Supporting Information for:

Direct comparison of a genetically encoded sensor and small molecule indicator: implications for quantification of cytosolic Zn^{2+}

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Methods

Plasmids

The following plasmids encoding ZapCY2 sensors were used: ZapCY in pocDNA3.1[1], NLS-ZapCY2 in pcDNA3.1[2]], Mito-ZapCY1 in pcDNA3.1[3], ER-ZapCY1[1], (e) Golgi-ZapCY1 in pcDNA3.1[1] and NES-ZapCY2[2] . GalT-mCherry in pEGFP-N1[4] was a gift from Jennifer Lippincott-Schwartz (NIH). VAMP2-mCherry was cloned by tagging VAMP2 with mCherry in pcDNA3.1 vector.

Cell culture and transfection conditions

HeLa cells were cultured in DMEM with 10% FBS; RWPE1 prostate cells were grown in keratinocyte serum free medium (K-SFM) containing 50 μg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor; LNCaP prostate carcinoma cells were cultured in RPMI 1640 medium with 15% FBS; mouse MIN6 insulinoma cells were grown in modified DMEM (DMEM, 10% FBS, 2mM L-glutamine, 1 mM Sodium pyruvate, 2×10^{-3} μl/ml Mercaptoethanol). Primary neurons were cultured from the dissociated cortex of P0 rat pups. Initially neurons were cultured for 24 hours in DMEM + 10% FBS at a density of 100,000 neurons/cm^2. After 24 hours, they were switched to Neurobasal

with 1X B-27 supplement (0.02% v/v). When plating cells in 3.5 cm imaging dishes, the dishes were coated with 0.01mg/ml polylysine for MIN6 and neurons.

Cells were plated onto 3.5 cm glass bottom dishes for imaging and transfected with plasmid DNA. Transfection of HeLa, RWPE1, and LNCaP cells was accomplished using TransIt (Mirus) according to the manufacturer's instructions (1 µg DNA per imaging dish). For MIN6 and primary cortical neurons, lipofectamine LTX was used for transfection (3.75 µg DNA per imaging dish). Cells were typically imaged 48-72 hrs after transfection.

Calculation of percent saturation and conversion to [Zn^{2+}]

The parameters obtained from in situ calibration (R, R_{TPEN}, and R_{Zn} for ZapCY2 and F, F_{TPEN}, F_{Zn} for FluoZin-3) were used to calculate the percent saturation according to the following equation (For FluoZin-3, F, F_{TPEN}, and F_{Zn} replaces R, R_{TPEN}, and R_{Zn}, respectively):

$$Fractional\ Saturation = \frac{R - R_{TPEN}}{R_{Zn} - R_{TPEN}} * 100\%$$

To calculate the resting cytosolic Zn^{2+} concentration ([Zn^{2+}]) in different cells using ZapCY2, the parameters from the *in situ* FRET ratios (R, R_{TPEN}, and R_{Zn}) were combined with the *in vitro* binding parameters (K_d = 811 pM, n = 0.44) and input into the following equation[1]:

$$[Zn^{2+}] = K_d' \sqrt[n]{\frac{(R - R_{TPEN})}{(R_{Zn} - R)}}$$

To calculate the corresponding [Zn^{2+}] to percent saturation using FluoZin3, we fitted the percent saturation as a function of [Zn^{2+}] by a one-site binding model (K_d=15 nM);

$$Percent\ saturation = \frac{[Zn^{2+}]}{Kd + [Zn^{2+}]} \times 100\%$$

To calculate the percent saturation of sensors, we added 10 µM Digitonin and 10 µM $ZnCl_2$ to cells to saturate the sensor. Digitonin is a detergent, which can permeabilize

the cell membrane and allow a high amount of Zn^{2+} influx into cells. It requires time to permeabilize plasma membrane (typically a few minutes), but ensures that high concentration of Zn^{2+} would saturate the sensors. Pyrithione is a cell permeable inophore which can bring Zn^{2+} into cells quickly; therefore it is used to compare the *in situ* kinetics of sensors. But pyrihione itself can also compete Zn^{2+} in cells, which limits its application in achieving a maximal signal in cells.

Measurement of sensor concentration in cells

Because direct excitation and emission of citrine is not affected by Zn^{2+} binding, the citrine channel can be used as an indicator of the intracellular sensor concentration. To estimate the sensor concentration of sensors in cells, standard curves were generated as follows. Microwells were generated in poly dimethylsiloxane (PDMS) based on previously published protocols [5]. Microwells were 8 μm in diameter and 10 μm in height and each array consisted of 100 x 100 wells, allowing multiple wells to be imaged simultaneously in one field of view. Wells were loaded with purified sensor protein of a known concentration and the citrine intensity was measured on the microscope using acquisition parameters analogous to live cell imaging experiments. Freshly purified sensor protein was subjected to a serial dilution with 10 mM MOPS/100 mM KCl pH=7.4. The protein concentration was determined by absorption spectroscopy in a 4 cm path length cuvette to allow more accurate determination of the optical density at low concentrations. To introduce protein into the well, 2 μl of sensor protein was sandwiched between a clean coverslip and the PDMS membrane, and the PDMS was gently pressed to seal the membrane coverslip interface. The average intensity of the wells at a given concentration of protein was measured for 3 different fields of view (multiple wells per field of view) after background correction. A standard curve was generated by plotting the citrine intensity as a function of the sensor concentration. Subsequently the intracellular citrine intensity measured under the same setting could be converted into sensor concentrations.

FluoZin-3 Staining.

FluoZin-3-AM (K_d = 15 nM; Life Technologies) stock solutions (2 mM) were prepared in DMSO. Cultured cells were incubated with 0.5-10 μM FluoZin-3-AM in phosphate free HEPES-buffered Hanks' Balanced Salt Solution (HHBSS) at room temperature or 37 ˚C for 30-40 minutes. Cells were then washed with HHBSS for 30-40 minutes to remove extracellular dye and allow time for cleavage of the AM ester before imaging.

Co-localization analysis

Images used for co-localization analysis were taken on a Nikon A1R Confocal microscope. Co-localization analyses were performed using Fiji/Image J with Colo 2 plugin.

Kinetic modeling

The concentration dependence of Zn^{2+} binding kinetics for the small-molecule FluoZin-3 was simulated using the computational systems biology toolkit, Simbiology, in Matlab. For each condition, the simulation was performed for 1000 seconds using the SUNDIALS solver type with an absolute and relative tolerance of 1×10^{-10} and 1×10^{-7}, respectively, with dimensional analysis enabled throughout.

Here, a mammalian cell was modeled as a 4.2 pL sphere containing an 80 pM intracellular concentration of labile Zn^{2+}, immersed in 2 mL of a solution containing 5μM Zn^{2+}. A set of coupled differential equations described transport into (Eq. 1) and out of the cell (Eq. 2), as well as formation (Eq. 3) and dissociation (Eq. 4) of the 1:1 FluoZin-3:Zinc complex. Subscripts in the equations denote whether the specified component is intracellular (I), or extracellular (E). The ratio of the *off* and *on* rates (Eq. 5) of the FluoZin-3:Zinc complex was chosen to maintain the experimental dissociation constant (15 nM) for FluoZin-3.

(Eq. 1) $$Zinc\ Input = kFwd\,[Zinc]_E[Pyrithione]_E^2$$

(Eq. 2) $$Zinc\ Output = kRev\ [Zinc]_I [Pyrithione]_I^2$$

(Eq. 3) $$Complex\ Formation = kOn\ [Zinc]_I [Sensor]_I$$

(Eq. 4) $$Complex\ Dissociation = kOff\ [Zinc\ Sensor\ Complex]_I$$

(Eq. 5) $$K_D = \frac{kOff}{kOn} = \frac{[Zinc]_I [Sensor]_I}{[Zinc\ Sensor\ Complex]_I} = 15\ nM$$

After 10 seconds of simulation time, 20 nanomoles of pyrithione (final concentration 10 μM) was added to the extracellular bath, triggering translocation of the zinc across the membrane and formation of the 1:1 FluoZin-3:Zinc complex. All of the simulation parameters were held constant while the intracellular concentration of FluoZin-3 was varied systematically from 1 nM to 1 mM. Simulation results suggest that the concentration of FluoZin-3 alone substantially alters the formation of FluoZin-3:Zinc complex, with higher concentrations of FluoZin-3 resulting in decreased rates of formation.

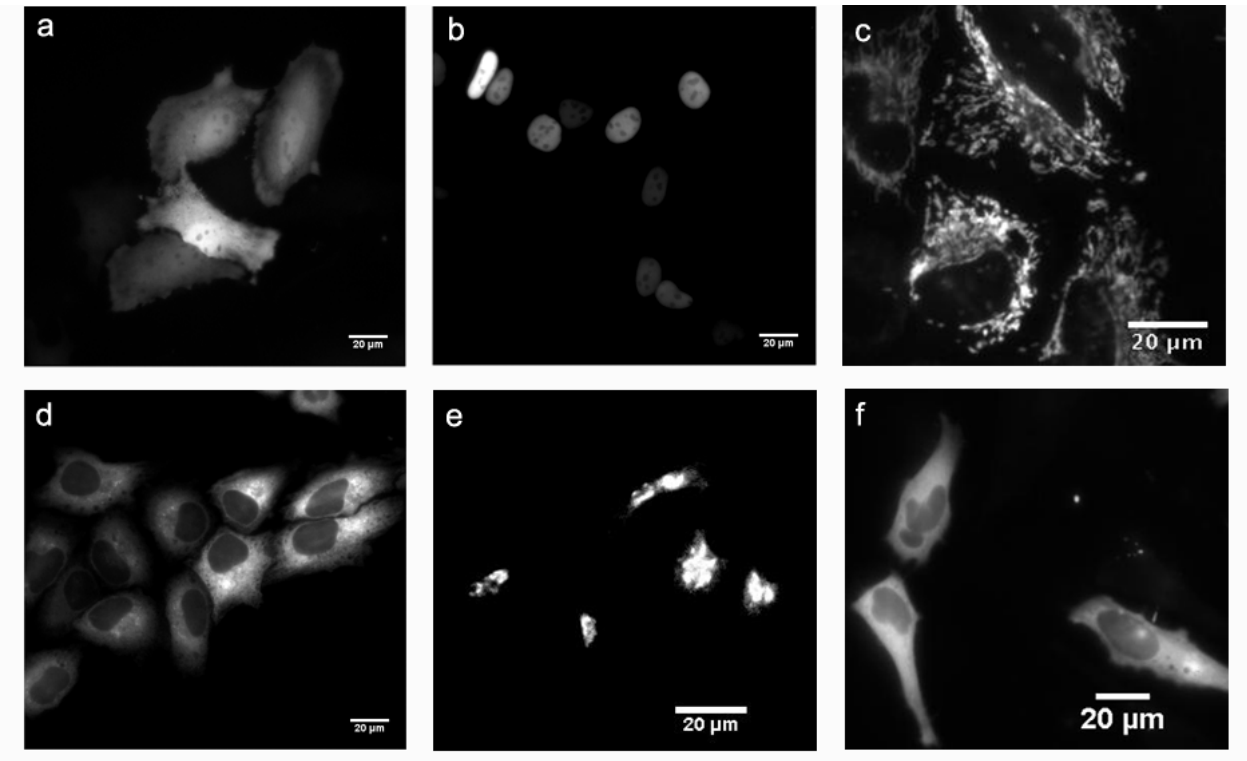

Figure S1. Localization of ZapCY sensors. ZapCY sensors were expressed in (a) cytosol and nucleus (ZapCY2 with no signal sequence)[1], (b) nucleus (NLS-ZapCY2), (c) mitochondria (Mito-ZapCY1)[3], (d) ER (ER-ZapCY1)[1], (e) Golgi (Golgi-ZapCY1)[1] and (f) cytosol only (NES-ZapCY2). Note that incorporation of a Nuclear Exclusion Signal (NES) leads to cytosolic localization of the sensor.

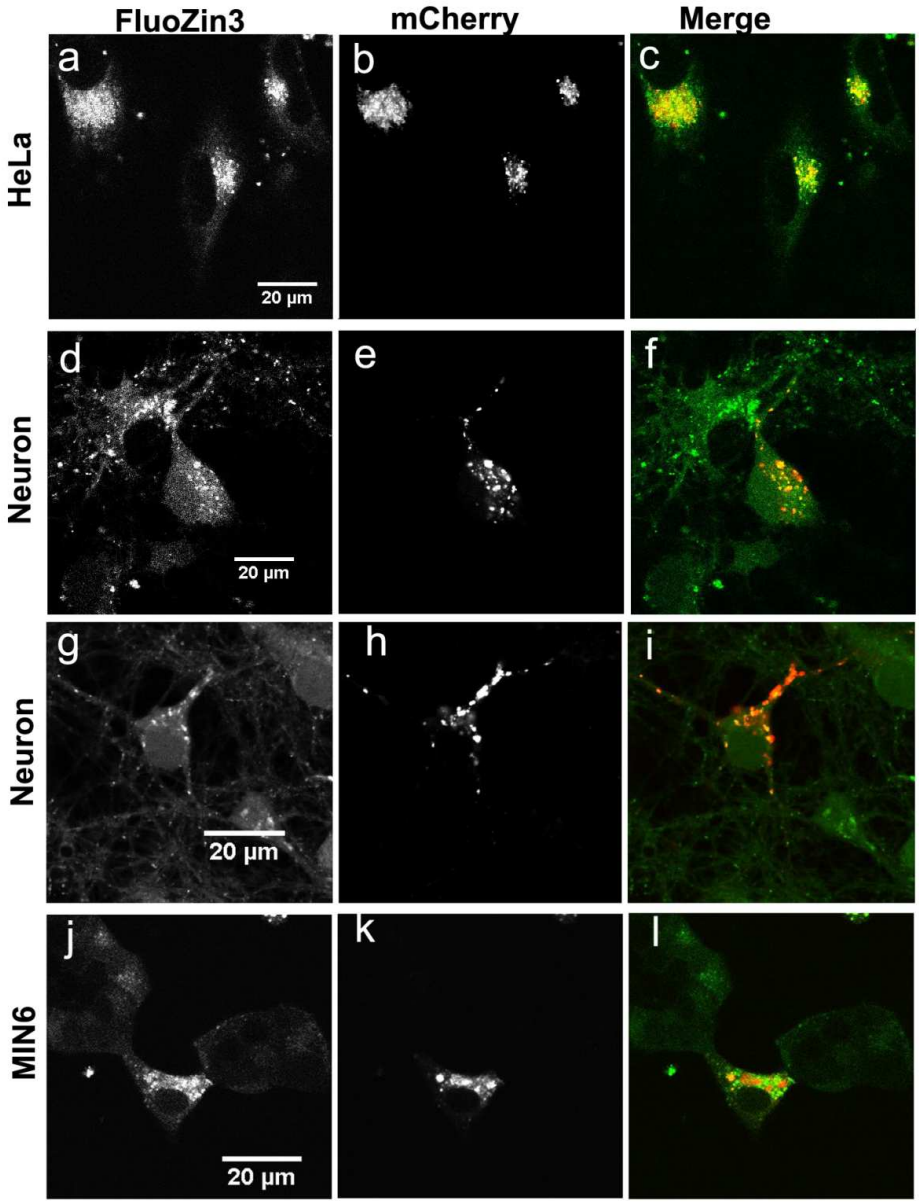

Figure S2. Localization of FluoZin-3 in cells. (a-c) Fluorescence images of HeLa cells depicting the baseline FluoZin-3 signal (a), the Golgi marker GalT-mCherry (b), and the overlay (c) with FluoZin-3 in green and mCherry in red. (d-f) Fluorescence images of cultured cortical neurons depicting the FluoZin-3 signal (d), GalT-mCherry (e), and the overlay of FluoZin-3 (green) with GalT-mCherry (red) (f). (g-i) Fluorescence images of cultured cortical neurons depicting the FluoZin-3 signal (g), VAMP2-mCherry (h), and the overlay (i). (j-l) Fluorescence images of MIN6 cells depicting FluoZin-3 (j), GalT-mCherry (k), and the overlay of the two (l).

There is strong co-localization of FluoZin-3 with GalT in HeLa cells, but poor colocalization in cortical neurons and MIN6 cells (see Table S1 for quantification).

Table S1. Summary of co-localization analysis.

Cell type	target	Pearson's coefficient	n (cell number)
HeLa	FluoZin-3 and GalT	0.60±0.18	20
Neurons	FluoZin-3 and GalT	0.41±0.32	5
Neurons	FluoZin-3 and VAMP2	0.66±0.19	11
MIN6	FluoZin-3 and GalT	0.37±0.40	16

Table S2: Statistics for analysis of resting Zn2+ levels in different cell types

	HeLa	RWPE1	LNCaP	MIN6	Neuron
Minimum	49.012482	28.924397	74.177086	29.521481	52.962463
Maximum	145.13544	90.725082	541.16571	220.61543	119.03281
Sum	1322.7579	1101.3806	3809.4006	1096.8606	401.254
Points	16	20	14	10	5
Mean	82.672366	55.06903	272.10004	109.68606	80.2508
Median	75.525723	51.404987	266.01373	90.467434	84.166283
RMS	86.65458	57.768899	304.90821	127.32104	83.851788
Std Deviation	26.818823	17.907589	142.78229	68.14702	27.178379
Variance	719.24929	320.68174	20386.782	4644.0163	738.66429
Std Error	6.7047059	4.0042586	38.160172	21.54998	12.154541

Skewness	0.83034588	0.69087001	0.40837248	0.40942313	0.33325368
Kurtosis	0.0090591172	-0.41027062	-0.62812973	-1.3012572	-1.1722951

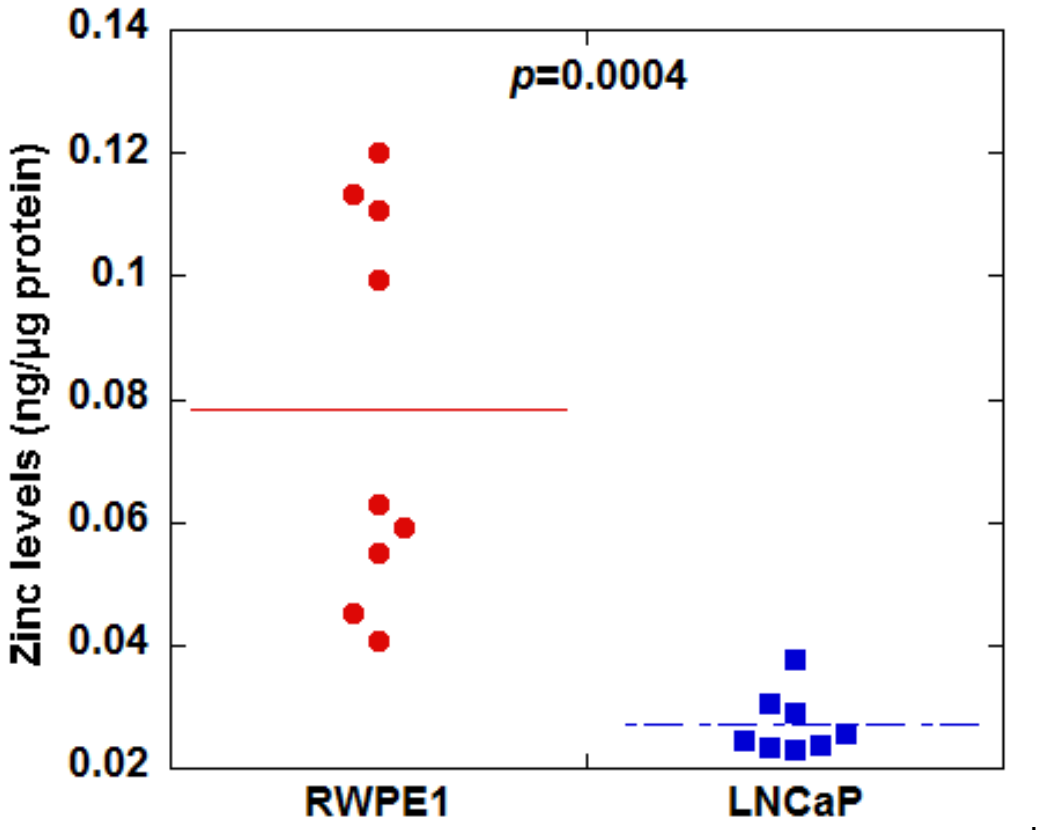

Figure S3. Comparison of the total zinc in normal prostate cells (RWPE1) and prostate cancer cells (LNCaP) by Inductively Coupled Mass Spectrometry. Each point represents an individual experiment. RWPE1 and LNCaP cell lines were collected and counted. Approximately 400,000 cells were treated with 200 µl of low grade nitric acid (Fisher Scientific) and subjected to 3 minutes of continuous vortex. Following the vortex period, tubes were placed on ice for 2 hours, and subsequently 5 mL of chelex-100 (Sigma Aldrich) treated water was added and mixed well. For each sample, 300 µl was used for a bicinchoninic acid (BCA) assay to quantify the protein concentration. The other samples were taken to The Laboratory for Environmental and Geological Studies (LEGS) ICP-MS facility for analysis at the University of Colorado at Boulder Geological Sciences Department. ICP-MS results showed that there is a significant reduction in total cell Zn^{2+} in the LNCaP prostate cancer cell line compared to the normal prostate cells. Note there is substantial variability in the total zinc for RWPE1 cells and at present we don't know whether this variability represents differences due to

passage number or cell state. We suspect more data points would be required to assess these possibilities.

References for Supporting Information

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