellular measurements of $R_{\text{final}} - R_{\text{free}}$ at different Ca^{2+} concentrations to fill in more of the binding curve.

Discussion

The FRET ratios reported by Ca^{2+} sensors can be converted to $[\text{Ca}^{2+}]_{\text{free}}$ using K_d' and n values derived from sensor titrations, but at a different temperature, pH, or salt

concentration, these values could differ significantly from the published values. This protocol describes methods that can be used to determine a sensor's K_d *in situ* when the accuracy of $[Ca^{2+}]$ estimation is critical. While there are many examples of no difference in the *in situ* and *in vitro* binding parameters, there are also well documented examples of significant differences that substantially influence conversion to $[Ca^{2+}]$ (Filippin et al. 2003; Rudolf et al. 2006; Thomas et al. 2000). Hence researchers are encouraged to carry out *in situ* titrations whenever possible.

This protocol also describes how to make and use metal chelate buffer solutions to set $[Ca^{2+}]_{free}$ to specific, sub-micromolar concentrations. These metal chelate buffer solutions can also be used to control extracellular or intracellular $[Ca^{2+}]_{free}$ for other applications (for example, to assess what $[Ca^{2+}]_{free}$ can trigger downstream cellular signaling). In fact, we find that these solutions, which control $[Ca^{2+}]_{free}$, have proven invaluable when using Ca^{2+} sensors, and that they can also be used to maintain low concentrations of other metal ions, such as Zn^{2+} .

Recipes

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.

EGTA stock solution (0.5 M)

EGTA = ethylene glycol-bis(2-aminoethylether)-*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, heating slightly (solution does not need to boil). 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⁺

Reagent	Final concentration
10X HBSS with 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. Mix well by stirring. 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 Ca^{2+} or Mg^{2+} ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HHBSS)

Reagent	Final concentration
10X HBSS <i>without</i> Ca^{2+} or 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 of 10X Ca^{2+} -, Mg^{2+} -free HBSS, 4.76 g HEPES and 2 g D-glucose. Mix well by stirring. 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 μL 100% DMSO to yield 10 mM stock solution. Mix well (vortex). Make 5 μL aliquots and store in the dark at -20°C . Dilute the 10 mM stock to 1 mM when preparing to use.

Metal chelate buffer system

Make two stock solutions for each metal chelate buffer system (refer to Tables 1-4). The procedure for making stock solutions for the Ca^{2+} -EGTA buffer system follows (Tsien and Pozzan 1989). Use the same procedure to make stock solutions of EDTA, HEDTA, or NTA. First, to make 10 mL of a 1 M EGTA solution, weigh out 3.84 g EGTA free acid (this is 1% over the theoretical weight of 10 mmol to account for 99% purity). Add 1.2 g KOH pellets and 6 mL water, stirring vigorously and heating slightly to get EGTA into solution. Cool to room temperature and add 10 N KOH to adjust pH to 8.0. Use a volumetric flask to bring the total volume up to 10 mL. We typically dilute this solution 100x to 10 mM EGTA in the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HHBSS solution.

Next, to use the pH titration method (Tsien and Pozzan 1989) to make 10 mL of a 1 M Ca^{2+} , 1 M EGTA solution. Weigh out 3.84 g EGTA free acid. Add 1.2 g KOH pellets, 0.95 g CaCO_3 (9.5 mmol), and 6 mL water, stirring vigorously and heating slightly to get EGTA into solution. Add 10N KOH, 10 μL at a time, until the pH is between 7 and 8. Add 20 μL 1 M CaCl_2 (0.2 mmol) and record the decrease in pH. Add ~30 μL 10N KOH to re-adjust the pH so that it stays between 6.5 and 8. Repeat the addition of CaCl_2 and KOH until the magnitude of the pH change after each addition of CaCl_2 decreases by 50%. Add 10N KOH to adjust pH to ~7.4. Use a volumetric flask to bring the total volume up to 10 mL. Using this method, the Ca^{2+} and EGTA concentrations should be within 0.5% of each other. This method can be performed with only a pH meter, but a Ca^{2+} electrode can also be used to accurately make this solution. We typically dilute this solution to 10 mM Ca^{2+} 10 mM EGTA in the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HHBSS solution.

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FIGURE 1.

Image analysis of one experiment in an *in situ* sensor titration. (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 FRET ratio of each cell is plotted over the course of the sensor calibration, and each line in the plot corresponds to FRET ratio of an ROI in a different cell. Notice the relatively stable FRET ratio before any perturbations, the increase in FRET ratio after the addition of the initial increase in FRET ratio after the addition of ionomycin and EGTA (Iono/EGTA) before stabilizing at R_{free} , and the gradual increase in FRET ratio after the addition of 10 μM and a defined $[\text{Ca}^{2+}]_{\text{free}}$, stabilizing at R_{final} .

FIGURE 2.

Fitted binding data from an *in situ* sensor titration. The same dataset was fit using the one-site or two-site model, with or without a Hill coefficient(s). The binding equation is shown in the lower right corner of each plot and the fitted parameter values are shown on the right side of each plot.

TABLE 1

$[\text{Ca}^{2+}]_{\text{free}}$ buffering ranges of some metal chelate buffer systems at 20°C and 25°C and 0.1 M ionic strength (Cheng et al. 1982; Smith et al. 2004; Tsien and Pozzan 1989). Each metal chelate buffer system can buffer $[\text{Ca}^{2+}]_{\text{free}}$ 10-fold above and below the K_d' . EDTA = ethylenedinitrilotetraacetic acid; EGTA = ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; HEDTA = *N*-(2-Hydroxyethyl)ethylenediamine-*N,N,N'*-triacetic acid.

Chelator	$K_d'(\text{Ca}^{2+})$ at pH 7.2 and 20°C	$K_d'(\text{Ca}^{2+})$ at pH 7.4 and 20°C	$K_d'(\text{Ca}^{2+})$ at pH 7.2 and 25°C	$K_d'(\text{Ca}^{2+})$ at pH 7.4 and 25°C
EDTA	32.2 nM	19.6 nM	32.6 nM	19.9 nM
EGTA	151 nM	60.5 nM	141 nM	56.7 nM
HEDTA	3.23 μM	2.03 μM	3.38 μM	2.12 μM

TABLE 2Specific $[\text{Ca}^{2+}]_{\text{free}}$ of Ca^{2+} -EDTA solutions at pH 7.2 and 25°C

Total $[\text{Ca}^{2+}]$ (mM)	10 mM EDTA (% volume)	10 mM Ca-EDTA (% volume)	Free $[\text{Ca}^{2+}]$ (nM)
1	90	10	3.62
2	80	20	8.15
3	70	30	14.0
4	60	40	21.7
5	50	50	32.6
6	40	60	48.9
7	30	70	76.1
8	20	80	130
9	10	90	293

TABLE 3Specific $[\text{Ca}^{2+}]_{\text{free}}$ of Ca^{2+} -EGTA solutions at pH 7.2 and 25°C

Total $[\text{Ca}^{2+}]$ (mM)	10 mM EGTA (% volume)	10 mM Ca-EGTA (% volume)	Free $[\text{Ca}^{2+}]$ (nM)
1	90	10	15.7
2	80	20	35.3
3	70	30	60.4
4	60	40	94.0
5	50	50	141
6	40	60	212
7	30	70	329
8	20	80	564
9	10	90	1.27×10^3

TABLE 4Specific $[\text{Ca}^{2+}]_{\text{free}}$ of Ca^{2+} -HEDTA solutions at pH 7.2 and 25°C

Total $[\text{Ca}^{2+}]$ (mM)	10 mM HEDTA (% volume)	10 mM Ca-HEDTA (% volume)	Free $[\text{Ca}^{2+}]$ (μM)
1	90	10	0.376
2	80	20	0.845
3	70	30	1.45
4	60	40	2.25
5	50	50	3.38
6	40	60	5.07
7	30	70	7.89
8	20	80	13.5
9	10	90	30.4