Published in final edited form as:

ACS Chem Biol. 2013 November 15; 8(11): 2366–2371. doi:10.1021/cb4003859.

¹Direct comparison of a genetically encoded sensor and small molecule indicator: implications for quantification of cytosolic Zn²⁺

Yan Qin¹, Jose G. Miranda¹, Caitlin I. Stoddard¹, Kevin M. Dean¹, Domenico F. Galati², and Amy E. Palmer¹

¹ Department of Chemistry and Biochemistry and BioFrontiers Institute, UCB 596, Boulder, CO 80309

² Department of Molecular, Cellular, and Developmental Biology, UCB 347, Boulder, CO 80309

Abstract

Fluorescent sensors are powerful tools for visualizing and quantifying molecules and ions in living cells. A variety of small molecule and genetically encoded sensors have been developed for studying intracellular Zn²⁺ homeostasis and signaling, but no direct comparisons exist making it challenging for researchers to identify the appropriate sensor for a given application. Here we directly compare the widely used small molecule probe FluoZin-3 and a genetically encoded sensor, ZapCY2. We demonstrate that, in contrast to FluoZin-3, ZapCY2 exhibits a well defined cytosolic localization, provides estimates of Zn²⁺ concentration with little variability, does not perturb cytosolic Zn²⁺ levels, and exhibits rapid Zn²⁺ response dynamics. ZapCY2 was used to measure Zn²⁺ concentrations in 5 different cell types, revealing higher cytosolic Zn²⁺ levels in prostate cancer cells compared to normal prostate cells (although the total zinc is reduced in prostate cancer cells), suggesting distinct regulatory mechanisms.

Keywords

Zinc; genetically encoded sensor; FluoZin-3; FRET

Fluorescent sensors have become instrumental tools in cell biology, permitting researchers to visualize specific ions, molecules, and biochemical reactions in the complex environment of a cell. There are now over 120 genetically encoded fluorescent sensors for different targets², and hundreds of small molecule fluorescent indicators. Increasingly, such tools are used to quantify the amount of a particular entity, such as concentration of a metal ion or the kinetics of ion fluxes. A substantial challenge for users is how to rigorously compare sensors

Corresponding Author Amy.Palmer@colorado.edu..

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

Other methods and results are listed in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

and evaluate quantitative estimates obtained using different probes, particularly given the vast array of probes with different molecular designs, biochemical, and photophysical characteristics²⁻⁴. For example, there is considerable disagreement among the measurements of accessible Zn^{2+} in cells using different tools, with estimates varying by three orders of magnitude and ranging from 5 to 1000 pM⁵⁻¹⁴. One possible explanation for this variability could be the use of different cell types. Indeed the above estimates include red blood cells, leukemic cells, splenocytes, thymocytes, cardiomyocytes, rat PC-12 cells, HT-29 cells, HEK 293 cells, insulin secreting INS-1 (832/13) cells, hepatocytes, neurons, and HeLa epithelial cells. Because few studies compare a range of cell types, it is difficult to evaluate whether these differences reflect natural variation in cells with different Zn^{2+} regulatory mechanisms, or whether estimates vary due to the use of different probes and measurement conditions.

Zn^{2+} has been documented to influence many cellular processes, including proliferation, differentiation, and apoptosis¹⁵⁻¹⁶, as well as inhibit enzymes such as caspases and phosphatases at concentrations ranging from pM to nM^{1, 17}. Yet the precise concentration of accessible Zn^{2+} , its spatial distribution, and the conditions under which levels are dynamic are poorly understood. One of the major challenges in the field is that there have been no quantitative measurements of the distribution of free Zn^{2+} in a diversity of cell types and an assessment of how this picture changes with cellular state. Thus, the range of physiological concentrations remains ill-defined.

Here we establish a general framework for comparing and validating fluorescent probes for Zn^{2+} , enabling newly developed tools and probes from different classes (e.g. small molecule versus genetically encoded) to be benchmarked against existing tools. By comparing the widely-used small molecule zinc sensor FluoZin-3¹⁸ with a representative genetically encoded sensor ZapCY2 (Kd: 811 pM, $n=0.44$)¹², we reveal that indeed different probes can yield substantially different results. Given the dizzying array of new probes and the increasing incidence with which they are used to measure biological processes such a framework will be critically important for the scientific community, both probe developers and the end-users. Lastly, using the validated genetically encoded sensor ZapCY2 we compare the cytosolic Zn^{2+} levels in five cell types, revealing an increase in accessible cytosolic Zn^{2+} in a prostate cancer cell line compared to a normal prostate cell line.

Results and Discussion

Establishing subcellular localization

There is growing evidence that the subcellular distribution of Zn^{2+} is inhomogeneous, with different concentrations in different organelles^{12-13, 19-20}. Therefore, when quantifying cellular Zn^{2+} levels it is essential to precisely define the identity of the compartment being measured. The location of genetically encoded sensors can be controlled by incorporation of a signal peptide into the sensor and the sensor can be explicitly targeted to the cytosol by incorporation of a nuclear exclusion signal (Figure S1). FluoZin-3 is a widely used small molecule Zn^{2+} indicator, employed in hundreds of publications. This membrane permeable dye is trapped inside cells upon cleavage of the acetoxymethyl (AM) ester by cellular esterases²¹. The localization of FluoZin-3 is not precisely defined and the dye is at least

partially sequestered into intracellular compartments. Moreover, the localization of the dye varies with cell type. Indeed, FluoZin-3 has been used as a cytosolic Zn^{2+} sensor²²⁻²⁵. But it has also been used as an indicator for vesicular Zn^{2+} ²⁶⁻²⁷, and lysosomal Zn^{2+} in oxidative neurons²⁸, and vesicles in HeLa cells when the expression of ZIP13 is reduced²⁹.

In our hands in HeLa cells, FluoZin-3 partially co-localizes with a Golgi marker (Fig 1, Fig S2a-c, Table S1, Pearson's coefficient = 0.60 ± 0.18), but is also present in the cytosol, as evidenced by the increase in cytosolic fluorescence upon treatment with Zn^{2+} (Fig 1 g). The high fluorescence signal in Golgi compared to other regions didn't decrease upon zinc chelation with 100 μM TPEN (Fig 1e), suggesting that the high signal in Golgi was not due to high Zn^{2+} , but rather to accumulation of the dye. In cultured cortical neurons and insulin-secreting MIN6 cells FluoZin-3 showed poor co-localization with a Golgi marker (Fig S2, Table S1, Pearson's coefficient = 0.41 ± 0.32 for neurons, and 0.37 ± 0.40 for MIN6).

Instead, in neurons, FluoZin-3 partially co-localized with the synaptic vesicle marker VAMP2 (Fig 1, Fig S2g-i, Table S1, Pearson's coefficient = 0.66 ± 0.19). However, the dye was also present in the cytosol and nucleus as treatment with 5 μM pyrithione and 100 μM ZnCl_2 (Fig 1h) yielded bright fluorescent signals in both locations.

Quantifying intracellular Zn^{2+}

. Comparing cytosolic Zn^{2+} concentration ($[\text{Zn}^{2+}]$) in different cells or in cells of different diseased states is an important step in evaluating how resting Zn^{2+} levels, and deviations from resting levels, influence cellular function. The most precise estimates of $[\text{Zn}^{2+}]$ involve calibration of the sensor in the cellular milieu and determination of the percent saturation of the sensor in each individual cell, followed by conversion to $[\text{Zn}^{2+}]$. Figure 2 presents representative calibrations of ZapCY2 and FluoZin-3. Each trace represents an individual cell and it is evident that even under resting conditions, there is substantial variability in the FRET ratio (ZapCY2) or fluorescence intensity (FluoZin-3) from cell-to-cell. However, when the parameters of the *in situ* calibration were used to determine the percent saturation of the sensor, ZapCY2 exhibited a narrow distribution (26.7-28.3%, Figure 2a), whereas the variability was much greater for FluoZin-3 in HeLa cells (15.1-41.1%, Fig 2b).

Another important consideration for accurately comparing cellular $[\text{Zn}^{2+}]$ is the extent to which the sensors themselves perturb free ion levels. In particular, if the [sensor] inside cells is comparable to the buffered Zn^{2+} pool, the sensor could lead to depletion of free Zn^{2+} ³⁰. To test whether sensors perturb the free Zn^{2+} pool, the fractional saturation was measured as a function of [sensor]. For ZapCY2, the percent saturation of sensor was maintained from 23% to 32% in different cells and was not greatly affected by [sensor] (Fig 2c). Conversely for FluoZin-3, the percent saturation varied from 45 to 20% and decreased with increasing [FluoZin-3] (Fig 2d), indicating that estimates of free Zn^{2+} with FluoZin-3 depend on the amount of dye internalized.

Comparison of intracellular Zn^{2+} in different cells

ZapCY2 was used to compare the free Zn^{2+} levels in 5 cell types under resting conditions. Full *in situ* calibrations were carried out for HeLa, RWPE, LNCaP, MIN6 and primary

cultured cortical neurons and the resting FRET ratio was converted into a $[\text{Zn}^{2+}]$, yielding estimates of mean cytosolic Zn^{2+} of ~ 60 pM for normal RWPE1 cells, ~ 270 pM for cancerous LNCaP cells, ~110 pM in MIN6 and 80 pM in Neurons (Fig 2e). While there can be substantial variability in $[\text{Zn}^{2+}]$ in some cell types (note LNCaP), on average, the cytosolic Zn^{2+} in cancerous LNCaP is significantly higher than normal RWPE ($P < 0.001$).

In vivo kinetic response

Lastly, we compared the temporal response of ZapCY2 and FluoZin-3 upon acute manipulation of intracellular Zn^{2+} . MIN6 cells were used to exploit their voltage-gated calcium channels (L-VGCC) that open upon depolarization and are permeable to Zn^{2+} ³¹. When MIN6 cells were treated with Zn^{2+} under depolarization conditions both sensors showed an increased signal and more rapid response compared to cells treated with ZnCl_2 alone (Fig 3a and b). In both conditions, ZapCY2 showed a quicker response compared to FluoZin-3. To rule out the possibility that this phenomenon was caused by the affinity difference (ZapCY2 is saturated at lower $[\text{Zn}^{2+}]$ than FluoZin-3), MIN6 cells were treated with pyrithione and ZnCl_2 to ensure rapid influx of high levels of Zn^{2+} . Under these conditions, saturation of ZapCY2 occurred more rapidly than FluoZin-3 (Fig 3c). One explanation for the kinetic difference could be that the $[\text{FluoZin-3}]$ is much higher than $[\text{ZapCY2}]$ in cells. To test this, we treated MIN6 cells with different $[\text{FluoZin-3}]$ (0.5-10 μM) for 40 minutes, then perfused the cells with 5 μM pyrithione and 100 μM ZnCl_2 . Cells with a lower amount of FluoZin-3 reached the maximal intensity faster than the cells with higher FluoZin-3 (Fig 3d). However the rate of entry was still slower than for ZapCY2. Computational simulations also provided supporting evidence that cells with higher $[\text{dye}]$ display slower kinetics (Fig S3).

In summary, ZapCY2 exhibited a number of distinct advantages when compared to FluoZin-3. First, the localization can be explicitly defined to be cytosolic, whereas FluoZin-3 reports on Zn^{2+} changes within the cytosol as well as membrane enclosed compartments, and that localization varies significantly with cell type (Fig.1 and Fig. S1). Hence interpretation of FluoZin-3 data is confounded by an inability to precisely define where the Zn^{2+} changes are occurring. This may explain why estimates of cytosolic Zn^{2+} using FluoZin-3^{6, 32} are typically higher than estimates using genetically encoded sensors¹²⁻¹³, particularly in cells containing vesicular Zn^{2+} . In addition, the accumulation of FluoZin-3 in subcellular compartments such as the Golgi leads to high fluorescence (Fig. 1) which may be incorrectly interpreted as elevated Zn^{2+} unless an in situ calibration is performed.

A second advantage is that in distinct contrast to FluoZin-3, ZapCY2 does not appear to perturb the resting free Zn^{2+} pool. **Scheme 1** presents a simplified model of Zn^{2+} homeostasis and illustrates the potential of an exogenous sensor to perturb steady state Zn^{2+} . This scheme suggests that if the $[\text{sensor}]$ approaches that of the buffer system, the sensor may deplete the cellular ion pool. In this work we measured the free Zn^{2+} as a function of $[\text{sensor}]$ for ZapCY2 and FluoZin-3. We found that while free Zn^{2+} estimates based on ZapCY2 were independent of the intracellular $[\text{sensor}]$ (Fig 2c), there was a strong dependence with increasing FluoZin-3, leading to a significant decrease in the measured

fractional saturation (Fig 2d), suggesting depletion of the cellular ion pool. This is consistent with previous studies using FluoZin-3 in human colon cancer (HT-29) cells and neurons^{6, 30}, and likely results from the fact that AM ester versions of Zn^{2+} indicators are typically concentrated at least 100-fold, yielding intracellular concentrations in the hundreds of micromolar^{6, 30}. The total buffered Zn^{2+} pool in mammalian cells has been estimated in the hundreds of μM ^{6, 33-34}, so it's possible that the lower levels of ZapCY2 (1-10 μM) compared to the amount of internalized FluoZin-3 (hundreds of μM) are not sufficient to perturb the equilibrium and deplete the Zn^{2+} pool. However another difference between the two probes is that measurements with genetically encoded sensors are performed days after introduction of the sensor into cells whereas those with FluoZin-3 are performed within a few hours of loading the dye, due to extrusion of the dye over time. Perhaps long term expression of ZapCY2 allows it to become part of the zinc buffer system, as represented in **Scheme 2**.

Lastly, a final advantage of ZapCY2 is the rapid in vivo response compared to FluoZin-3 (Fig 4). We suspect that the relatively rapid response of ZapCY2 results from the fact that there is significantly less sensor within the cell and hence saturation of the sensor occurs more quickly. This was supported by kinetic modeling showing that higher concentrations of sensor lead to slower kinetic responses. These data suggest that ZapCY2 might be a better tool for studying zinc uptake and efflux kinetics compared to FluoZin-3.

Given the wide range of estimates for resting free Zn^{2+} levels and the scarcity of studies that directly compare Zn^{2+} in a range of diverse cell types, we used ZapCY2 to compare relative Zn^{2+} levels in 3 types of epithelial cells: HeLa, normal prostate RWPE1, and cancerous prostate LNCaP, as well as neurons and a model pancreatic beta cell line (MIN6). We found no difference in cytosolic Zn^{2+} between HeLa, MIN6, and cortical neurons, but a significant decrease in RWPE1, and an increase in the LNCaP. The higher level of free Zn^{2+} in the cytosol of LNCaP versus RWPE1 is particularly intriguing given that it is well established that *total* Zn^{2+} levels in prostate cancer cells are lower than normal cells and that Zn^{2+} content strongly correlates with metastasis³⁵. Indeed, using inductively coupled mass spectrometry (ICP-MS) we verified that total cellular Zn^{2+} in LNCaP is 65% lower than in RWPE1 cells (Fig S3). Thus, our study reveals the importance of defining Zn^{2+} levels in explicit subcellular locations and reveals for the first time that cytosolic Zn^{2+} is actually higher in the prostate cancer cell line, despite lower levels of total Zn^{2+} . It is interesting to speculate that high cytosolic Zn^{2+} in cancer cells may contribute to these cells being more refractory toward apoptosis, as Zn^{2+} can inhibit caspase 3 activity and repress apoptosis¹. If this is the case, addition of a Zn^{2+} chelator might be used to induce apoptosis in cancer cells, an approach that has been used in the development of new agents for reducing tumor size in animal models³⁶.

In conclusion, direct comparisons between ZapCY2 and FluoZin-3 showed that the genetically encoded sensor can overcome many limitations of this small molecule sensor when the experimental goal is to define Zn^{2+} in a precise location (such as the cytosol), to minimize perturbation of the cellular ion pool, facilitate Zn^{2+} quantification, and report the kinetics of cellular Zn^{2+} dynamics.

Methods

Details of the plasmids, culture, transfection conditions for all cells, method for quantifying sensor concentration in cells and FluoZin3 staining colocalization analysis are provided in Supporting Information.

Cellular Zn^{2+} Measurements

Imaging experiments were performed on a Zeiss Axiovert 200M inverted fluorescence microscope configured as previously described¹². Images were collected with a 40X 1.3NA oil objective, using a5% light transmission, and 300 msec exposure time. Images of ZapCY2 were background corrected by generating a region of interest (ROI) on a blank area of the coverslip and subtracting the fluorescence intensity of each channel, e.g. $I_{FRET}(\text{sample}) - I_{FRET}(\text{background})$, and $I_{CFP}(\text{sample}) - I_{CFP}(\text{background})$. The background corrected FRET and CFP images were used to calculate the FRET ratio (R, i.e. FRET/CFP). Images of FluoZin-3 were not background corrected because the resting signal was very low and close to the background.

Cells were imaged in phosphate-free HHBSS, pH = 7.4 to prevent precipitation of zinc. To carry out in situ calibrations, cells were treated with 150 μM cell permeable zinc chelator N,N,N',N'-tetrakis (1-pyridylmethyl) ethylenediamine (TPEN) to obtain the FRET ratio at minimum Zn^{2+} ($R_{\min}$) and addition of 5 μM pyrithione and 10 or 100 μM $ZnCl_2$ to establish the FRET ratio at maximum Zn^{2+} ($R_{\max}$).

Statistical analysis

Statistical analysis was performed using the t-test in the KaleidaGraph program. The error bars indicate SEM (standard error of the mean).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

NIH Predoctoral Fellowship (F31 GM093443 (J.G.M)), UROP and HHMI Undergraduate Research Fellowship (C.I.S.), NIH Molecular Biophysics Training Grant (T32 GM-065103) and NSF Computational Optical Sensing and Imaging IGERT 0801680 (K.M.D.), and NIH GM084027 and Alfred P. Sloan Foundation (A.E.P.).

ABBREVIATIONS

FRET	Förster resonance energy transfer
K_D	dissociation constant
Zn^{2+}	free Zn^{2+} concentration
sensor	sensor concentration

REFERENCES

1. Perry DK, Smyth MJ, Stennicke HR, Salvesen GS, Duriez P, Poirier GG, Hannun YA. Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. *J Biol Chem.* 1997; 272:18530–18533. [PubMed: 9228015]
2. Newman RH, Fosbrink MD, Zhang J. Genetically encodable fluorescent biosensors for tracking signaling dynamics in living cells. *Chem Rev.* 2011; 111:3614–3666. [PubMed: 21456512]
3. Dean KM, Qin Y, Palmer AE. Visualizing metal ions in cells: An overview of analytical techniques, approaches, and probes. *Biochim Biophys Acta.* 2012
4. Que EL, Domaille DW, Chang CJ. Metals in neurobiology: probing their chemistry and biology with molecular imaging. *Chem Rev.* 2008; 108:1517–1549. [PubMed: 18426241]
5. Bozym RA, Thompson RB, Stoddard AK, Fierke CA. Measuring picomolar intracellular exchangeable zinc in PC-12 cells using a ratiometric fluorescence biosensor. *ACS Chem Biol.* 2006; 1:103–111. [PubMed: 17163650]
6. Krezel A, Maret W. Zinc-buffering capacity of a eukaryotic cell at physiological pZn. *J Biol Inorg Chem.* 2006; 11:1049–1062. [PubMed: 16924557]
7. Simons TJ. Intracellular free zinc and zinc buffering in human red blood cells. *J Membr Biol.* 1991; 123:63–71. [PubMed: 1774775]
8. Adebodun F, Post JF. Role of intracellular free Ca(II) and Zn(II) in dexamethasone-induced apoptosis and dexamethasone resistance in human leukemic CEM cell lines. *J Cell Physiol.* 1995; 163:80–86. [PubMed: 7896902]
9. Zalewski PD, Forbes IJ, Betts WH. Correlation of apoptosis with change in intracellular labile Zn(II) using zinquin [(2-methyl-8-p-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II). *Biochem J.* 1993; 296(Pt 2):403–408. [PubMed: 8257431]
10. Brand IA, Kleineke J. Intracellular zinc movement and its effect on the carbohydrate metabolism of isolated rat hepatocytes. *J Biol Chem.* 1996; 271:1941–1949. [PubMed: 8567642]
11. Sensi SL, Canzoniero LM, Yu SP, Ying HS, Koh JY, Kerchner GA, Choi DW. Measurement of intracellular free zinc in living cortical neurons: routes of entry. *J Neurosci.* 1997; 17:9554–9564. [PubMed: 9391010]
12. Qin Y, Dittmer PJ, Park JG, Jansen KB, Palmer AE. Measuring steady-state and dynamic endoplasmic reticulum and Golgi Zn²⁺ with genetically encoded sensors. *Proc Natl Acad Sci U S A.* 2011; 108:7351–7356. [PubMed: 21502528]
13. Vinkenborg JL, Nicolson TJ, Bellomo EA, Koay MS, Rutter GA, Merks M. Genetically encoded FRET sensors to monitor intracellular Zn²⁺ homeostasis. *Nat Methods.* 2009; 6:737–740. [PubMed: 19718032]
14. Turan B, Fliss H, Desilets M. Oxidants increase intracellular free Zn²⁺ concentration in rabbit ventricular myocytes. *Am J Physiol.* 1997; 272:H2095–2106. [PubMed: 9176274]
15. Li Y, Maret W. Transient fluctuations of intracellular zinc ions in cell proliferation. *Exp Cell Res.* 2009; 315:2463–2470. [PubMed: 19467229]
16. Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T. Zinc homeostasis and signaling in health and diseases: Zinc signaling. *J Biol Inorg Chem.* 2011; 16:1123–1134. [PubMed: 21660546]
17. Wilson M, Hogstrand C, Maret W. Picomolar concentrations of free zinc(II) ions regulate receptor protein-tyrosine phosphatase beta activity. *J Biol Chem.* 2012; 287:9322–9326. [PubMed: 22275360]
18. Gee KR, Zhou ZL, Ton-That D, Sensi SL, Weiss JH. Measuring zinc in living cells. A new generation of sensitive and selective fluorescent probes. *Cell Calcium.* 2002; 31:245–251. [PubMed: 12098227]
19. Sensi SL, Paoletti P, Bush AI, Sekler I. Zinc in the physiology and pathology of the CNS. *Nat Rev Neurosci.* 2009; 10:780–791. [PubMed: 19826435]
20. Park JG, Qin Y, Galati DF, Palmer AE. New sensors for quantitative measurement of mitochondrial Zn(2+). *ACS Chem Biol.* 2012; 7:1636–1640. [PubMed: 22850482]

21. Tsien RY, Pozzan T, Rink TJ. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J Cell Biol.* 1982; 94:325–334. [PubMed: 6980885]
22. Nicolson TJ, Bellomo EA, Wijesekara N, Loder MK, Baldwin JM, Gyulkhandanyan AV, Koshkin V, Tarasov AI, Carzaniga R, Kronenberger K, Taneja TK, da Silva Xavier G, Libert S, Froguel P, Scharfmann R, Stetsyuk V, Ravassard P, Parker H, Gribble FM, Reimann F, Sladek R, Hughes SJ, Johnson PR, Masseboeuf M, Burcelin R, Baldwin SA, Liu M, Lara-Lemus R, Arvan P, Schuit FC, Wheeler MB, Chimienti F, Rutter GA. Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. *Diabetes.* 2009; 58:2070–2083. [PubMed: 19542200]
23. Mato S, Sanchez-Gomez MV, Bernal-Chico A, Matute C. Cytosolic zinc accumulation contributes to excitotoxic oligodendroglial death. *Glia.* 2013
24. Kiedrowski L. Cytosolic acidification and intracellular zinc release in hippocampal neurons. *J Neurochem.* 2012; 121:438–450. [PubMed: 22339672]
25. Kiedrowski L. Cytosolic zinc release and clearance in hippocampal neurons exposed to glutamate--the role of pH and sodium. *J Neurochem.* 2011; 117:231–243. [PubMed: 21255017]
26. McCormick N, Velasquez V, Finney L, Vogt S, Kelleher SL. X-ray fluorescence microscopy reveals accumulation and secretion of discrete intracellular zinc pools in the lactating mouse mammary gland. *PLoS One.* 2010; 5:e11078. [PubMed: 20552032]
27. Qian J, Noebels JL. Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse. *J Physiol.* 2005; 566:747–758. [PubMed: 15919713]
28. Hwang JJ, Lee SJ, Kim TY, Cho JH, Koh JY. Zinc and 4-hydroxy-2-nonenal mediate lysosomal membrane permeabilization induced by H₂O₂ in cultured hippocampal neurons. *J Neurosci.* 2008; 28:3114–3122. [PubMed: 18354014]
29. Jeong J, Walker JM, Wang F, Park JG, Palmer AE, Giunta C, Rohrbach M, Steinmann B, Eide DJ. Promotion of vesicular zinc efflux by ZIP13 and its implications for spondylocheiro dysplastic Ehlers-Danlos syndrome. *Proc Natl Acad Sci U S A.* 2012; 109:E3530–3538. [PubMed: 23213233]
30. Dineley KE, Malaiyandi LM, Reynolds IJ. A reevaluation of neuronal zinc measurements: artifacts associated with high intracellular dye concentration. *Mol Pharmacol.* 2002; 62:618–627. [PubMed: 12181438]
31. Gyulkhandanyan AV, Lee SC, Bikopoulos G, Dai F, Wheeler MB. The Zn²⁺-transporting pathways in pancreatic beta-cells: a role for the L-type voltage-gated Ca²⁺ channel. *J Biol Chem.* 2006; 281:9361–9372. [PubMed: 16407176]
32. Haase H, Hebel S, Engelhardt G, Rink L. Flow cytometric measurement of labile zinc in peripheral blood mononuclear cells. *Anal Biochem.* 2006; 352:222–230. [PubMed: 16545333]
33. Takeda A. Movement of zinc and its functional significance in the brain. *Brain Res Brain Res Rev.* 2000; 34:137–148. [PubMed: 11113504]
34. Colvin RA, Bush AI, Volitakis I, Fontaine CP, Thomas D, Kikuchi K, Holmes WR. Insights into Zn²⁺ homeostasis in neurons from experimental and modeling studies. *Am J Physiol Cell Physiol.* 2008; 294:C726–742. [PubMed: 18184873]
35. Franklin RB, Feng P, Milon B, Desouki MM, Singh KK, Kajdacsy-Balla A, Bagasra O, Costello LC. hZIP1 zinc uptake transporter down regulation and zinc depletion in prostate cancer. *Mol Cancer.* 2005; 4:32. [PubMed: 16153295]
36. West DC, Qin Y, Peterson QP, Thomas DL, Palchaudhuri R, Morrison KC, Lucas PW, Palmer AE, Fan TM, Hergenrother PJ. Differential Effects of Procaspase-3 Activating Compounds in the Induction of Cancer Cell Death. *Mol Pharm.* 2012

Figure 1. Localization of FluoZin-3 in HeLa cells and cultured cortical neurons

Fluorescence image of mCherry-GalT Golgi marker in HeLa (a) and mCherry-VAMP2 in neurons (f). FluoZin-3 staining at rest in HeLa (b) and neurons (g), after treatment with 100 μ M TPEN (c, h), and after incubation with 100 μ M ZnCl_2 and 5 μ M pyithione (d, i). Overlay of Golgi (red) with FluoZin-3 (green) for HeLa (e) and VAMP2 (red) with FluoZin-3 (green) for neurons (j). Threshold of images at low zinc level (b,c and g,h) is adjusted lower than images for zinc saturation (d, i) to enable visualization with naked eyes. Images b and c are thresholded at the same level, and g and h are thresholded at the same level.

Figure 2. Reduced signal variability and zinc buffering of ZapCY2 compared to FluoZin-3
a) HeLa cells expressing ZapCY2(a) or loaded with FluoZin-3 (b) and subjected to calibration. Percent saturation of ZapCY2 and estimated $[Zn^{2+}]$ at rest as a function of intracellular [sensor] (c) and for FluoZin-3 as a function of the extracellular [FluoZin-3] used to treat cells (fit to an exponential decay) (d). Intracellular [FluoZin-3] is typically ~100x higher than the extracellular concentration. Data are from 6 cells at each concentration. e) Cytosolic $[Zn^{2+}]$ in different cell types. Box represents middle quartiles and whiskers represent upper and lower quartile (full statistics in Table S2).

Figure 3. Comparison of the temporal response of FluoZin-3 and ZapCY2 in MIN6 cells
 The increase in intracellular Zn^{2+} was induced by incubation with 100 μM $ZnCl_2$ (a), 50 mM KCl and 100 μM $ZnCl_2$ (b), or 5 μM pyrithione and 100 μM $ZnCl_2$ (c). MIN6 cells loaded with 0.5 μM , 2 μM , 5 μM or 10 μM FluoZin-3 were treated with pyrithione/100 μM $ZnCl_2$ (d). Data are reported as saturation percentage $[(F-F_0)/(F_{max}-F_0)\%]$. Each plot represents the average and S.E.M. of at least five cells. (e) Simulation of Zn^{2+} binding kinetics of FluoZin-3. In this simulation model, higher concentrations of FluoZin-3 would take longer time to reach the saturation.

Scheme 1.

Scheme 2.