Genetically Encoded Sensors to Elucidate Spatial Distribution of Cellular Zinc

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Transition metals are essential enzyme cofactors that are required for a wide range of cellular processes. Paradoxically, whereas metal ions are essential for numerous cellular processes, they are also toxic. Therefore cells must tightly regulate metal accumulation, transport, distribution, and export. Improved tools to interrogate metal ion availability and spatial distribution within living cells would greatly advance our understanding of cellular metal homeostasis. In this work, we present genetically encoded sensors for Zn2+ based on the principle of fluorescence resonance energy transfer. We also develop methodology to calibrate the probes within the cellular environment. To identify both sources of and sinks for Zn2+, these sensors are genetically targeted to specific locations within the cell, including cytosol, plasma membrane, and mitochondria. Localized probes reveal that mitochondria contain an elevated pool of Zn2+ under resting conditions that can be released into the cytosol upon glutamate stimulation of hippocampal neurons. We also observed that Zn2+ is taken up into mitochondria following glutamate/Zn2+ treatment and that there is heterogeneity in both the magnitude and kinetics of the response. Our results suggest that mitochondria serve as a source of and a sink for Zn2+ signals under different cellular conditions.

Although mammalian cells are known to concentrate transition metals, it is now well established that under resting conditions, “free” (e.g. unbound) metals are maintained at extremely low levels. Estimates of the total Zn2+ concentration in mammalian cells typically range from 100 to 500 μM (1); yet free Zn2+ concentrations are tightly buffered by proteins such as metallothionein to maintain cytosolic Zn2+ concentrations in the picomolar to nanomolar range (2–5). However, there is emerging evidence that this static picture is dramatically the picomolar to nanomolar range (2–5). However, there is emerging evidence that this static picture is dramatically

These sensors reveal differences in the spatial distribution of Zn2+ and highlight the power and utility of localized probes.

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2 The abbreviations used are: FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; MOPS, 4-morpholinepropanesulfonic acid; TPEN, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine.

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EXPERIMENTAL PROCEDURES

In Vitro Characterization—Details of sensor construction, protein purification, and buffered metal solutions are presented in the supplemental text. Purified sensor protein (0.5 μM) was buffer exchanged into 10 mM MOPS, 100 mM KCl (pH 6.8) and titrated with Zn2+ to obtain the apparent dissociation constant, Kd. We determined the Kd using two approaches. In one approach, the fluorescence intensity at 529 nm (emission maximum of YFP) was plotted as a function of Zn2+, and in the second approach, the change in FRET ratio (R - Rmin) was plotted as a function of Zn2+, where the FRET ratio (R) is defined as the emission maximum of YFP divided by the emission maximum of CFP upon excitation of CFP, and Rmin is the minimum FRET ratio observed under Zn2+-free conditions. These measurements yielded Kd values within experimental error of one another. It should be noted that we measure the apparent Kd because we are monitoring the conformational change that leads to FRET as opposed to directly measuring the Zn2+ binding event. Mixed metal buffer solutions (15) were used to buffer Zn2+ from 9 nm to 1.3 μM (Zn2+/Sr2+/EGTA) and 2–134 μM (Zn2+/Ca2+/EGTA). The Cys2His2 sensor was purified and maintained under anaerobic conditions (see supplemental text for more details). A standard Ellman’s assay (16) was used to quantify the percentage of reduced Cys. The observed FRET ratio change (R - Rmin) for the Cys2His2 sensor varied with the percentage of reduced Cys (typically between 50 and 80% of Cys were reduced). The lack of complete Cys reduction likely resulted from our inability to maintain a completely anoxic environment. Normalization to the amount of active, reduced protein yielded the maximal dynamic range for the Cys2His2 sensor of 2.2-fold. Fluorescence measurements were made on a Safire-II fluorescence plate reader (Tecan). FRET ratios (R) were calculated for each titration point by dividing the YFP intensity (λmax = 529 nm) by CFP intensity (λmax = 475 nm) upon CFP excitation at 420 nm. The emission bandwidth was 10 nm.

Cellular Zn2+ Measurements—The His4 sensor was targeted to the extracellular surface of mammalian cells using PDisplay (Invitrogen). The Cys2His2 sensor was targeted to the mitochondrial matrix, referred to as mito-Cys2His2, by attaching four tandem repeats of the cytochrome c oxidase signal sequence (MSVLTPLLRLGLTGSAARRLPVPRAKHSLGDP) to the N terminus of the sensor (17). Sensor constructs were transfected into HeLa cells using TransIT (Mirus) according to the manufacturer’s instructions and imaged 48 h post-transfection. The imaging experiments were performed on an Axiovert 200M inverted fluorescence microscope (Zeiss) with a Cascade 512B CCD camera (Roper scientific), and equipped with CFP (430/24 excitation, 455 dichroic, 470/24 emission), YFP (495/10 excitation, 515 dichroic, 535/25 emission), and YFP FRET (430/24 excitation, 455 dichroic, 535/25 emission) filters controlled by a Lambda 10-3 filter changer (Sutter Instruments) and analyzed using Metafluor software (Universal Imaging). Experiments on cytosolically expressed sensors were collected at 40× magnification, whereas mitochondrial experiments were collected at 100× magnification.

The cells were imaged in phosphate-free HEPES-buffered Hanks’ balanced salt solution. To determine the in situ Kd values, the cells were treated with 150 μM TPEN to obtain the minimum FRET ratio (Rmin) followed by a wash and the addition of increasing concentrations of a well defined Zn2+ solution (Zn2+/Sr2+/EGTA or Zn2+/Ca2+/EGTA-buffered solutions). For intracellularly expressed sensors (cytosol and mitochondria), the cells were treated with 150 μM TPEN to obtain the minimum FRET ratio (Rmin) followed by a wash and the addition of 15 μM digitonin (Calbiochem) and a saturating Zn2+ solution (1.5 mM) to establish the maximum FRET ratio (Rmax). The digitonin was used to permeabilize the plasma membrane and facilitate Zn2+ entry into the cell. The Rmax treatment was generally toxic to cells and therefore was always performed at the end of an experiment. For mitochondrial calibrations, the Zn2+ ionophore pyritione (25 μM) was included along with digitonin to facilitate Zn2+ entry across the mitochondrial membrane. Because for the Cys2His2 sensor, the sensor concentration was similar in magnitude to the Kd, and the FRET ratio versus [Zn2+] was fit with the following expression (18),

\[
FRET = \frac{[\text{Zn}^{2+}]_i + [\text{sensor}]_i + K_d}{2[\text{sensor}]_i} \frac{-1/2 \sqrt{-1/2 [\text{Zn}^{2+}]_i - [\text{sensor}]_i - K_d^2 - 4[\text{Zn}^{2+}]_i [\text{sensor}]_i}}{1/2 [\text{sensor}]_i}
\]

where [sensor] was taken to be 6 μM. This sensor concentration was determined experimentally as described below. Still, [sensor] was varied from 0.5 to 10 μM in the above fits with no change in the calculated Kd. For the His4 sensor, the data were fit to a one site binding equation.

Citrine YFP intensity was converted to sensor protein concentration using an established method (19). Briefly, a protein standard curve was generated by measuring the citrine intensities of purified sensor protein of known concentrations in a 50-μm-tall rectangular glass capillary (VitroCom) using identical settings as the imaging experiments.

The FRET ratio (R) was calculated from background corrected FRET and CFP fluorescence images and corrected for photobleaching if necessary. The FRET ratios were converted to Zn2+ concentrations using the following equation (20) along with experimentally derived Rmin, Rmax, and in situ Kd values.

\[
[Zn^{2+}] = \frac{K_d (R_{\text{max}} - R)}{(R_{\text{min}} - R)}
\]

It should be noted that Rmin and Rmax are obtained in each experiment and in each individual cell.

Neuronal Culturing and Transfection—Primary hippocampal neurons were prepared from postnatal day 1 Sprague-Dawley rats, with slight modifications (21). Briefly, the hippocampi were dissociated and plated on 35-mm glass-bottomed dishes coated with poly-d-lysine (Sigma) and laminin (Invitrogen) and grown in neurobasal A supplemented with B27 and Glutamax medium (Invitrogen). The neurons were transfected prior to plating by Amaxa nucleofection at 2,000,000 cells/transfection with 2 μg of DNA
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(S1021-A2011006908599d32)(Cys2His2), a mutant zinc finger containing four histidines (His4), and a mutant zinc finger containing four alanines (Ala4) to abrogate Zn2+ binding.

In Vitro Characterization—To characterize the sensors in vitro, the sensor protein was purified by affinity and size exclusion chromatography. Fig. 1B demonstrates that Zn2+ binding to the His4 sensor causes a decrease in CFP and an increase in YFP emission upon CFP excitation caused by an increase in FRET. The same spectral changes were observed for the Cys2His2 sensor (data not shown). As expected, the sensor is ratiometric, and therefore the sensor response is presented as the FRET ratio (R). The dynamic range of the sensor is defined as \( R_{\text{max}}/R_{\text{min}} \), and was 4-fold for the His4 sensor and 2.2-fold for the Cys2His2 sensor, making these some of the more sensitive FRET sensors based on conformational change. For comparison, redesigned sensors for Ca2+ exhibit a 5-fold ratio change (17, 23), kinase sensors typically exhibit a 0.2–0.6-fold ratio change (24), and glutamate sensors exhibit a 0.25-fold ratio change (25). We suspect that the difference in the dynamic range between the His4 and Cys2His2 sensors results from slight variation in the overall geometry and hence orientation of the fluorescent proteins with respect to one another. There is some precedence for this because a systematic analysis of analogous Ca2+ sensors revealed large changes in the dynamic range for sensors with different orientations but similar distances between the two fluorescent proteins (23).

To determine the apparent dissociation constant for Zn2+ (\( K_d \)), the FRET intensity and FRET ratio for each sensor were measured as a function of Zn2+ concentration. These measurements yielded identical \( K_d \) values. The FRET ratio (R) binding curves are presented in Fig. 1 (C and D). The in vitro zinc affinities for the Cys2His2 and His4 sensors were determined to be 1.7 ± 0.2 and 160 ± 4 \( \mu M \), respectively. The Cys2His2 sensor exhibited a lower Zn2+ affinity than the isolated zinc finger (1.7 \( \mu M \) versus 10 \( \mu M \)). We suspect that this is because attachment of the fluorescent proteins alters the apparent zinc affinity. As expected, mutation of Cys2His2 to His4 led to a reduction in Zn2+ affinity, enabling us to generate both a high and low affinity sensor. Proteolytic removal of the His6 tag did not alter the \( K_d \) of either sensor, indicating that the His tag does not participate in Zn2+ binding. Mutation of Cys2His2 to Ala4 abrogates

Results

Sensor Design—The sensor design consists of two fluorescent proteins and a “sensing domain” that undergoes a conformational change upon Zn2+ binding. A sensor schematic is presented in Fig. 1A. Binding of Zn2+ to the sensing domain changes the distance and orientation between the cyan fluorescent protein (CFP) and the citrine fluorescent protein (YFP), leading to increased FRET from CFP to YFP.

The Zn2+ sensing domain is a canonical Cys2His2 zinc finger from the mammalian transcription factor, Zif268. The zinc finger was selected because extensive structural data indicates that these domains are largely unstructured in the absence of metal ion and fold into a compact structure upon Zn2+ binding (22). Three sensor variants were generated: a wild type zinc finger

manufacturer’s recommendations (Amaza) and grown to 9 days in vitro. The neurons were imaged at 9 days in vitro as above using 100× magnification in a modified Tyrode’s salts solution.

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Genetically Targeted Zinc Sensors

Although genetically encoded FRET-based sensors for Zn\textsuperscript{2+} have been reported previously, they have either not been demonstrated to function in cells (27–29) or were not used explicitly to measure cellular Zn\textsuperscript{2+} (30). Therefore we sought to express our Cys\textsubscript{2}His\textsubscript{2} and His\textsubscript{4} sensors in mammalian cells and characterize the in situ response. We chose to target the low affinity His\textsubscript{4} sensor to the extracellular surface of the plasma membrane and the higher affinity Cys\textsubscript{2}His\textsubscript{2} sensor to both the cytosol and the mitochondrial matrix. Fig. 2A presents pseudo color FRET ratio images of HeLa cells expressing the cytosolic Cys\textsubscript{2}His\textsubscript{2} sensor. The addition of TPEN, a Zn\textsuperscript{2+}-specific chelator, results in a decrease in the FRET ratio, whereas an addition of Zn\textsuperscript{2+} results in an increase. Fig. 2 (B and C) demonstrates sensors targeted to mitochondria and the plasma membrane, respectively. Table 1 summarizes the sensors generated. To measure the apparent dissociation constant in situ, the cells were treated with TPEN to establish the minimum FRET ratio in zero Zn\textsuperscript{2+} (R\textsubscript{min}) followed by a washout and successive additions of ZnCl\textsubscript{2} along with 15 \(\mu\text{M}\) digitonin to obtain each titration point. In situ calibration for Cys\textsubscript{2}His\textsubscript{2} in the cytosol (○), His\textsubscript{4} on the plasma membrane (□) and Ala\textsubscript{4} in the cytosol (■). The FRET ratio change \((R - R\textsubscript{min})\) is plotted as a function of Zn\textsuperscript{2+}. The data were fit as described in methods yielding the following parameters: \(K_f = 1.5 \pm 0.2 \mu\text{M}\) (■) and 200 \(\pm 10 \mu\text{M}\) (○). The scale bar represents 10 \(\mu\text{m}\).

FIGURE 2. Cellular response and sensor calibration in HeLa cells. A, pseudo color FRET ratio images under resting conditions (left panel), following 150 \(\mu\text{M}\) TPEN treatment (middle panel), and after treatment with ZnCl\textsubscript{2}/15 \(\mu\text{M}\) digitonin (right panel). B, the Cys\textsubscript{2}His\textsubscript{2} sensor targeted to mitochondria. C, the His\textsubscript{4} sensor targeted to the plasma membrane. D, representative microscope trace for a typical calibration experiment of the Cys\textsubscript{2}His\textsubscript{2} sensor in the cytosol. The trace shows the initial treatment of 150 \(\mu\text{M}\) TPEN (R\textsubscript{min}), followed by a washout and successive additions of ZnCl\textsubscript{2} along with 15 \(\mu\text{M}\) digitonin to obtain each titration point. E, in situ calibration for Cys\textsubscript{2}His\textsubscript{2} in the cytosol (○), His\textsubscript{4} on the plasma membrane (□) and Ala\textsubscript{4} in the cytosol (■). The FRET ratio change \((R - R\textsubscript{min})\) is plotted as a function of Zn\textsuperscript{2+}. The data were fit as described in methods yielding the following parameters: \(K_f = 1.5 \pm 0.2 \mu\text{M}\) (□) and 200 \(\pm 10 \mu\text{M}\) (○). The scale bar represents 10 \(\mu\text{m}\).

the sensor response, indicating that Zn\textsuperscript{2+} binding to the Zn\textsuperscript{2+} finger portion of the sensor is required for the observed FRET ratio change (Fig. 1D).

Although the cellular environment is generally reducing, cellular compartments may be oxidizing, or the cell may experience transient changes in redox potentials. The cysteines of the Cys\textsubscript{2}His\textsubscript{2} sensor are sensitive to oxidation. To test the effects of oxidation on the FRET ratio, we measured the in vitro FRET ratios of the reduced and oxidized Cys\textsubscript{2}His\textsubscript{2} sensor. R\textsubscript{min} was not affected by Cys\textsubscript{2}His\textsubscript{2} sensor oxidation; however, the oxidized form of the sensor was unable to achieve the maximum FRET ratio with the addition of saturating Zn\textsuperscript{2+}, resulting in a decreased dynamic range.

For sensors to report Zn\textsuperscript{2+} dynamics accurately within the complex environment of the cell, it is important that they be specific for Zn\textsuperscript{2+} over other abundant divalent ions (Mg\textsuperscript{2+} and Ca\textsuperscript{2+}) and biologically relevant transition metals (Cu\textsuperscript{2+}, Mn\textsuperscript{2+}, Fe\textsuperscript{2+}, Fe\textsuperscript{3+}, and Ni\textsuperscript{2+}). To determine the metal specificity, we examined whether other metals could elicit a FRET ratio change in the Cys\textsubscript{2}His\textsubscript{2} and His\textsubscript{4} sensors and/or interfere with the Zn\textsuperscript{2+} response (supplemental Fig. S2). The majority of metals did not elicit a FRET change and did not interfere with the Zn\textsuperscript{2+} response. The two exceptions were Fe\textsuperscript{2+} and Cu\textsuperscript{2+}. Fe\textsuperscript{2+} caused a small perturbation of Zn\textsuperscript{2+} binding to both sensors but only at the highest concentration tested (250 \(\mu\text{M}\)). Fe\textsuperscript{2+} itself did not cause an artificial FRET change. Cu\textsuperscript{2+} interfered with Zn\textsuperscript{2+} binding to the His\textsubscript{4} sensor such that the maximum Zn\textsuperscript{2+} response could only be achieved at the lowest Cu\textsuperscript{2+} concentration (5 \(\mu\text{M}\)). The 5 \(\mu\text{M}\) Cu\textsuperscript{2+} level is still significantly higher than the estimated cytosolic free copper concentration of less than 10\textsuperscript{-18} M or attomolar (26). Cu\textsuperscript{2+} only bound to and elicited a FRET change at the highest concentration tested (250 \(\mu\text{M}\)), suggesting that Cu\textsuperscript{2+} signals would be unlikely to give false FRET increases. The metal specificity results suggest that metal cross-reactivity will not be a problem in the cellular environment. However, given that copper ions pose the greatest potential for interference, we further experimentally verified Zn\textsuperscript{2+} selectivity over copper in cells (see below).

Genetically Targeted Zinc Sensors

Characterization—Although genetically encoded FRET-based sensors for Zn\textsuperscript{2+} have been reported previously, they have either not been demonstrated to function in cells (27–29) or were not used explicitly to measure cellular Zn\textsuperscript{2+} (30). Therefore we sought to express our Cys\textsubscript{2}His\textsubscript{2} and His\textsubscript{4} sensors in mammalian cells and characterize the in situ response. We chose to target the low affinity His\textsubscript{4} sensor to the extracellular surface of the plasma membrane and the higher affinity Cys\textsubscript{2}His\textsubscript{2} sensor to both the cytosol and the mitochondrial matrix. Fig. 2A presents pseudo color FRET ratio images of HeLa cells expressing the cytosolic Cys\textsubscript{2}His\textsubscript{2} sensor. The addition of TPEN, a Zn\textsuperscript{2+}-specific chelator, results in a decrease in the FRET ratio, whereas an addition of Zn\textsuperscript{2+} results in an increase. Fig. 2 (B and C) demonstrates sensors targeted to mitochondria and the plasma membrane, respectively. Table 1 summarizes the sensors generated. To measure the apparent dissociation constant in situ, the cells were treated with TPEN to establish the minimum FRET ratio in zero Zn\textsuperscript{2+} (R\textsubscript{min}) followed by membrane permeabilization with digitonin and the addition of known concentrations of Zn\textsuperscript{2+}. Fig. 2D presents a representative trace illustrating a Zn\textsuperscript{2+} titration, and Fig. 2E
reports the full binding curves. The in situ Zn\(^{2+}\) affinity of each sensor was comparable with that observed in vitro (K\(_d\) = 1.5 ± 0.2 μM in situ versus 1.7 ± 0.2 μM in vitro for Cys\(_2\)His\(_2\)) and 200 ± 10 μM in situ versus 160 ± 4 μM in vitro for His\(_4\)). As expected, the cytosolic Ala\(_4\) sensor showed no significant FRET ratio change upon addition of Zn\(^{2+}\) to cells. The dynamic range of both sensors was reduced in cells as compared with in vitro (0.25-fold in situ versus 2.2-fold for Cys\(_2\)His\(_2\) and 4-fold for His\(_4\) in vitro), even though the affinity for Zn\(^{2+}\) was unchanged. The dynamic range for Cys\(_2\)His\(_2\) was identical in the cytosol and mitochondria, suggesting that possible redox differences in these locations do not affect the sensor functionality and Zn\(^{2+}\) measurements. We speculate that the reduced dynamic range in situ may be due to molecular crowding in the cellular environment, causing the fluorophores of the sensor to be closer together in the absence of Zn\(^{2+}\), resulting in an overall higher R\(_{\text{min}}\). This is supported by our observation that R\(_{\text{min}}\) is generally higher in cells (~4) than in purified protein (R\(_{\text{min}}\) = ~1.5) when measured under the same experimental conditions on the microscope. Although the dynamic range was reduced in cells, the sensors still yielded robust responses to changes in Zn\(^{2+}\) levels and enabled visualization of Zn\(^{2+}\) dynamics in neurons.

Next, we determined whether cellular Cu\(^{2+}\) or Cu\(^{2+}\) ions interfered with Zn\(^{2+}\) measurements in cells. Resting FRET ratios were not affected by the addition of either neocuproine hydrochloride monohydrate (a Cu\(^{2+}\) specific chelator) or bathocuproinedisulfonic acid disodium salt (a Cu\(^{2+}\) chelator) but decreased to a minimum FRET ratio upon addition of TPEN, indicating that the in situ resting ratios were specific to Zn\(^{2+}\) and not influenced by Cu\(^{2+}\) or Cu\(^{+}\) (supplemental Fig. S3).

The mitochondrially targeted sensor (mito-Cys\(_2\)His\(_2\)) exhibited a marked improvement in localization over the small molecule probe RhodZin-3 (Invitrogen; supplemental Fig. S4). Moreover, mito-Cys\(_2\)His\(_2\) stayed localized within mitochondria upon membrane depolarization, whereas RhodZin-3 leaked out into the cytosol under these conditions.

The first step in characterizing cellular Zn\(^{2+}\) homeostasis is to define the levels of Zn\(^{2+}\) in different locations within the cell. There has been some controversy over whether mitochondria contain a labile pool of Zn\(^{2+}\) that is elevated over the cytosol (9, 31). To address this, we measured resting Zn\(^{2+}\) concentrations in the cytosol and mitochondria using our Cys\(_2\)His\(_2\) sensor. Because the addition of a sensor may perturb cellular Zn\(^{2+}\) levels, determinations of the “free” Zn\(^{2+}\) concentrations should be conducted over a range of sensor concentrations, enabling mathematical extrapolation to the “free” [Zn\(^{2+}\)] at zero sensor. This variation in sensor concentrations is a natural consequence of transient transfection where the numbers of gene copies can vary from cell to cell. Tighter control of sensor expression will likely be possible with the use of inducible promotors, and the establishment of stably transfected cell lines. Fig. 3 (A and B) presents the concentration of Zn\(^{2+}\) as a function of sensor concentration in the cytosol and mitochondria, respectively. For the cytosolic sensor citrine YFP intensity was converted to protein concentration using a protein standard curve (19) (supplemental Fig. S5). In mitochondria, the citrine intensity was not converted to a protein concentration because of the uncertainty in predicting mitochondrial volume.

We observe a direct correlation between sensor protein expression and the calculated Zn\(^{2+}\) values in the cytosol, where higher sensor concentrations give rise to higher estimated Zn\(^{2+}\) levels. A simple saturation model yields the best fit and projects

### TABLE 1
Summary of sensors developed in this work

<table>
<thead>
<tr>
<th>Sensor</th>
<th>In situ K(_d)</th>
<th>Targeted locations</th>
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</thead>
<tbody>
<tr>
<td>Cys(_2)His(_2)</td>
<td>1.5 μM</td>
<td>Cytosol and mitochondria</td>
</tr>
<tr>
<td>His(_4)</td>
<td>200 μM</td>
<td>Cytosol, mitochondria,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and plasma membrane</td>
</tr>
<tr>
<td>Ala(_4)</td>
<td>Control; does not bind Zn(^{2+})</td>
<td>Cytosol</td>
</tr>
</tbody>
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FIGURE 3. Comparison of cytosolic and mitochondrial Zn\(^{2+}\). A and B, cytosolic resting Zn\(^{2+}\) concentrations from 46 individual cells (A, ○) and mitochondrial resting Zn\(^{2+}\) concentrations from 10 individual cells (B, ■), as a function of sensor expression. In the cytosol, there is a wide variation in sensor expression from cell to cell, whereas the variation is markedly reduced in mitochondria. C, representative traces of the cytosolic (○), and mitochondrial (■) Cys\(_2\)His\(_2\) sensor. Resting ratios are established at the start, followed by treatment with 150 μM TPEN to obtain R\(_{\text{min}}\), and 2 mM ZnCl\(_2\), 15 μM digitonin (○) or 2 mM Zn\(^{2+}\), 15 μM digitonin, 25 μM pyrithione (■) to obtain R\(_{\text{max}}\). The number of R\(_{\text{max}}\) data points we can collect is limited by the fact that the R\(_{\text{max}}\) experimental conditions are toxic to the cells.
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A, treatment of neurons with 100 μM glutamate, 250 μM Zn²⁺ leads to an increase in mitochondrial Zn²⁺ as demonstrated by the mito-Cys₂His₂ response (black circle). The low affinity mito-His₄ sensor (blue square) yielded no response as expected given that this probe would be sensitive to Zn²⁺ concentrations between 20 and 2000 μM. Zn²⁺ uptake into mitochondria is Ca²⁺-dependent as mito-Cys₂His₂ yielded no response when the stimulation was carried out in Ca²⁺-free/phosphate-free HEPES-buffered Hanks’ balanced salt solution (red diamond). The left axis is for circles and squares. The right axis is for diamonds. B, heterogeneity in different regions of interest in response to 100 μM glutamate, 250 μM Zn²⁺ addition (at time 0). C, pseudo color FRET ratio images showing specific regions of interest. Left panels, zoomed in images under resting conditions (top panel), 10 s following treatment (middle panel), and 25 s following treatment (bottom panel).

In hippocampal neurons, upon treatment with glutamate and exogenous Zn²⁺, we observed an increase in Zn²⁺ within mitochondria for the high affinity mito-Cys₂His₂ sensor but not the lower affinity mito-His₄ sensor (Fig. 4). Supplemental Fig. S6 presents data traces of seven individual cells from three different experiments. Although there was some variability in the magnitude of the response (percentage of ratio change, i.e. \( R/R_{\text{min}} \), 3.2 ± 0.8%, n = 7), 100% of the cells measured showed an increase in mitochondrial Zn²⁺ upon treatment with glutamate plus Zn²⁺. Interestingly, the transient mitochondrial Zn²⁺ signals are dependent on the presence of extracellular Ca²⁺, because stimulation with glutamate and Zn²⁺ in Ca²⁺-free HEPES-buffered Hanks’ balanced salt solution lead to no observable increase in mitochondrial Zn²⁺. The mechanism of Zn²⁺ uptake is unclear, although the Ca²⁺ uniporter has been shown to transport Zn²⁺ (31). We are currently exploring the mechanism of uptake using inhibitors of different transport mechanisms. As depicted in Fig. 4 and supplemental movies, we observe heterogeneity in the magnitude and kinetics of Zn²⁺ uptake. Fig. 4B depicts the FRET ratio as a function of time for four regions of interest, whereas Fig. 4C shows the actual images. The two cells responded at different rates, with the bottom cell peaking at ~20 s after stimulation, and the top cell peaking at ~40 s after stimulation. Likewise there is variability in the response within a given cell. Additionally we often observed transient hot spots (depicted by the white arrows) for one or two frames. These could correspond to individual mitochondria or small clusters of mitochondria. Although we are still exploring the cellular consequences of this heterogeneity, the experiments demonstrate the richness of information possible by subcellular analysis of Zn²⁺ signals.
Treatment with Zn\(^{2+}\) alone showed no change to the FRET ratio in the mitochondria of hippocampal neurons. However, treatment of hippocampal neurons with glutamate alone resulted in a transient decrease in mitochondrial Zn\(^{2+}\) (Fig. 5). The small molecule Zn\(^{2+}\) indicator FluoZin-3-AM (Invitrogen) indicates that this mitochondrial Zn\(^{2+}\) is released into the cytosol. Importantly, the decrease in mitochondrial Zn\(^{2+}\) was not observed with the low affinity mito-His4 sensor.

Glutamate-stimulated Zn\(^{2+}\) release was slightly affected by extracellular Ca\(^{2+}\) because glutamate treatment in Ca\(^{2+}\)-free buffer altered the kinetics (but not the magnitude) of the response. Zinc release was measured in six individual cells from two separate experiments (supplemental Fig. S6), where the percentage of ratio change (\(R/R_{\text{min}}\)) was 3.5 ± 1.0% (\(n = 6\)), and every cell measured showed release of Zn\(^{2+}\) upon treatment with glutamate alone. Glutamate stimulation of neurons leads to acidification followed by alkalinization of the cytosol. Given that fluorescent proteins, particularly the citrine YFP, can be pH-sensitive, we felt it was important to evaluate the pH sensitivity of the mitochondrial targeted sensor and the extent of acidification in mitochondria. As shown in Fig. 6, the mito-Cys\(_2\)His\(_2\) sensor was unaffected by pH changes between 7.4 and 6.5, but the FRET ratio decreased at pH 6.0. The decrease in FRET ratio is likely caused by H\(^+\) quenching of citrine YFP fluorescence as the \(pK_a\) of citrine is 5.7 (34). To characterize the pH changes within the mitochondrial matrix upon glutamate stimulation in the presence and absence of exogenous Zn\(^{2+}\), we targeted ecliptic pHluorin (35) to mitochondria. Following glutamate treatment in the presence and absence of Zn\(^{2+}\), the pH sensor was calibrated by adding H\(^+\) ionophores with buffers of known pH values. As seen in Fig. 6, glutamate stimulation lead to a slight acidification in mitochondria; the pH dropped from >7.4 to ~7.0. There is no observed effect on the mito-Cys\(_2\)His\(_2\) sensor in this pH range.

**DISCUSSION**

Transition metals such as Zn\(^{2+}\) are both essential and toxic. The canonical view of metal homeostasis is that mammalian cells concentrate metal ions from their environment, but the vast majority of these metals are bound to proteins or cellular buffers. An alternate paradigm is emerging in which metals may be mobilized from labile pools, such as metallothionein or organelles. The possibility of transient metal “signals” is forcing us to re-evaluate cellular control of metal availability and its influence on cellular function. To obtain a comprehensive picture of Zn\(^{2+}\) regulation, it is necessary to define reservoirs of labile Zn\(^{2+}\) as well as the fate of mobilized Zn\(^{2+}\).

In this work, we present genetically targeted Zn\(^{2+}\) sensors and demonstrate their utility in monitoring the spatial distribution of Zn\(^{2+}\). Our mitochondrially targeted sensor reports an elevated resting Zn\(^{2+}\) level in mitochondria relative to the cytosol. Moreover, treatment of hippocampal neurons with

![FIGURE 5. Neuronal mitochondria release Zn\(^{2+}\) upon treatment with glutamate alone. Cytosolic FluoZin3 response (black triangle), mito-Cys\(_2\)His\(_2\) response (black circle), mito-His4 response (gray diamond), and mito-Cys\(_2\)His\(_2\) response in the absence of extracellular Ca\(^{2+}\) (light gray square) upon treatment with 100 \(\mu\)M glutamate are shown.](image1)

![FIGURE 6. Characterization of mitochondrial pH and the effect of pH on the sensor. A, mito-Cys\(_2\)His\(_2\) sensor FRET ratio as a function of pH. B, measurement of mitochondrial pH at rest and upon treatment with 100 \(\mu\)M glutamate stimulation (Glu). C, measurement of mitochondrial pH at rest and upon treatment with 100 \(\mu\)M glutamate, 250 \(\mu\)M Zn\(^{2+}\). For B and C, the pH calibration was performed immediately following stimulation using the same region of interest from three individual cells as described in the supplemental text.](image2)
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glutamate results in transient release of this zinc into the cytosol, indicating that mitochondria can indeed serve as a source of Zn\(^{2+}\) signals. This is consistent with the finding by Sensi et al. (9) that mitochondrial Zn\(^{2+}\) pools may be mobilized independently of cytosolic pools. We also extended the work of Nolan et al. (32) and demonstrate that upon treatment with glutamate plus Zn\(^{2+}\), Zn\(^{2+}\) is taken up into neurons and is sequestered into mitochondria, thus creating a transient Zn\(^{2+}\) signal in mitochondria. Our results indicate that the role of mitochondria in modulating Zn\(^{2+}\) signals is context-dependent; glutamate treatment alone leads to Zn\(^{2+}\) release, whereas treatment with glutamate plus Zn\(^{2+}\) leads to Zn\(^{2+}\) uptake. This finding may have important biological implications because some hippocampal neurons contain only glutamate in presynaptic vesicles, whereas others contain both glutamate and Zn\(^{2+}\) (4). Our results suggest that perhaps different neurons will elicit different cellular responses, although it is important to note that the cellular consequences of these Zn\(^{2+}\) signals are still unclear.

There is a great challenge in accurately quantifying cellular Zn\(^{2+}\) concentrations. In this work we demonstrate that cellular Zn\(^{2+}\) levels are perturbed, even by expression of the relatively low affinity (K\(_d\) = \(\sim 1\) \(\mu M\)) Cys\(_2\)His\(_2\) sensor. This perturbation is not surprising given that our sensor concentration is also in the low micromolar range, and hence [sensor] is approximately equal to the K\(_d\). By measuring Zn\(^{2+}\) levels as a function of [sensor], we were able to extrapolate to an estimate of 180 nM Zn\(^{2+}\) in the cytosol. This estimate is higher than reports using other probes in which Zn\(^{2+}\) levels are predicted to be in the picomolar range, and hence [sensor] is approximately equal to the K\(_d\). As illustrated in Fig. 6, they are quenched at low pH (6.0 and below), which would impede measurement of Zn\(^{2+}\) in acidic compartments. The Cys\(_2\)His\(_2\) sensor relies on Cys to bind Zn\(^{2+}\), and thus its use is restricted to reducing environments. Lastly, although we were able to observe reproducible signals, the sensors would benefit from an expanded dynamic range.

In summary, we have developed genetically targeted sensors for Zn\(^{2+}\) and used these to demonstrate that mitochondria contain a labile and releasable pool of Zn\(^{2+}\) under resting conditions. In neurons, mitochondria can serve as a source of Zn\(^{2+}\) signals by releasing Zn\(^{2+}\) into the cytosol, as well as a sink by sequestering elevated cytosolic Zn\(^{2+}\). The observation of a mitochondrial pool of Zn\(^{2+}\) raises the intriguing question of whether other organelles modulate Zn\(^{2+}\) availability by serving as either sources or sinks for Zn\(^{2+}\).

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REFERENCES

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