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Optical Recording of Zn^{2+} Dynamics in the Mitochondrial Matrix and Intermembrane Space with the GZnP2 Sensor

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Abstract

The zinc ion (Zn^{2+}) is emerging as an important signaling molecule. Here, we engineered an improved Zn^{2+} probe GZnP2 based on a previously developed fluorescent sensor GZnP1 to provide a higher fluorescent readout (2-fold higher) that is proportional to cellular labile Zn^{2+} concentrations. We further developed a set of GZnP2 derived imaging tools to determine the labile Zn^{2+} concentrations in the mitochondrial matrix, mitochondrial intermembrane space (IMS), and cytosol in four different cell lines (HeLa, Cos-7, HEK293, and INS-1). The labile Zn^{2+} concentration in the matrix was less than 1 pM, while the labile Zn^{2+} concentration in the IMS was comparable to the cytosol (~100 pM). With these sensors, we showed that upon exposure to high Zn^{2+} , only the cytosol and the IMS were overloaded with Zn^{2+} , while the mitochondrial matrix was unable to sequester excess labile Zn^{2+} in depolarized INS-1 cells. This work highlighted the importance of distinguishing the labile Zn^{2+} concentrations and dynamics between the mitochondrial matrix and IMS.

Graphical Abstract

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Author Contributions

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Supporting Information

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Additional detailed methods, Figures S1–S7, and Tables S1 (PDF)

The authors declare no competing financial interest.

Emerging studies have shown that zinc affects certain cellular processes such as enzymatic reactions,¹ mitochondrial function,^{2–4} cell division,⁵ and apoptosis.⁵ The desire to further elucidate the roles of zinc in these essential cellular functions has driven the development of numerous quantitative and qualitative tools for both total zinc and labile zinc ions (referred to as “Zn²⁺” for the remainder of this paper unless stated otherwise) over the past few decades. In particular, the ability to measure labile Zn²⁺ accurately in various subcellular locations is paramount to understanding how labile Zn²⁺ modulates various functions within a cell. A number of such functions occur as a complex spatiotemporal mechanism related to the dynamic changes of labile Zn²⁺. Changes in labile Zn²⁺ such as flux between different subcellular compartments, communication with the extracellular environment, and buffering from Zn²⁺ binding proteins/ligands all could affect cellular function. For these reasons, a probe that can be reliably localized to specific regions of a cell can offer insight into how Zn²⁺ concentrations are changed in cells and how Zn²⁺ dynamics can alter cellular function.

Both small molecule sensors and genetically encoded sensors have been utilized to monitor and measure Zn²⁺ concentrations and dynamics in different subcellular compartments. Among these tools, a variety of Zn²⁺ sensors have been targeted to the mitochondria with great effort. Small molecule mitochondrial Zn²⁺ sensors such as RhodZin 3,⁶ DA-ZP1-TPP,^{7,8} DQZn2,⁹ M-Zn,¹⁰ and SZn-mito¹¹ rely on the negative membrane potential of the mitochondria for targeting. Genetically encoded mitochondrial Zn²⁺ sensors including mito-ZapCY1,¹² mito-eCALWY-4 and 6,¹³ mito-eZinCh-2,¹⁴ mito-GZnP1,¹⁵ and mito-CA-DSRed2¹⁶ utilize a mitochondrial targeting peptide for more accurate localization.

In spite of these great efforts on developing mitochondrial Zn²⁺ sensors, there are still two unresolved questions regarding mitochondrial Zn²⁺. First, it is still debated if the mitochondrial matrix stores a pool of labile Zn²⁺. Indirect evidence has suggested that the mitochondria could act as a source or sink of labile Zn²⁺.^{17,18} Various studies have shown that the total zinc concentrations in mitochondria are high in several cell types such as mammary gland cells,¹⁷ prostate cells,¹⁹ and neurons,^{7,20} suggesting that the labile Zn²⁺ might be high in these cells as well. In addition, Zn²⁺ might be released from mitochondria to the cytosol because cytosolic Zn²⁺ concentrations were increased when mitochondria were depolarized in cultured neurons.⁷ Studies have also reported large amounts of mitochondrial Zn²⁺ sequestration using the small molecule Zn²⁺ probe FluoZin-3. Although FluoZin-3 is not specifically localized to mitochondria, scientists have shown that high FluoZin-3 fluorescence overlapped with a mitochondrial marker after lysosomal permeabilization²¹ and ischemia stimulation.^{7,22} However, direct quantitative measurements have reported that the free, labile Zn²⁺ in the mitochondrial matrix is very low, nearly zero measured with several probes such as mito-eZinCh-2 (3.3 pM),¹⁴ Mito-CA-DSRed2 (0.2 pM),¹⁶ and mito-ZapCY1 (0.14 pM)¹² in HeLa cells and PC12 cells. This ambiguity of Zn²⁺ levels in the mitochondrial matrix requires further examination among different cell types.

The second unresolved question is, what is the concentration of labile Zn²⁺ in the mitochondrial intermembrane space (IMS)? All previously reported mitochondrial sensors were localized in the mitochondrial matrix, and no sensors have been designed for recording Zn²⁺ in the IMS. It is still unclear if the Zn²⁺ dynamics in the mitochondrial IMS are a separate moiety from the cytosol. Given that the voltage-dependent anion channel (VDAC)/

porin protein is a nonselective ion channel present on the outer membrane of the mitochondria, it has been inferred that Zn^{2+} ions can diffuse freely across the mitochondrial outermembrane, and the Zn^{2+} concentrations in the IMS are synonymous with the cytosolic Zn^{2+} concentration.^{23,24} However, conflicting studies have shown that the VDAC channel is selective and only allows certain solutes through the pore.²⁵ Moreover, the IMS is a critical site for oxidative phosphorylation reactions which create free radicals such as superoxide and hydrogen peroxide, and these oxidative radicals could liberate Zn^{2+} from metallothionein (MT).^{26,27} Due to the high amount of oxidation–reduction reactions within the IMS, the free Zn^{2+} concentration could potentially be much higher than the cytosol.

In order to address these two unresolved questions, we created a Zn^{2+} sensor GZnP2 with increased sensor response and specifically targeted the sensor to the mitochondrial matrix and IMS. Previously, we developed an intensimetric turn-on genetically encoded sensor called Green Zn^{2+} Probe (GZnP1).¹⁵ This GZnP1 sensor possesses three different sections: the circularly permuted GFP (cpGFP), the Zn^{2+} sensing domains (zinc fingers ZF1 and ZF2 from Zap1 transcription factor), and the linker regions connecting the cpGFP to the two zinc fingers (Figure 1a). In this work, we optimized GZnP1 by saturation mutagenesis and cell-lysate-based screening and engineered a new sensor GZnP2, which displayed twice the dynamic range as GZnP1's dynamic range, allowing for higher sensor response to changes in Zn^{2+} concentrations. Based on GZnP2, we developed sensors to record Zn^{2+} in the cytosol, mitochondrial matrix, and IMS by incorporation of a red fluorescent protein mCherry that is not sensitive to Zn^{2+} (as an internal control) and mitochondrial targeting sequences (to matrix and IMS).

We measured and compared cellular Zn^{2+} distributions among the cytosol, the mitochondrial matrix, and the IMS in four cell types (HeLa, HEK293, INS-1, and COS-1) with our new sensors. Our results provided supporting evidence that the mitochondrial matrix only contains very low concentrations of Zn^{2+} . In addition, our results settled the debate concerning the Zn^{2+} concentration in IMS by showing that the Zn^{2+} concentration in the IMS is comparable to the cytosolic concentration of Zn^{2+} at the resting state. Finally, we recorded and compared Zn^{2+} transport to the cytosol, IMS, and the mitochondrial matrix in INS-1 pancreatic β cells upon cell depolarization and showed that mitochondria did not sequester excess Zn^{2+} when INS-1 cells were activated. This work developed new sensors to distinguish Zn^{2+} levels between mitochondrial matrix and IMS, addressed two fundamental questions about mitochondrial Zn^{2+} distributions in the matrix and IMS, and revealed new aspects about mitochondrial Zn^{2+} uptake in pancreatic β cells.

RESULTS AND DISCUSSION

GZnP Sensor Optimization and Characterization.

Our previous work developed a single fluorescent protein-based genetically encoded sensor GZnP1, which displayed a 2-fold dynamic range in response to Zn^{2+} in the mitochondrial matrix.¹⁵ To optimize GZnP1 and increase its dynamic range, we created sensor libraries with saturation mutagenesis in the linker regions between the Zn^{2+} binding motif and the fluorescent protein (linker 1 and linker 2, Figure 1a). Lysates were collected from bacterial cells expressing sensor variants and then were treated with 100 μM of the specific Zn^{2+}

chelator N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) and 130 μM buffered Zn^{2+} separately in 96-well plates. The minimal fluorescence intensities of cell lysates with Zn^{2+} chelation (F_{min}) and the maximal fluorescence with Zn^{2+} saturation (F_{max}) were read by a Tecan fluorescence plate reader (Figure 1a). The sensor variant with increased dynamic range as measured by the ratio between maximal and minimal intensity ($F_{\text{max}}/F_{\text{min}}$) or increased brightness as measured by F_{max} were further examined by *in situ* characterization and then selected for next round of screening (Figure 1b). Through this cell-lysate-based screening, we identified a new sensor variant GZnP2. When expressed in HeLa cells, GZnP2 yielded increased fluorescence intensity by 4–5-fold between Zn^{2+} saturation and TPEN chelation (Figure 1b and c). The purified GZnP2 sensor protein displayed a 6-fold rise in fluorescence in response to Zn^{2+} (Figure 1d).

In vitro biophysical characterization demonstrated that GZnP2 is a specific, bright, and sensitive sensor for Zn^{2+} . The purified sensor protein showed a specific increased response to Zn^{2+} over other biological divalent metals such as Cu^{2+} , Mg^{2+} , Fe^{3+} , Mn^{2+} , Co^{2+} , and Ni^{2+} (Figure S1). GZnP2 displayed a binding affinity in the picomolar range with the apparent dissociation constant (K_d') for Zn^{2+} to be 352 pM at pH 7.4 (cytosol) and 112 pM at pH 8 (mitochondria; Table 1, Figure S2). The quantum yield (QY) of GZnP2 when saturated with Zn^{2+} (QY = 0.64) is close to the Ca^{2+} sensor GCaMP3 (QY = 0.65; Table 1, Figure S3),²⁸ demonstrating that this sensor is bright enough for live cell imaging. The sensor QY increased from 0.21 to 0.64 and the sensor extinction coefficient increased from 10 000 to 34 000 between the apo and the Zn^{2+} -saturated state, suggesting that Zn^{2+} binding enhanced sensor fluorescence by increasing both light absorption and fluorescent photon emission. The GZnP2 fluorescence was not significantly affected by 0–2 mM H_2O_2 (Figure S4), suggesting that mild redox such as the mitochondrial environment would not affect sensor signals.

Compare Zn^{2+} Quantification in the Cytosol, Mitochondrial Matrix, and Mitochondrial Intermembrane Space (IMS).

In order to measure cellular Zn^{2+} concentrations, we incorporated further modification to GZnP2 to account for z-axis drift and sensor expression variations. The mitochondria are highly dynamic, undergoing morphology changes by frequent fission and fusion as well as redistribution throughout the cells by trafficking. The sensor expression level also varies among different cells. Both mitochondrial motility and sensor expression variations can affect the recorded sensor intensity. For this reason, we attached GZnP2 with a red fluorescent protein mCherry, which is not sensitive to Zn^{2+} for normalization. This strategy has been utilized for labile Ca^{2+} quantification previously²⁹ and allows the single fluorescent protein-based sensor to be more amenable to quantitative analysis, providing better measurements of labile Zn^{2+} . Using this strategy, we created three sensors, mCherry-GZnP2 for the cytosol, mito-mCherry-GZnP2 for the mitochondrial matrix, and SMAC-mCherry-GZnP2 for the IMS (Figure 2a). The cytochrome c subunit 8, which has been frequently utilized for mitochondrial matrix localization,¹² was used to construct mito-mCherry-GZnP2 (Figure 2a and b). The leader sequence from the second mitochondria derived activator of caspases (SMAC) was used to target SMAC-mCherry-GZnP2 to mitochondrial IMS (Figure 2a and c) as this SMAC signal peptide has been reported for IMS targeting previously.³⁰

Both mito-GZnP2 and SMAC-GZnP2 demonstrated high colocalization with the mitochondrial markers (Pearson coefficient >0.85 ; Figure S7). The sensor mCherry-GZnP2 resides in the cytosol and nucleus (Figure 2a and d).

Calibrations of each sensor in three different subcellular compartments (mitochondrial matrix, IMS, and cytosol) were performed to compare Zn^{2+} levels in each compartment (Figure 2e–g) in HeLa cells. After cellular Zn^{2+} was chelated with $100\ \mu\text{M}$ TPEN for 5 min, the sensor signal was reduced to the minimal saturation (0%), while treatment of $5\ \mu\text{M}$ pyrithione and $20\ \mu\text{M}$ ZnCl_2 resulted in 100% sensor saturation. Mito-mCherry-GZnP2 was close to 0% saturation by matrix Zn^{2+} in the resting state, while both SMAC-mCherry-GZnP2 and mCherry-GZnP2 were $\sim 20\%$ saturated by Zn^{2+} in the resting state (Figure 2e–g). Although both mito-mCherry-GZnP2 and SMAC-mCherry-GZnP2 presented the same pattern of mitochondrial localization (Figure 2b and c), their calibration plots were different (Figure 2e and f), confirming that these two sensors were targeted to the two distinct regions within mitochondria. The calibration results suggest that free, labile Zn^{2+} in the mitochondrial matrix is lower than cytosol and IMS Zn^{2+} , while Zn^{2+} concentrations in IMS are the same as the cytosol.

We further determined the concentration of free Zn^{2+} in the mitochondrial matrix, IMS, and cytosol in four different cell types. Full in situ calibrations were carried out using HeLa cells, African green monkey kidney fibroblast (Cos-7) cells, human embryonic kidney 293 (HEK 293) cells, and rat pancreatic insulin secreting β (INS-1) cells. The sensor fluorescence intensity was normalized to the mCherry intensity, and the normalized signal was used to calculate Zn^{2+} concentrations. In all four cell types, Zn^{2+} concentrations in the mitochondrial matrix were estimated to be less than $1\ \text{pM}$ (Figure 3a, Figure S5 and Table S1). These results provided additional quantitative evidence in favor of the opinion that the mitochondrial matrix stores minimal labile Zn^{2+} , much lower than the cytosol. Given that a handful of mitochondrial matrix enzymes such as glutamate dehydrogenase, α -ketoglutarate dehydrogenase, and aconitase can be inhibited by Zn^{2+} , low Zn^{2+} concentrations in the mitochondria can maintain these important enzymes in the active state and ensure efficient energy production required for proper mitochondrial function.^{19,31–35} High Zn^{2+} concentrations in the mitochondria would be deleterious to cells by decreasing or halting production of ATP.³⁶

Among all four cell types, the Zn^{2+} concentrations in the IMS were the same as in the cytosol. The Zn^{2+} concentrations in the cytosol were between 75 and $115\ \text{pM}$, and Zn^{2+} concentrations in the IMS were between 65 and $100\ \text{pM}$ (Figure 3 and Table S1). No significant difference was found between the cytosol and the IMS Zn^{2+} concentration across all four cell types (Table S1). The Zn^{2+} homogeneity between these two regions of the cell suggests the outer mitochondrial membrane is permeable to Zn^{2+} allowing for passive diffusion of the ion, likely mediated by the VDAC protein.

Next, we compared Zn^{2+} quantification among four cell types. Interestingly, we found that the Zn^{2+} concentration within mitochondrial matrix presented high cell heterogeneity with significant differences among different cell types: highest in HEK293 cells and lowest in Cos-7 cells (Figure 3a, Figure S5 and Table S1). The precise reason for HEK293 cells

containing a substantially higher concentration of mitochondrial Zn^{2+} is not known. However, studies have demonstrated that HEK293 cells carry neuronal properties and might be neuronal in origin, while neurons have been thought to store higher mitochondrial labile Zn^{2+} than other cells.^{12,37} Nevertheless, there was less cell heterogeneity in Zn^{2+} concentration within either the IMS or the cytosol among four different cell types (Figure 3b,c and Table S1). Studies have reported that both cytosol and IMS contain high capacity Zn^{2+} buffering proteins like glutathione and metallothionein, which can maintain static Zn^{2+} concentrations in these two compartments.^{38–41}

Zinc Transport into the Matrix, IMS, and Cytosol.

Mitochondria have been reported as a site for Zn^{2+} sequestration for cells under stress.^{7,18} For example, mitochondrial Zn^{2+} uptake has been reported in neurons during ischemia/reperfusion.^{7,22} Here, we will further examine if mitochondria can act as a sink to sequester excess Zn^{2+} and lower cytosolic Zn^{2+} concentrations. Previous studies have shown that a high amount of Zn^{2+} can flux into pancreatic β -cells through voltage-dependent Ca^{2+} channels when cells are depolarized.^{42,43} Here, we induced Zn^{2+} influx in pancreatic β -cells and compared and analyzed Zn^{2+} uptake into the cytosol, IMS, and mitochondrial matrix. INS-1 cells were depolarized with high amounts of KCl (100 mM) in the presence of 100 μM ZnCl_2 , while the control cells were treated with 100 μM ZnCl_2 alone in the absence of extracellular Ca^{2+} (Figure 4a,b). With the presence of Ca^{2+} , 100 μM ZnCl_2 with high KCl caused cells to die quickly, so that we used 50 μM ZnCl_2 along with the depolarization (Figure 4e). We found that depolarization induced higher Zn^{2+} influx in the cytosol and mitochondrial IMS (Figure 4b,c), and the increased levels were similar between these two subcellular regions (Figure 4f), further supporting that the labile Zn^{2+} diffuses freely between the IMS and the cytosol. However, depolarization did not induce more Zn^{2+} uptake into the mitochondrial matrix, which is independent of extracellular Ca^{2+} (Figure 4a,e and Figure S6). These results indicated that the higher cytosolic Zn^{2+} only induced more Zn^{2+} uptake in the IMS, but not in the mitochondrial matrix (Figure 4b,c).

Two transport mechanisms have been proposed for Zn^{2+} uptake into the mitochondrial matrix: the mitochondrial Ca^{2+} uniporter (MCU) and mitochondrial Ca^{2+} uniporter independent mechanisms such as ZnT2 protein.^{44–47} How Zn^{2+} is transported out of the mitochondria still remains uncertain. The MCU on the inner membrane of the mitochondria was believed to be permeable to Zn^{2+} ions mainly from two studies on isolated mitochondria.^{45,46} The MCU can be activated by higher cytosolic Ca^{2+} concentrations.⁴⁸ When INS-1 cells were depolarized, higher Ca^{2+} would influx into the cells, activating the MCU and causing more Ca^{2+} to flow into the mitochondrial matrix.⁴⁸ Consistent with previous studies, we found that the mitochondrial Ca^{2+} increased rapidly then plateaued, while cytosolic Ca^{2+} increased but rapidly dropped off after depolarization in the presence of Ca^{2+} buffer (Figure 4d), confirming that mitochondria can buffer excess Ca^{2+} to reduce cytosolic Ca^{2+} levels. Unlike Ca^{2+} , no more Zn^{2+} was sequestered by the mitochondrial matrix in depolarized cells, and the cytosolic Zn^{2+} was not buffered immediately (Figure 4a–c). Even with the presence of Ca^{2+} influx, which can activate MCU, the mitochondria did not uptake excess Zn^{2+} (Figure 4e), which is conflicting with previous results that the MCU can mediate mitochondrial Zn^{2+} uptake. Since the evidence of permeability of MCU to Zn^{2+}

was collected from isolated mitochondria, we believe that the differences between native mitochondria and isolated mitochondria may cause different results. Further studies are needed to clarify the roles of MCU in regulating mitochondrial Zn^{2+} in live cells.

CONCLUSIONS

In this work, we engineered a new genetically encoded sensor GZnP2 with improved sensor response to Zn^{2+} and constructed three probes for recording Zn^{2+} concentrations and dynamics within the mitochondrial matrix, IMS, and cytosol. The GZnP2 sensor displayed twice the dynamic range compared to the previous sensor GZnP1. An increased dynamic range in the GZnP2 sensor enhanced the signal-to-noise in response to Zn^{2+} , allowing for more precise measurement of Zn^{2+} dynamics. In addition, we designed the first Zn^{2+} sensor targeted to the IMS of the mitochondria, filling in the gap of our understanding about subcellular Zn^{2+} distributions. We also developed two more GZnP2 based sensors localized to the mitochondrial matrix and the cytosol of the cell. Using the same set of sensors in different cellular compartments avoids variations in biophysical features of different types of sensors, so that offers better comparisons of Zn^{2+} levels among different subcellular regions.

We measured and compared labile Zn^{2+} concentrations and dynamics among the mitochondrial matrix, IMS, and cytosol in mammalian cells at a resting state as well as in cells exposed to excess Zn^{2+} . First, we found that the IMS Zn^{2+} concentration is the same as the cytosolic Zn^{2+} concentration across all four cell types. This continuity between the cytosol and IMS was confirmed again by recording the same level of Zn^{2+} uptake into the cytosol and IMS in depolarized pancreatic β -cells. The nonselective VDAC channels localized on the outer mitochondrial membrane might facilitate the diffusion of Zn^{2+} between the cytosol and IMS. Second, the mitochondrial matrix Zn^{2+} levels displayed high heterogeneity between different cell types. Despite the cell heterogeneity, mitochondrial matrix Zn^{2+} concentrations were estimated to be less than 1 pM among all four different cell types. The low concentrations of Zn^{2+} could be a result of the mitochondria containing higher amounts of citrate than in the cytosol and other small zinc binding molecules buffering the labile Zn^{2+} to nearly zero.⁴⁹ Last, we found that Zn^{2+} does not flow at a faster rate into the mitochondrial matrix despite the higher cytosolic and IMS Zn^{2+} concentration in depolarized cells compared to nondepolarized cells. These data suggest that mitochondrial IMS, not matrix, can sequester Zn^{2+} when the cytosol is overloaded with Zn^{2+} . However, with the interpretations that arose from time-lapse experiments within 30 min, we could not rule out the possibility that over longer periods of time the mitochondrial matrix could act as a storage site for Zn^{2+} .

In conclusion, we developed new sensors to map Zn^{2+} distributions among the mitochondrial matrix, IMS, and cytosol; provided the first direct measurement of Zn^{2+} concentrations within the IMS; and exemplified the benefits of these new probes by recording Zn^{2+} dynamics. Most importantly, we demonstrated that mitochondrial matrix and IMS played different roles in regulating cellular Zn^{2+} homeostasis. Our new tools will enable scientists to distinguish the Zn^{2+} levels between these two distinct compartments despite the resolution limitation of the light microscope that cannot distinguish the subcellular localizations between mitochondrial matrix and IMS.

METHODS

Details of the sensor plasmids construction, sensor characterization, cell culture, and transfection conditions for all cells are provided in the Supporting Information.

GZnP2 Development.

The Zn^{2+} sensor GZnP2 was created by cell-lysate-based screening from sensor libraries derived from GZnP1 according to previously described methods for GCaMP sensor development.⁵⁰ Sensor libraries were generated by incorporating mutations to two residues in the linker regions or Zn^{2+} binding motifs in GZnP1 by saturation mutagenesis, which yielded a library size of 400 each time. For each library, 800 Top 10 *Escherichia coli* colonies expressing sensor variants were picked and grown in deep well plates. Sensor expression was induced by 0.2% arabinose. After 2 days of growing in the shaker at RT, the cells were collected and lysed with BPER buffer. After lysis, the cells were centrifuged at 2250g for 20 min. The supernatant containing sensor proteins was added into 96-well black plates and incubated with 135 μM buffered Zn^{2+} and 100 μM TPEN separately to yield the minimal and maximal fluorescence ($F_{\min}$ and $F_{\max}$), which were read by the Tecan plate reader (E_x , 488 nm; E_m , 515 nm) and normalized to the fluorescence before treatment. The sensor dynamic range ($F_{\max}/F_{\min}$) and sensor brightness ($F_{\max}$) were compared among different sensor variants.

Quantification of Zn^{2+} Concentration in the Cytosol, IMS, and Matrix.

Sensor constructs were transfected into cells and imaged 48 h post-transfection. Imaging experiments were performed on a Nikon/Solamere CSUX1 spinning disc microscope. Images were collected with a 40 $\times$ 1.3 NA oil objective, and imaging data were acquired using the MicroManager software. The acquisition times were 20 s with exposure times of 100 ms and a laser power of 10% from the 488 nm laser, and exposure times of 50 ms and laser power of 3% by the 561 nm laser. Cells were imaged in phosphate-free HHBSS, at pH = 7.4. For sensor calibration, cells were treated with 100 μM TPEN to give the minimum signal and 1.25–5 μM pyrithione and 20 μM ZnCl_2 to establish the maximum signal. Because unhealthy cells could demonstrate compromised sensor response, we only chose the cells with dynamic ranges from 3 to 4.5 for mitochondrial matrix quantification and dynamic ranges 4–6 for cytosolic measurement.

Imaging analysis was performed by Fiji (ImageJ). The image stacks GZnP2 and mCherry sensor were split. Every image in an image stack was aligned to account for drift using the Stackreg plugin in Fiji. For the quantitative analysis, images of both GZnP2 and mCherry 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., $I_{\text{GZnP2}}(\text{cell}) - I_{\text{GZnP2}}(\text{background})$, and $I_{\text{mCherry}}(\text{cell}) - I_{\text{mCherry}}(\text{background})$. The background corrected GZnP2 and mCherry images are used to calculate the normalization ratio (R , i.e., GZnP2/mCherry). The calibration of the sensors was plotted in the KaleidaGraph program. The baseline mCherry-GZnP2 ratio (R), minimal ratio achieved with TPEN ($R_{\min}$), and maximal ratio ($R_{\max}$) obtained with Zn^{2+} saturation were used for cytosolic Zn^{2+} quantification using the equation $[\text{Zn}^{2+}] = K_d [(R - R_{\min})/(R_{\max} - R)]^{1/n}$ (K_d and n were used for specific region

pH = 8 for the matrix and pH = 7.4 for cytosol and IMS). All in situ sensor calibration was performed at RT. Statistical analysis was performed using the t-test in the KaleidaGraph program. The error bars represent the standard error of the mean.

Monitor cellular Zn²⁺ Dynamics in the Mitochondrial Matrix, IMS, and Cytosol.

To monitor Zn²⁺ uptake when cells are loaded with high Zn²⁺, sensor constructs were transfected into INS-1 pancreatic β -cells and imaged 48 h post-transfection. Imaging experiments were performed using the same microscope and microscope settings as stated above. Cells were imaged in phosphate-free HHBSS buffer, at pH = 7.4 with or without extracellular CaCl₂. To record the cellular Zn²⁺ transport under physiological conditions, cells were maintained in an environmental chamber with 37 °C, 5% CO₂, and 50–70% humidity. Cell depolarization was induced by a high KCl buffer (100 mM KCl, 1.1 mM MgCl₂, 16.8 mM D-Glucose, 20 mM HEPES, and 41.14 mM NaCl, at pH 7.4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

IMS	intermembrane space
ZF	zinc finger
FP	fluorescent protein
GZnP	green zinc protein
RT	room temperature
pM	picomolar
HHBSS	Hanks Balanced Salt Solution

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Figure 1.

Engineering GZnP2 with a higher dynamic range compared to GZnP1. (a) Optimize GZnP1 sensors by cell lysate based high throughput screening assays. Mutations were introduced into the Zn^{2+} binding motifs and the linker regions to generate several sensor libraries by saturation mutagenesis. The sensor library was then screened using cell lysates, high-throughput assays based on sensor fluorescence read with Zn^{2+} and the chelator TPEN. (b) The in situ response of the selected sensor variant in HeLa cells. Sensor fluorescence was decreased when cells were treated with 100 μM TPEN, and the fluorescence was increased with the addition of 5 μM pyrithione and 100 μM ZnCl_2 . (c) Comparison of in situ dynamic range ($F_{\text{max}}/F_{\text{min}}$) of GZnP1 and GZnP2. The dynamic range of GZnP2 was increased over 100% greater than GZnP1. (d) The *in vitro* emission spectrum of GZnP2 in the apo and the Zn^{2+} saturated state. GZnP2 displayed an emission peak at 510 nm, and the peak fluorescence intensity increased ~6 fold in response to Zn^{2+} .

Figure 2.

Compare Zn^{2+} measurements in the mitochondrial matrix, IMS, and cytosol. (a) Diagram of subcellular locations of GZnP2 in the mitochondrial matrix, IMS, and cytosol. The mito-mCherry-GZnP2 is targeted to the mitochondrial matrix with the cytochrome c oxidase subunit 8a; the SMAC-mCherry-GZnP2 sensor localizes to the IMS with the SMAC/DIABLO protein, while the mCherry-GZnP2 sensor without targeting sequence resides in the cytosol. (b–d) Images of the sensors in HeLa cells at the baseline for mito-mCherry-GZnP2 (b), SMAC-mCherry-GZnP2 (c), and mCherry-GZnP2 (d). (e–g) Representative calibration curves showing the percent saturation of the sensor in the mitochondrial matrix (e), IMS (f), and cytosol (g) in HeLa cells. The specific Zn^{2+} chelator TPEN (100 μM) was used to yield 0% saturation, while 5 μM pyrithione and 20 μM ZnCl_2 were applied to yield 100% saturation.

Figure 3.

Comparing Zn²⁺ concentrations among four cell types within the mitochondrial matrix, IMS, and cytosol. (a) In the mitochondrial matrix, the highest Zn²⁺ concentrations were in HEK293 cells, and the lowest Zn²⁺ concentrations were in Cos-7 cells. (b) The labile Zn²⁺ concentrations in IMS were between 60 and 100 pM. There were no differences for IMS Zn²⁺ between cell types except that INS-1 cells had higher IMS Zn²⁺ than Cos-7 cells. (c) There was no difference among different cell types for cytosolic concentration of labile Zn²⁺. The error bars indicate SEM (standard error of the mean). A * indicates a $p < 0.05$, and ** indicates $p < 0.01$. p values were determined using the t-test with unpaired data and unequal variance. Error bars are standard error of mean.

Figure 4.

Recording of Zn²⁺ uptake in the mitochondrial matrix, IMS, and cytosol of INS-1 cells with and without depolarization. 100 mM KCl was used to induce depolarization. (a–c) The Zn²⁺ uptake in the mitochondrial matrix (a), IMS (b) and cytosol (c) in INS-1 cells treated with 100 μM ZnCl₂ with or without 100 mM KCl in the absence of extracellular Ca²⁺. Higher Zn²⁺ was uptaken into the cytosol and IMS but not matrix when cells were depolarized. (d) The Ca²⁺ uptake in the cytosol and mitochondrial matrix recorded with GGECO1 and mito-RGECO1 in INS-1 cells treated with 100 mM KCl. The mitochondria rapidly sequestered a substantial amount of Ca²⁺ while the cytosolic Ca²⁺ increased and dropped back to baseline. (e) The Zn²⁺ uptake in mitochondrial matrix in INS-1 cells treated with 50 μM ZnCl₂ with or without 100 mM KCl in the presence of extracellular Ca²⁺. Extracellular Ca²⁺ did not induce more Zn²⁺ uptake into the mitochondrial matrix when cells were depolarized. (f) A comparison of the maximal Zn²⁺ levels in the mitochondrial matrix, IMS, and cytosol of INS-1 cells incubated with 50 or 100 μM ZnCl₂ with or without depolarization. There is a significant difference between the control and depolarized cells for Zn²⁺ levels in the cytosol and IMS, but no statistically significant difference was recorded in the mitochondrial matrix between the control and the depolarized cells with and without extracellular Ca²⁺. ***p < 0.001.

Table 1.

In vitro Characterization of GZnP2 Sensor

apparent dissociation constant (K_d' , pH 7.4)	apparent dissociation constant (K_d' , pH 8)	Hill coefficient (n , pH 7.4)	Hill coefficient (n , pH 8)	quantum yield (QY, Apo)	quantum yield (QY, Zn^{2+} -bound)	extinction coefficient (Apo)	extinction coefficient (Zn^{2+} -bound)
352 pM	112 pM	0.49	0.264	0.21	0.64	10,000	34,000