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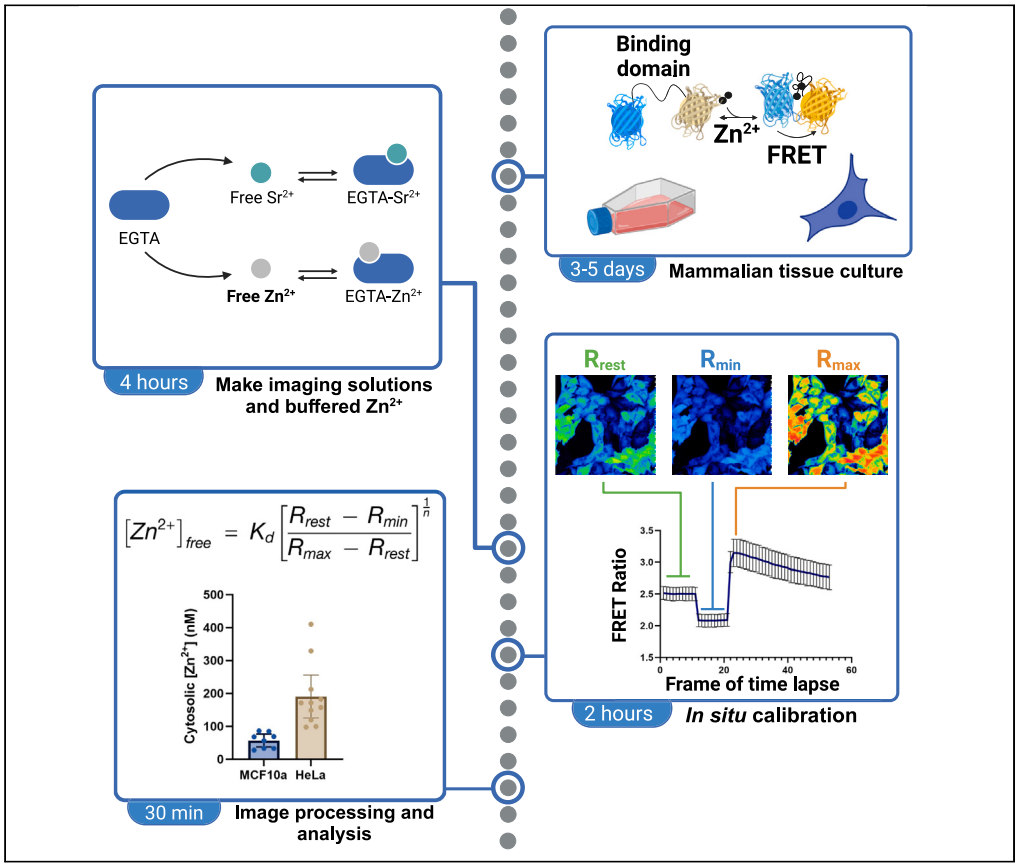
Protocol for measuring labile cytosolic Zn^{2+} using an *in situ* calibration of a genetically encoded FRET sensor



Zinc (Zn^{2+}) plays roles in structure, catalysis, and signaling. The majority of cellular Zn^{2+} is bound by proteins, but a fraction of total Zn^{2+} exists in a labile form. Here, we present a protocol for measuring labile cytosolic Zn^{2+} using an *in situ* calibration of a genetically encoded Förster resonance energy transfer (FRET) sensor. We describe steps for producing buffered Zn^{2+} solutions for performing an imaging-based calibration and analyzing the imaging data generated to determine labile Zn^{2+} concentration in single cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Generate a highly precise buffered Zn^{2+} solution using a two-ion buffer system

Calibrate a Zn^{2+}-specific FRET sensor via fluorescence microscopy

Analyze microscopy images and calculate cytosolic-free Zn^{2+}

Holtzen et al., STAR Protocols 5, 103130
June 21, 2024 © 2024 The Author(s). Published by Elsevier Inc.
https://doi.org/10.1016/j.xpro.2024.103130

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Protocol for measuring labile cytosolic Zn^{2+} using an *in situ* calibration of a genetically encoded FRET sensor

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https://doi.org/10.1016/j.xpro.2024.103130

SUMMARY

Zinc (Zn^{2+}) plays roles in structure, catalysis, and signaling. The majority of cellular Zn^{2+} is bound by proteins, but a fraction of total Zn^{2+} exists in a labile form. Here, we present a protocol for measuring labile cytosolic Zn^{2+} using an *in situ* calibration of a genetically encoded Förster resonance energy transfer (FRET) sensor. We describe steps for producing buffered Zn^{2+} solutions for performing an imaging-based calibration and analyzing the imaging data generated to determine labile Zn^{2+} concentration in single cells.

For complete details on the use and execution of this protocol, please refer to Rakshit and Holtzen et al.[1]

BEFORE YOU BEGIN

The metal ion zinc (Zn^{2+}) is an essential micronutrient. It binds thousands of proteins and plays roles in catalysis and protein structure.[2,3] Although there are hundreds of micromolar total Zn^{2+} in a typical mammalian cell, most of this is bound, buffered, or otherwise sequestered.[4] The labile Zn^{2+} levels in cells vary by cell type but are typically on the order of hundreds of picomolar.[1,5–7] In response to extracellular and intracellular signals, cells can increase or decrease labile Zn^{2+} by releasing it from intracellular stores such as buffering proteins and organelles.

In recent years, fluorescent biosensors based on Förster resonance energy transfer (FRET) have become prevalent for quantifying labile metal ion levels and dynamics.[8] To precisely assess cytosolic labile Zn^{2+} levels, we used the Zn^{2+} specific FRET-based biosensor ZapCV2, which consists of a Zn^{2+}-responsive element sandwiched between two fluorescent proteins capable of participating in FRET.[9,10] The Zn^{2+}-responsive element in ZapCV2 consists of two zinc fingers from the yeast Zap1 transcription factor. When the Zn^{2+} concentration increases, the zinc finger domains bind Zn^{2+} and undergo a conformational change that changes the distance and orientation between the two fluorescent proteins (in this case ECFP and circularly permuted mVenus). In such biosensors, researchers typically measure the FRET channel (excitation of ECFP, emission from cpVenus) and the donor channel (excitation of CFP, emission from CFP) and report the ratio of FRET/CFP. An increase in the FRET ratio (FRET signal / CFP signal) is proportional to an increase in free Zn^{2+} concentration in the compartment to which the sensor is targeted.

Since sensor expression varies from cell to cell, it is crucial to conduct an *in situ* calibration of the sensor to accurately quantify labile Zn^{2+} concentrations in single cells. Calibration of ZapCV2 involves depleting cells of Zn^{2+} using a metal ion chelator tris(2-pyridylmethyl) amine (TPA) to get the lowest FRET ratio of the sensor in that cell (R_{min}), then forcing Zn^{2+} into the cell by using

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pyrithione, saponin, and excess Zn^{2+} to get the highest FRET ratio of the sensor (R_{max}). It is critical to optimize *in situ* calibration conditions for your particular cell type as inaccurate calibration can lead to erroneous estimates of labile Zn^{2+}.[11] This protocol will outline the steps for preparing the buffers necessary for a FRET sensor calibration, conducting the calibration in MCF10a cells, and analyzing the data to calculate labile Zn^{2+} in single cells. While this protocol focuses on MCF10a cells expressing a nuclear excluded version of ZapCV2, the approach should work in any cell type.

Cell culture of MCF10a cells expressing NES-ZapCV2

Timing: 5–7 days

1. Thaw one vial of MCF10a cells expressing NES-ZapCV2 quickly in a 37°C water bath.
   a. Transfer to a 15 mL conical tube containing 5 mL of MCF10a media.
   b. Centrifuge at 500 × *g* for 5 min.
   c. Aspirate supernatant and resuspend in 10 mL of media.
   d. Transfer cell suspension to a 10 cm dish.

   Note: MCF10a media is composed of 1:1 DMEM/F12 supplement, 5% horse serum, 20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, and 1% penicillin/streptomycin antibiotics.

2. Culture cells in a 10 cm dish at 37°C, 5% CO_2 and at 90% humidity until 80% confluent.
3. Passage cells 1:5 every 2–3 days and record passage number.

   Alternatives: Researchers may not have access to resources to create stable cell lines. In this case, transient transfection such as lipofectamine or electroporation is a simple way of expressing the NES-ZapCV2 sensor. Please refer to the manufacturer's protocol for transfection parameters.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Tris(2-pyridylmethyl) amine 98% (TPA)	Sigma-Aldrich	Cat# 723134
Zinc chloride, anhydrous, 99.95% (metals basis)	Alfa Aesar	Cat# 87900
Chelex-100, sodium form	Sigma-Aldrich	Cat# C7901
DMEM/F12, HEPES	Thermo Fisher Scientific	Cat# 11330057
Horse serum, New Zealand origin	Thermo Scientific	Cat# 16050122
Hydrocortisone	Sigma-Aldrich	Cat# H4001
Gibco EGF recombinant human protein	Thermo Fisher Scientific	Cat# PHG0313
HEPES	Sigma-Aldrich	Cat# H4034-1KG
Calcium chloride, 99.9%	Sigma-Aldrich	Cat# 449709-10G
Potassium chloride	Sigma-Aldrich	Cat# P9541-500G
Magnesium chloride hexahydrate	Sigma-Aldrich	Cat# 63068-250G
Sodium chloride	Sigma-Aldrich	Cat# S9625-1KG
Dextrose (D-glucose)	Sigma-Aldrich	Cat# D9434-500G
Pyrithione	MedChem Express	Cat# HY-B1747
Saponin	Sigma-Aldrich	Cat# 47036-50G-F
Cholera toxin	Sigma-Aldrich	Cat# C8052
Potassium hydroxide, ACS reagent >85%	Sigma-Aldrich	Cat# 221473-25G
Pen/Strep	Gibco	Cat# 15140–122
0.05% Trypsin-EDTA(1X)	Gibco	Cat# 25300–120

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
EGTA, molecular biology grade	MilliporeSigma	Cat# 324626-25G
Strontium chloride, anhydrous, 99.99+%	Sigma-Aldrich	Cat# 439665-25G
Sodium hydroxide pellets	Fisher Scientific	Cat# S318-500
Experimental models: Cell lines		
Human: MCF10a + PB-NES ZapCV2 and PB-H2B- mCherry (stable)	Lo et al.[5]	N/A
Software and algorithms		
Nikon Elements Software	Nikon, Inc.	N/A
ImageJ/Fiji	NIH	N/A
Microsoft Excel	Microsoft, Inc.	N/A
BioRender	BioRender	https://biorender.com/
Interactive Shiny App	This work	https://holtzy.shinyapps.io/free_metals_app
Recombinant DNA		
pcDNA3.1(+)-NES-ZapCV2 (cpV143) for transient transfection	Fiedler et al.[9]	Addgene: #112060
Other		
Glass bottom dish 35 mm, #1.5 glass	ibidi	81218-200
Nikon Ti-E wide-field microscope (or any wide-field microscope) outfitted with an emission filter wheel capable of detecting FRET	Nikon, Inc.	N/A

MATERIALS AND EQUIPMENT

HHBSS (PO_4^{3-} free; pH 7.4)		
Component	Final concentration	Amount (500 mL)
$CaCl_2$ (1 M)	1.26 mM	0.63 mL
KCl (1 M)	5.4 mM	2.7 mL
$MgCl_2$*$6H_2O$ (1 M)	1.1 mM	0.55 mL
NaCl	137 mM	4 g
D-Glucose	16.8 mM	1.5 g
HEPES	30 mM	3.572 g
Chelex-treated water	N/A	500 mL
Total	N/A	500 mL
Prepare and adjust pH using NaOH. After sterile filtration, store at 15°C–25°C indefinitely.		

HHBSS (PO_4^{3-}, Ca^{2+}, Mg^{2+} free; pH 7.4)		
Component	Final concentration	Amount
KCl (1 M)	5.4 mM	2.7 mL
NaCl	137 mM	4 g
D-Glucose	16.8 mM	1.5 g
HEPES	30 mM	3.57 g
Chelex-treated water	N/A	500 mL
Total	N/A	500 mL
Prepare and adjust pH using NaOH. After sterile filtration, store at 15°C–25°C indefinitely.		

R_{min} solution (2X)		
Component	Volume	Final concentration
HHBSS (PO_4^{3-} free)	9.95 mL	-
TPA (20 mM stock)	50 μL	100 μM
Total	10 mL	-
Prepare solution fresh before each calibration.		

R_{max} solution (2X)		
Component	Volume	Final concentration
HHBSS (Ca^{2+} / Mg^{2+} / PO_4^{3-} free)	9.75 mL	-
Saponin (0.1 % stock)	200 μL	0.02%
Pyrithione (500 μM stock)	30 μL	1.5 μM
Solution A	100 μL	-
Solution B	100 μL	-
Total	10 mL	-

Prepare fresh before each calibration.

⚠ CRITICAL: To acquire FRET images, the microscope must be able to acquire images for the donor fluorescent protein and the FRET channel nearly simultaneously (a filter wheel will enable images to be collected within ~ 20 ms, moving the dichroic turret will allow images to be collected within ~ 500 ms). The best approach is to use an emission filter wheel to swap between the donor emission wavelength and the acceptor emission wavelength. Researchers should identify whether their microscope setups are able to change filters this quickly.

Note: Many common buffering systems such as phosphate or Tris will interact detrimentally with Zn^{2+} ions in solution. For example, zinc phosphate is highly insoluble in water, and Tris can act as a chelator of metal ions in solution.[12] As such, we use a HEPES-buffered solution, which does not interact with Zn^{2+} in solution.

STEP-BY-STEP METHOD DETAILS

Prepare the calibration solutions and buffered Zn^{2+} solutions

Timing: 4 h

Here, we describe steps for making a buffered Zn^{2+} solution using the pH jump method,[13,14] EGTA as the chelator, and Sr^{2+} as the counterion. These solutions will be used in the final *in situ* calibration to deliver controlled levels of labile Zn^{2+} to the cells during the R_{max} phase of the calibration.

1. Treat MilliQ water with Chelex-100 to remove any free metal ions in solution.
   a. Add 1 g/L of Chelex-100 to MilliQ water in a plastic container and stir for at least 16 h. Store in plastic bottles indefinitely.
2. Soak glassware in metal-free nitric acid for at least 1 h.
   a. Rinse glassware thoroughly with Chelex-treated MilliQ water.
   b. Dry at least 16 h on paper towels before use.

⚠ CRITICAL: All water in the following steps must be treated with Chelex-100 to remove excess metal ions. Since these solutions are highly precise, small changes in metal ion composition will change the overall free Zn^{2+} concentration. In addition, all glassware must be washed with nitric acid and rinsed with Chelex-100 treated MilliQ water to remove residual metal ions.

3. Make Solution A (EGTA, $ZnCl_2$, and $SrCl_2$ buffered solution).
   a. Weigh out 5 mmol of high purity EGTA, 4.5 mmol of high purity $ZnCl_2$, and 1.225 g KOH pellets.
   b. Combine EGTA, $ZnCl_2$, and KOH in a clean metal-free beaker and add 15 mL Chelex-treated MilliQ water.
   c. Heat slightly while stirring. Add 1.0 M KOH in Chelex-treated water incrementally until pH is between 7 and 8.5.

d. Prepare a 50 mL 1.0 M $ZnCl_2$ solution in Chelex-treated water in a volumetric flask.
e. Prepare 50 mL of a 1.0 M $SrCl_2$ solution in Chelex-treated water using a volumetric flask.
f. Note the pH of the Zn-EGTA solution to the thousandths place.
g. Add a known number of mmol of $ZnCl_2$ solution by pipetting between 20–40 μL of the $ZnCl_2$ solution into the Zn-EGTA solution.

Note: While adding $ZnCl_2$ solution, make sure to dip the pipette tip into the solution and mix it properly. Always use a new pipette tip for mixing.

h. Calculate the ΔpH/ΔZn. This is the starting value.
i. Add the $ZnCl_2$ solution, record the pH and the ΔpH/ΔZn with each addition of $ZnCl_2$.
j. Add aliquots of the KOH solution as necessary to keep the pH within the 7–8.5 regime. An example titration is shown in Table 1.
k. When the ΔpH/ΔZn is less than $^1/_2$ the original value, the titration is complete.
l. Add aliquots of the KOH solution until the pH of solution A is 7.0, then transfer the titrated solution to a 50 mL volumetric flask.
m. Add 10.0 mL of the $SrCl_2$ solution and bring the volume of the solution to the 50 mL mark with Chelex-treated water.
n. Transfer the solution to a clean plastic container. This solution can be stored at room temperature (15–25°C) indefinitely.

⚠ CRITICAL: All chemicals contain impurities. Due to the precise nature of this titration, researchers must consider impurity when measuring chemical masses. For example, if the EGTA used is 95% purity, the mass used will be 1.05 times the "ideal" mass of 5 mmol 100% purity EGTA.

Note: The pH must be in the neutral range for EGTA to dissolve completely. This will require heating and stirring to dissolve completely. A slightly acidic pH may be required for dissolving $ZnCl_2$.

4. Make Solution B (EGTA and $SrCl_2$ buffered solution).
   a. Weigh out 5 mmol of high purity EGTA.
   b. Weigh out 1.2 g of KOH pellets.
   c. Place in a clean, dry flask and add 10.0 mL of 1.00 M $SrCl_2$ solution. Note the pH.
   d. Add aliquots of 1.0 M KOH until the pH is 7.0.
   e. Transfer solution to a clean, dry volumetric flask and bring volume to 50 mL with Chelex-treated MilliQ water.
   f. Transfer to a clean dry plastic container. This solution can be stored at room temperature (15–25°C) indefinitely.

Pause point: Solutions A and B may be prepared well in advance and can be stored at 15°C–25°C indefinitely.

Note: The ratio of solution A and solution B in R_{max} may need to be optimized for your cell line, but a good starting place is a 5:5 ratio of A:B (Figure 1C).

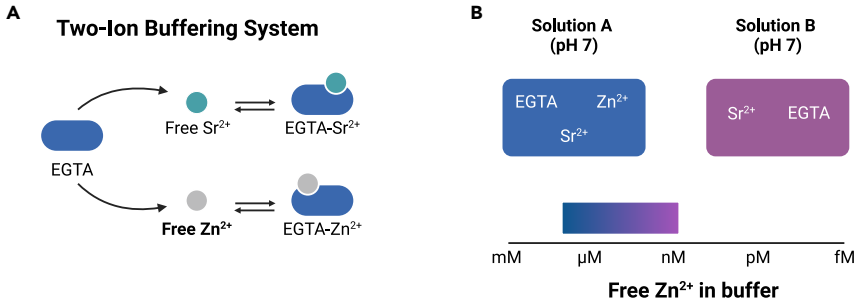
⚠ CRITICAL: Simply weighing out the masses of each chemical and salt will result in a large error, and therefore an imprecise free Zn^{2+} concentration in the final buffer. In order to make these solutions, we use a modified version of the pH jump method.[13,14] When Zn^{2+} binds to EGTA at neutral pH, it displaces two H^+ ions, which lowers the pH. Since H^+ concentration can be very precisely calculated using a pH meter, we use pH as a readout for the titration. We then titrate in Zn^{2+} and record the pH to monitor the titration progression. We maintain the pH of the solution between 7–8 using potassium hydroxide as needed. As the Zn^{2+}

Table 1. Example buffered Zn^{2+} titration

Added KOH/$ZnCl_2$ (mmol)	pH	ΔpH	ΔpH/ΔZn
+ 0 $ZnCl_2$	7.47	–	–
+ 20 $ZnCl_2$	7.27	0.2	10
+ 40 KOH	7.46	–	–
+ 20 $ZnCl_2$	7.2	0.2	10
+ 40 KOH	7.45	–	–
+ 20 $ZnCl_2$	7.27	0.18	9
+ 40 KOH	7.45	–	–
+ 20 $ZnCl_2$	7.26	0.19	9.5
+ 40 KOH	7.43	–	–
+ 20 $ZnCl_2$	7.25	0.18	9
+ 40 KOH	7.42	–	–
+ 40 $ZnCl_2$	7.10	0.32	8
+ 100 KOH	7.51	–	–
+ 40 $ZnCl_2$	7.18	0.33	8.25
+ 100 KOH	7.61	–	–
+ 40 $ZnCl_2$	7.26	0.35	8.75
+ 100 KOH	7.72	–	–
+ 40 $ZnCl_2$	7.33	0.39	9.75
+ 100 KOH	7.80	–	–
+ 40 $ZnCl_2$	7.43	0.37	9.25
+ 0 KOH	7.43	–	–
+ 40 $ZnCl_2$	7.15	0.28	7
+ 80 KOH	7.42	–	–
+ 40 $ZnCl_2$	7.17	0.25	6.25
+ 100 KOH	7.53	–	–
+ 40 $ZnCl_2$	7.26	0.27	6.75
+ 100 KOH	7.64	–	–
+ 40 $ZnCl_2$	7.36	0.28	7
+ 80 KOH	7.66	–	–
+ 40 $ZnCl_2$	7.42	0.26	6.5
+ 80 KOH	7.71	–	–
+ 40 $ZnCl_2$	7.47	0.24	6
+ 80 KOH	7.77	–	–
+ 20 $ZnCl_2$	7.55	0.22	5.5

concentration approaches the EGTA concentration, the number of H^+ ions it displaces will decrease, indicating that the Zn^{2+} concentration is within the buffering regime of EGTA (Table 1). The addition of Sr^{2+} acts as a counterion to sequester EGTA to tune the free Zn^{2+} concentration to the desired value. It is important to use plastic containers to store components, since glass can leach and retain metal ions. In addition, chemical weights must be corrected for the purity of the chemical to ensure precise molarity.

Note: It is necessary to ensure a stable concentration of labile Zn^{2+} throughout the R_{max} phase of the calibration. To do this, we use a buffer system that has a precise Zn^{2+} concentration, which has been shown to yield more accurate R_{max} values during the final stage of the calibration than high concentrations of unbuffered zinc.[11] We combine a solution of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) Zn^{2+} and Sr^{2+} (Solution A) with a solution of Sr^{2+} and EGTA (Solution B) (Figures 1A and 1B). The ratio of these two solutions will determine the concentration of free Zn^{2+} in the R_{max} buffer (Figure 1C). It is important to note that any chelator-counterion pair can be used in this system to accurately tune the free Zn^{2+} as long as the affinity of the counterion to the chelator is less than that of Zn^{2+}. It is also crucial that solutions A and B are at the exact same pH, since free Zn^{2+} in these solutions is pH dependent. For most cell lines, we use Sr^{2+} and EGTA as the chelator-counterion pair



C

Solution A	Solution B	Total Zn	Free Zn
1 part	9 parts	0.1 mM	8.26 nM
2 parts	8 parts	0.2 mM	20.2 nM
3 parts	7 parts	0.3 mM	37.7 nM
4 parts	6 parts	0.4 mM	63.1 nM
5 parts	5 parts	0.5 mM	101.4 nM
6 parts	4 parts	0.6 mM	162.1 nM
7 parts	3 parts	0.7 mM	187.8 nM
8 parts	2 parts	0.8 mM	485.2 nM
9 parts	1 part	0.9 mM	1.14 μM

Figure 1. Buffered Zn^{2+} system

(A) Example of a two-ion buffering system with EGTA as the chelating agent, and Sr^{2+} as the counterion. Varying Sr^{2+} concentration will act to sequester EGTA to tune free Zn^{2+} in the buffer.

(B) Components of solutions A and B (top) and the range of free Zn^{2+} that can be achieved using this buffering system

(C) A chart illustrating varying Solution A:Solution B ratios and their corresponding free Zn^{2+} concentrations at pH 7.0 in the final R_{max} buffer. Figure created with BioRender.

since this pair can buffer Zn^{2+} between 8 nM and 1.1 μM at pH 7.0. Another option for an ion-counterion-chelator system is Zn^{2+}/Ca^{2+}/EGTA.

Note: To precisely calculate the free metal concentration of this two-ion buffer system, we use a set of equations, established apparent dissociation constants for each metal/chelator pair ($K_{D_{ML}}^{app}$), ionic strengths, and pK_a values for the chelators at a constant temperature and pH:

$$[M_2] = \frac{\Delta - K_{D_{ML2}}^{app}}{2(1+R)} + \sqrt{\left(\frac{\Delta - K_{D_{ML2}}^{app}}{2(1+R)}\right)^2 + \frac{[M_2]_T \times K_{D_{ML2}}^{app}}{1+R}} \quad \text{(Equation 1)}$$

$$\Delta = [M_1]_T + [M_2]_T - [L]_T \quad \text{(Equation 2)}$$

$$R = \frac{K_{D_{ML1}}^{app}}{K_{D_{ML2}}^{app}} \times \frac{[ML1]}{[ML2]} \quad \text{(Equation 3)}$$

$$K_{D_{ML}}^{app} = \left[1 + 10^{(pKa_1 - 0.11 - pH)} + 10^{(pKa_1 + pKa_2 - 0.22 - 2pH)}\right] \times 10^{pK_M} \quad \text{(Equation 4)}$$

$$\frac{[ML1]}{[ML2]} = \frac{[L]_T}{[M_2]_T - [M_2]} - \frac{K_{D_{ML2}}^{app}}{[M_2]} - 1 \quad \text{(Equation 5)}$$

where M_2 is the metal whose free concentration we are varying (in this case Zn^{2+}), M_1 is the competing metal with lower affinity for the chelator (in this case Sr^{2+}), and L is the chelator (in this case EGTA). Additionally, any variable with a subscript "T" refers to the total concentration of that species in solution. These equations and sample calculations are presented in Table S1. For our purposes, we will be using the affinity constants of EGTA for Zn^{2+} (12.6) and Sr^{2+} (8.43) at 25°C, as well as the two relevant pK_a values for EGTA (pK_{a1} = 9.40, pK_{a2} = 8.78) at 25°C. Table S2 presents the K_d and pK_a values for a series of metal chelators. Since all $K_{D_{ML}}^{app}$ values were

Table 2. Phases and imaging frequencies for the *in situ* calibration

Phase	Treatment	Capture frequency	Imaging duration
R_{rest}	Unperturbed cells in a resting state	1 frame every 30 s	5–10 min
R_{min}	Treatment of cells with a Zn^{2+} chelator	1 frame every 30 s	5 min
R_{max}	Treatment of cells with buffered Zn^{2+}, an ionophore, and a detergent	1 frame every 10 s	5–20 min

determined empirically at ionic strength 0.1 M, we will correct the $K_{D_{ML}}^{app}$ to reflect this different ionic strength and solution pH in Equation 4. For this protocol, all of the calculations have been done assuming free Zn^{2+} as M_2 and Sr^{2+} as M_1, and free Zn^{2+} values are available in Figure 1C. It is worth noting that the $K_{D_{ML}}^{app}$ calculation assumes two relevant pK_a values. If your chelator has only one, re-factor Equation 4 to reflect this. Note that Equation 5 contains $[M_2]$, which is the unknown value for which we are solving. To resolve this, we include an initial "guess" of the free $[M_2]$, then iteratively calculate $[M_2]$ based on the initial guess until the value converges on the true $[M_2]$ value. We have attached an interactive spreadsheet in supporting information that contains logK and pK_a values of commonly used chelators and counterions as well as example calculations. In addition, we have deployed an interactive Shiny App to easily calculate the range of free metals values for several chelator/ion/counterion systems (https://holtzy.shinyapps.io/free_metals_app/).

Live cell *in situ* calibration of the cytosol-targeted ZapCV2 sensor

Timing: 2 h (plus cell plating and recovery)

Here, we outline steps to conduct *an in* situ calibration of the NES-ZapCV2 sensor in order to calculate the concentration of resting labile cytosolic Zn^{2+}. This is done by first imaging the cells in their native resting state, then depleting labile Zn^{2+} from the cells using a membrane permeable chelator TPA. The media is changed, and cells are treated with buffered Zn^{2+}, pyrithione, and saponin, which floods the cells with excess Zn^{2+} and saturates the sensor (Table 2, Methods video S1).

5. Trypsinize MCF10a cells expressing the NES-ZapCV2 sensor.
   a. Remove the 10 cm maintenance dish from the incubator, aspirate off the media and wash the cells two times with 7–10 mL of 1X PBS. Cells should be 80% confluent and will yield approximately 5 million cells.
   b. Add 1 mL of 0.05% trypsin-EDTA to the dish and allow to incubate for 15 min at 37°C in the tissue culture incubator.
   c. Quench the trypsin with 8 mL of growth media and wash the plate with the cell suspension to remove remaining attached cells.
   d. Centrifuge cell suspension at 500 × g for 5 min to pellet.
   e. Resuspend cells in 1 mL growth media and count using a hemocytometer or an automated cell counter.
6. Plate 300,000 cells per 35 mm dish in 1 mL treatment media.
7. Allow to adhere to the dish at least 16 h in the tissue culture incubator.
8. Thirty minutes before the imaging experiment, remove one dish from the tissue culture incubator.
9. Wash the imaging dish three times with 2 mL of imaging media (HHBSS, PO_4^{3-} free pH 7.4).
10. Aspirate media and replace with 1 mL of fresh imaging media.
11. Allow the dish to sit at room temperature (20–25°C) for 30 min.

Note: Do not place the dishes back in the tissue culture incubator. HEPES buffer cannot buffer CO_2 and will quickly acidify and kill the cells.

Note: The imaging media must be equilibrated to room temperature when washes and imaging take place. Perform this imaging experiment at room temperature. Fluctuations in temperature can cause changes in the FRET response.

12. Make HHBSS (both PO_4^{3-}, Ca^{2+}, Mg^{2+} free, and PO_4^{3-} free), 2X R_{min} solution, and 2X R_{max} solution as defined in the materials and equipment section.
13. Set up acquisition parameters while the imaging dish is equilibrating.

Note: Depending on the acquisition software available, researchers may need to manually acquire images at these time points or set up an automatic acquisition. There are three phases to the calibration R_{rest} (resting state of ZapCV2), R_{min} (unbound state of ZapCV2) and R_{max} (bound state of ZapCV2) as described in Methods video S1.

14. Place cells on the microscope using a 35 mm dish holder, ensuring the metal arms hold the dish firmly in place.

Note: During the calibration, there is a high chance of moving the dish during buffer addition and washing which can result in the loss of the field of view and therefore the calibration of that dish. To minimize the odds of bumping the dish, use a 35 mm dish holder with metal arms to keep the dish firmly in place. While removing solution from the imaging dish try to stabilize the dominant hand with other hand and try to avoid touching the base of dish with the pipette tip.

15. Switch to a 20X air objective and focus the cells using the donor channel (in this case, CFP_{ex}, CFP_{em}), ensuring that the field of view contains multiple cells (problem 1).
16. Draw regions of interest (ROI) in the cytosol of each cell, in addition to at least three regions of interest in the empty space between cells, which will be recorded as background (Methods video S1).

Optional: You may acquire the calibration before drawing the ROIs. It is suggested that you draw ROIs to monitor sensor response throughout the calibration. Adding an ROI and tracking the signals across time will be different in each acquisition software. Consult the user's manual for more information.

17. Monitor the donor channel (CFP_{ex}, CFP_{em}) and the FRET channel (CFP_{ex}, YFP_{em}) and ensure the exposure times are the same for both channels.
18. Ensure the optical configurations are set as follows:
    a. CFP Ex: 440/20, 455 dichroic, Em: 480/20.
    b. CFP/YFP FRET Ex: 440/20, 455 dichroic, Em: 540/21.

Optional: YFP Ex: 510/25, 518 dichroic, Em: 540/21.

19. Begin the first phase R_{rest} and monitor for at least 5 min and ensure that the FRET ratio does not change appreciably before beginning the next phase of the calibration.
    a. If the resting FRET ratio is unstable for 5 min, the R_{rest} phase can be extended until the FRET ratio is stable (Methods video S1).
20. Pause the acquisition and add 1 mL of the 2X R_{min} solution (final concentration of 50 μM TPA).
21. Resume the acquisition.
22. Monitor the R_{min} for at least 3 min and ensure that the R_{min} value is not changing before beginning the next phase of the calibration (problem 2).

Note: Upon addition of R_{min} solution, there will be a sudden drop in FRET ratio and then R_{min} value will remain unchanged. The donor channel (YFP) will not change appreciably upon addition of the R_{min} solution (Figures 2B and 2C, Methods video S1).

23. Pause the acquisition.
24. Remove as much of the calibration solution as possible making sure not to touch the dish with the pipette tip.

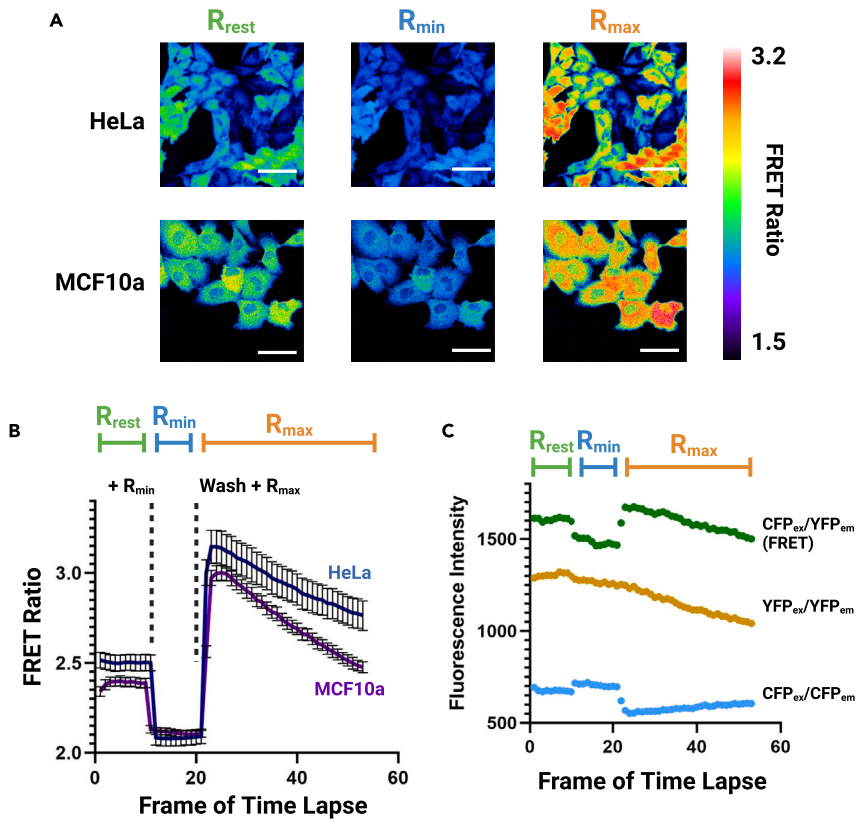


Figure 2. *In situ* calibration of NES-ZapCV2 in HeLa and MCF10a cells

(A) Sample FRET ratio image of cells in R_{rest} buffer, after exposure to R_{min} buffer, and after exposure to R_{max} buffer in both HeLa and MCF10a cells. Scale bar = 60 μm.

(B) A plot of the average FRET ratio of cells after each perturbation in both MCF10a and HeLa cells. Phases of the calibration are labeled above the curves. Error bars represent 95% confidence intervals. n = 7 cells.

(C) The corresponding CFP, YFP, and FRET intensities during each phase of the calibration. Phases of the calibration are labeled above the data points. Figure created with BioRender.

25. Quickly wash twice with 1 mL of imaging buffer (HHBSS, PO_4^{3-}, Ca^{2+}, Mg^{2+} free; pH = 7.4).
26. Remove the buffer each time and resupply with 1 mL of imaging buffer to the dish.
27. Start acquiring the R_{max} phase of the calibration and add 1 mL of R_{max} buffer.
28. Collect one frame every 10 s until the signal peaks and begins to decline (Figures 1B and 1C) (problem 2, problem 3).

Note: The donor channel (YFP) will not change appreciably upon addition of the R_{max} solution but may decrease slightly due to photobleaching from a higher acquisition rate (Figures 2B and 2C, Methods video S1).

Note: The R_{max} buffer will require optimization in new cell lines. If the FRET ratio peaks very quickly and immediately drops off after adding the R_{max} it is likely that the true R_{max} has not been achieved. Therefore, researchers will need to change concentrations of pyrithione, saponin, or the ratio between solutions A and B to yield a steady rise and constant plateau in FRET signal after R_{max} addition (Figure 2B). Using high concentrations of unbuffered Zn^{2+} is not encouraged, since this can lead to a quick plateau and decay. For more information and examples of reagent optimization, refer to Carter et al., 2017, Analytical Chemistry.[11]

Note: During R_{max}, cells will begin to change shape and peel off the dish and become apoptotic due to the pyrithione, Zn^{2+} and Sr^{2+} in the R_{max} solution. This is normal. Once cell death becomes apparent, end the acquisition. The cells cannot be calibrated again.

29. Save each time-lapse image in either the native format (.nd2, .rwl, .orf) or as a TIFF stack (.tif) for downstream analysis.

Image processing and analysis

Timing: 30 min

This step enables researchers to extract FRET ratios of each cell, background correct the FRET signal, and calculate the labile Zn^{2+} ion concentration in cells.

30. Open one time-lapse image in ImageJ/Fiji.
31. Draw ROIs by selecting the "Oval ROI" tool.
    a. Draw several ROIs in different cells, as well as at least one ROIs in an area of the image devoid of cells (this will serve as a background ROI).
    b. After drawing each ROI, right click and select "Add to ROI Manager." A new window will pop up and list your ROIs.
    c. Alternatively, select *Analyze* > *Tools* > *ROI Manager…* to open the ROI manager and manually add each ROI by pressing the hotkey "t".

Note: Since cells may move or change shape over time, ensure that the region covers the cytosol of one cell throughout the time lapse image.

32. Select *Analyze* > *Set Measurements…* and ensure that only "mean gray value" is selected.
33. Deselect all other measurements.
34. In the ROI manager, select *More* > *Multi Measure*.
35. Check "Measure all X slices" and "One row per slice" then click OK.
36. Copy data to an Excel spreadsheet.
    a. Measurements from each ROI at one frame are listed on the rows of the table, and each ROI is listed in each column.
    b. The measurements of one channel are in rows 1, 3, 5, etc. and the other are in 2, 4, 6, etc.
37. Subtract the background intensities at each timepoint from the intensities of the test ROIs.

Note: The background intensities will change over the time lapse, so ensure the background is subtracted with its time-paired ROI.

38. Calculate the FRET ratio by dividing the background-subtracted FRET channel by the background-subtracted donor channel.
39. Find the mean R_{rest} ratio of each ROI by averaging the FRET ratios during the R_{rest} phase of the calibration.

Note: If the R_{rest} was unstable (increasing or decreasing), only average the FRET ratios for the region of the curve that was stable, typically right before changing to the R_{min} solution.

40. Find the mean R_{min} ratio of each ROI by averaging the FRET ratios for the last few data points of the R_{min} phase of the calibration.
41. Find the R_{max} by finding the maximum FRET ratio during the R_{max} phase of the calibration.
42. Use the following equations to calculate the $[Zn^{2+}]_{cytosol}$ for each ROI, where K_d = 5.3 nM, and n = 0.29[10], which were determined empirically for this specific sensor, ZapCV2, for Zn^{2+}.

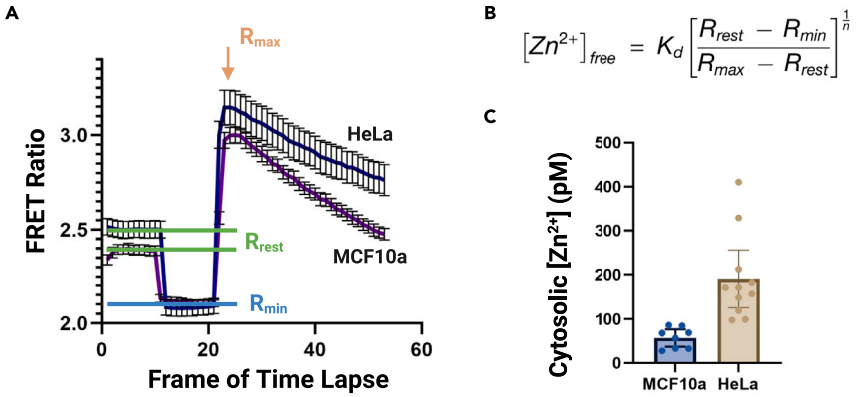


Figure 3. Image processing and analysis examples

(A) Average traces of FRET ratio throughout *an in situ* calibration for both MCF10a and HeLa cells. R_{rest}, R_{min}, and R_{max} are labeled on the graph.

(B) The equation for calculating labile Zn^{2+} using the R_{rest}, R_{min} and R_{max} parameters, the Hill coefficient (n) and the sensor's apparent K_d.

(C) Quantification of resting cytosolic Zn^{2+} concentration in MCF10a and HeLa cells using the equation in (B) Error bars represent the 95% confidence interval. Figure created with BioRender.

$$FRET\ ratio = \frac{I_{CFP\ ex\ YFP\ em} - I_{CFP\ ex\ YFP\ em\ background}}{I_{CFP\ ex\ CFP\ em} - I_{CFP\ ex\ CFP\ em\ background}}$$

$$[Zn^{2+}] = K_d\left[\frac{R_{rest} - R_{min}}{R_{max} - R_{rest}}\right]^{\frac{1}{n}}$$

EXPECTED OUTCOMES

The labile Zn^{2+} pool is highly dynamic in cells and can be measured using a FRET-based fluorescent biosensor. In this protocol, we describe steps to perform an *in situ* calibration of this biosensor using a low and high Zn^{2+} solution to find the dynamic range of the sensor. Depending on the available Zn^{2+} in the growth media, MCF10a cells can have a labile Zn^{2+} concentration of between 1 pM and 1 nM, with an average of 80 pM in Zn^{2+} adequate media (Figure 3C). This protocol can be used to determine the resting labile Zn^{2+} concentration in treated or untreated cells, or in a variety of cell types.

LIMITATIONS

Sensor limitations

The ZapCV2 sensor was optimized in HeLa cells and has been used in several cell types such as MCF10a cells, MDA-MB-231 cells, primary hippocampal neurons, and primary macrophages.[1,15,16] We have found that the calibration solutions outlined in this proposal work for most cell types. That said, researchers may need to optimize the calibration conditions (see problem 2 and problem 3 below). In HeLa cells, the sensor dynamic range is 1.7–2.5[10], in MCF10a cells the dynamic range is typically 1.4–1.7[1]. If the dynamic range is less than 1.35, the Zn^{2+} concentration is often overestimated.[17] We recommend limiting analysis to cells in which the dynamic range is over 1.4 (Methods video S1). The sensor expression can vary from cell to cell and the expression level can impact the dynamic range and therefore the signal to noise ratio of the sensor. We recommend avoiding very bright and very dim cells in the analysis.

Microscope limitations

Imaging FRET sensors comes with its own challenges. First, the most important part of the microscope setup is the ability to acquire the donor channel and the FRET channel almost simultaneously.

The optimal system involves the use of an emission filter wheel, which is not as common as filter turrets that hold filter cubes. Ensuring the capability of your microscope to capture FRET is critical for the success of this protocol. Any perturbation in temperature can influence FRET efficiency, so it is important that cells are always kept at room temperature (20–25°C) and are allowed to equilibrate to atmospheric conditions before the calibration.

TROUBLESHOOTING

Problem 1

Not enough cells in the imaging dish for calibration experiment.

Potential solution

Insufficient adhesion of cells to the imaging dish may result in their detachment during multiple washing steps. It would be advisable to allow the cells to adhere for an additional day after plating. If the cell type being studies is only weakly adherent, coating the imaging dish with poly-lysine, collagen or gelatin can increase adhesion.

Problem 2

Poor dynamic range of the sensor during R_{min} and R_{max} phases of the calibration.

Potential solution

Optimize the chemical components in the R_{max} solution. Pyrithione is an ionophore that enables Zn^{2+} to pass through the plasma membrane and intracellular membranes. But pyrithione can be quite toxic to cells and high concentrations tend to give poor *in situ* calibrations.[11] In addition, we find that the concentration of Zn^{2+} may need to be optimized. This protocol outlines the use of a solution in which Zn^{2+} is buffered at 101.4 nM. In general, we have found that buffered Zn^{2+} solutions in the nM range give better calibrations than high concentrations of unbuffered Zn^{2+}.[11] While not strictly necessary for Zn^{2+} to enter a cell, we have found that low concentrations of saponin, a membrane permeabilizing detergent, often yield a higher dynamic range. The concentration of saponin may need to be adjusted for different cell types.

Problem 3

The R_{max} step peaks very quickly and decays without a plateau.

Potential solution

When the R_{max} peaks and rapidly decays, it is likely the true R_{max} of the sensor has not been achieved. This requires optimizing pyrithione, saponin, and the buffered zinc solution. We often find that the rapid peak and decay is a sign of the cells dying very rapidly in the R_{max} solution conditions. Therefore, we advise lowering the pyrithione concentration and lowering the concentration of buffered Zn^{2+} to try to achieve a slower rise and plateau. If it is not possible to achieve a full calibration (R_{min} followed by R_{max}), you can conduct two "half calibrations" in two different dishes. This involves using one dish to get the R_{min} value, by first measuring R_{rest}, followed by R_{min}. In the other dish, measure the R_{rest}, then add the Rmax solution to obtain the R_{max} value.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to the lead contact, Amy Palmer (amy.palmer@colorado.edu).

Technical contact

Requests for further technical information should be directed to the technical contact, Amy Palmer (amy.palmer@colorado.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any new datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103130.

ACKNOWLEDGMENTS

We thank the University of Colorado Biochemistry Cell Culture Core (specifically Theresa Nahreini) for assistance with cell culture. We would like to acknowledge NIGMS MIRA R35 GM139644 (A.E.P.) for generous financial support.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, A.E.P., A.R., and S.E.H.; investigation, A.R. and S.E.H.; writing, S.E.H. and A.E.P.; funding acquisition, supervision, and project administration, A.E.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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