Reaction-based fluorescent sensor for investigating mobile Zn$^{2+}$ in mitochondria of healthy versus cancerous prostate cells

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Chelatable, mobile forms of divalent zinc, Zn(II), play essential signaling roles in mammalian biology. A complex network of zinc import and transport proteins has evolved to control zinc concentration and distribution on a subcellular level. Understanding the action of mobile zinc requires tools that can detect changes in Zn(II) concentrations at discrete cellular locales. We present here a zinc-responsive, reaction-based, targetable probe based on the dicysteyled form of Zinpyr-1. The compound, (6-amidoethyl)triphenylphosphonium Zinpyr-1 diacetate (DA-ZP1-TPP), is essentially nonfluorescent in the metal-free state; however, exposure to Zn(II) triggers metal-mediated hydrolysis of the acetyl groups to afford a large, rapid, and zinc-induced fluorescence response. DA-ZP1-TPP is insensitive to intracellular esterases over a 2-h period and is impervious to proton-induced turn-on. A TPP unit is appended for targeting mitochondria, as demonstrated by live cell fluorescence imaging studies. The practical utility of DA-ZP1-TPP is demonstrated by experiments revealing that, in contrast to healthy epithelial prostate cells, tumorigenic cells are unable to accumulate mobile zinc within their mitochondria.

Significance

Mobile zinc plays important roles in mammalian physiology. Understanding the action of mobile zinc requires tools to follow it within and between live cells. Zinc-selective fluorescent probes offer a facile means for detecting such mobile zinc, but small molecules constructed to perform this task typically have an unpredictable cellular distribution. Subtle changes in chemical structure can radically alter subcellular localization. To overcome this challenge, we installed a chemical unit to direct the sensor specifically to mitochondria and discovered that tumorigenic cells lose their ability to accumulate mobile zinc within these organelles. To carry out this work, we devised a reaction-based sensor that undergoes zinc-mediated chemistry, converting a nonfluorescent molecule into one that emits brightly and avoiding undesired sequestration in endo/lysosome.

Author contributions: S.J.L. and R.J.R. designed research; W.C., D.Y.Z., and R.J.R. performed research; W.C., D.Y.Z., S.J.L., and R.J.R. analyzed data; and W.C., S.J.L., and R.J.R. wrote the paper.

The authors declare no conflicts of interest.

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analog. Not only did this conversion result in the desired loss of endosomal/lysosomal entrapment, but the resulting probe, (6-amidoethyl)triphosphophonium Zinpyr-1 diacete (DA-ZP1-TPP), proved to have several additional highly desirable properties. The presence of the acetyl groups provided nearly complete fluorescence quenching that was rapidly reversed on exposure to Zn(II), the Lewis acidity of which mediated hydrolysis of the ester groups (28) to afford a large, rapid, zinc-induced fluorescence response (Fig. 1B). Moreover, DA-ZP1-TPP is insensitive to intracellular esterases over a 2-h period, impervious to proton-induced turn-on, and an excellent probe for targeting mitochondria in live cells. Unlike most reaction-based probes (29, 30), DA-ZP1-TPP also retains a measure of reversibility, owing to photo-induced electron transfer (PeT) from the dipicolylamine (DPA) groups in the apo state of the sensor (Fig. 1C).

To demonstrate the practical utility of DA-ZP1-TPP, we conducted live cell imaging studies of three different prostate cell lines. The results of these experiments revealed that, in contrast to healthy epithelial prostate cells, tumorigenic cells are unable to accumulate mobile zinc within their mitochondria.

Results and Discussion

ZP1-TPP is Sequestered in Endosomes/Lysosomes and Unable to Respond to Mobile Zinc. In targeting mitochondria, we relied on an aminoethyl derivative of triphenylphosphine to generate the lipophilic TPP cation (31). TPP delivers payloads, including fluorescent sensors (21–27), to the mitochondrial matrix by exploiting the negative potential maintained by actively respiring mitochondria (32). The free amino group on the amine groups of the TPP derivative provided a convenient synthetic handle for attachment of -6-COOH ZnP1 (33). The resulting construct, ZP1-TPP, has photophysical and zinc-binding properties similar to those of other ZP1 derivatives, yielding an approximate sevenfold increased zinc-induced fluorescence response with an apparent $K_d$ of 0.60 ($\pm$ 0.03) nM in cuvette studies (Chyan et al. 2015), but not at all with Zwitterion Endosome/lysosome

nts for TPP-Mediated Targeting of Mitochondria.

To better understand the limitations of ZP1-TPP in directing fluorophores to mitochondria, we conducted live cell imaging studies of three different prostate cell lines. The results of these experiments revealed that, in contrast to healthy epithelial prostate cells, tumorigenic cells are unable to accumulate mobile zinc within their mitochondria.

Fig. 2. Fluorescence imaging of ZP1-TPP in live HeLa cells. HeLa cells were pretreated with a 5-μM solution of ZP1-TPP in dye- and serum-free DMEM for 30 min (37 °C, 5% CO$_2$). Initial fluorescence images reveal a distinct punctate pattern that does not respond significantly to medium supplemented with 50 μM ZnPT or 100 μM TPEN (Scale bar: 25 μm).

Table 1. Lipophilicity, charge of predominate species in solution at physiological pH, and observed cellular localization of various dyes in live HeLa cells

<table>
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<th>Compound</th>
<th>log $P$ value</th>
<th>Charge</th>
<th>Localization</th>
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<tr>
<td>MitoTracker Red</td>
<td>1.15 ± 0.04</td>
<td>+1</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>DA-ZP1-TPP</td>
<td>0.74 ± 0.18</td>
<td>+1</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>C343-TPP</td>
<td>0.72 ± 0.05</td>
<td>+1</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>MitoTracker Green</td>
<td>0.56 ± 0.02</td>
<td>+1</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>ZP1-TPP</td>
<td>−0.11 ± 0.01</td>
<td>+1</td>
<td>Endosome/lysosome</td>
</tr>
<tr>
<td>FL-TPP</td>
<td>−0.39 ± 0.03</td>
<td>−1</td>
<td>Extracellular</td>
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$log P$ values were measured in octanol/water, buffered at pH 7 with 10 mM Tris, using a modified shake-flask procedure (SI Appendix).

Charge of the predominate species in solution at pH 7.4.

**Assessing the Physical Requirements for TPP-Mediated Targeting of Fluorophores to Mitochondria.** To better understand the limitations of ZP1-TPP in directing fluorophores to mitochondria, we prepared two additional TPP derivatives, one based on 6-carboxylfluorescein (FL-TPP) and the other based on coumarin 343 (C343-TPP) (SI Appendix, Fig. S4). We postulated that, under physiological conditions, insufficient hydrophobicity and cationic charge would prevent ZP1-TPP from accumulating in mitochondria (35, 36). At pH 7.4, ZP1-TPP [pK$_a$ = 6.96 (34); SI Appendix, Fig. S5] would be a zwitterion, owing to the triphenylphosphonium ion and the 2-carboxylate anion. The negative charge of the latter would reduce ZP1-TPP’s effectiveness in targeting mitochondria by mitigating the positive charge on the TPP moiety and decreasing the hydrophobicity of the probe. To test this hypothesis, we conducted live cell fluorescence imaging experiments with FL-TPP. Without the appended dipicolylamine (DPA) arms, FL-TPP is predominately anionic at physiological pH [phenolic oxygen pK$_a$ = 6.7 (37)]. Not only did FL-TPP fail to accumulate in mitochondria, it was cell-impermeable under our imaging conditions (SI Appendix, Fig. S6). In contrast, the cationic C343-TPP strongly colocalized with MitoTracker Red in live HeLa cells (Pearson’s $r = 0.72 ± 0.02$) (SI Appendix, Fig. S7).

To quantify the effect of hydrophobicity in mitochondrial targeting, we measured the octanol/aqueous buffer partition coefficient (log $P$) for our TPP constructs and two MitoTracker dyes. We then tabulated the log $P$, expected charge of the predominant species at physiological pH in solution, and cellular localization for each dye (Table 1). Based on these data, we conclude that TPP alone is insufficient to ensure mitochondrial targeting. Along with cationic charge, a minimum level of lipophilicity is required to achieve effective mitochondrial uptake.

Fig. 1. Schematic representation of zinc-induced fluorescence from DA-ZP1-TPP. (A) ZP1-TPP is rendered nonfluorescent by addition of acetyl groups on the phenolic oxygen atoms of the xanthene ring. (B) Coordination of Zn(II) enhances fluorescence intensity by promoting ester hydrolysis and alleviating PeT originating from the two DPA arms. (C) Fluorescence enhancement is partially reversed by removing Zn(II), which restores the fluorescence-quenching ability of the DPA groups.
must be achieved to evade endosomal/lysosomal sequestration and enable effective mitochondrial targeting.

Design, Synthesis, and Evaluation of DA-ZP1-TTP, a Zinc Reaction-Based Probe that Targets Mitochondria. To create a ZP1-TTP derivative optimized for mitochondrial localization, we developed a reaction-based probe based on fluorescein diacetate (Scheme 1). Modifying the fluorescein scaffold with esters or ether derivatives is a common strategy to increase the cell permeability, retention, and sensitivity of fluorescein-based probes (33, 38). In traditional applications, acetyl or acetoxymethyl groups are added to the phenolic oxygen atoms of the xanthene ring structure, resulting in formation of the nonfluorescent lactone isomer (39). These modifications neutralize the negative charge from the fluorescein carboxylate, increase the overall hydrophobicity of the probe, and allow the fluorophore to readily cross the plasma membrane. Once in the cytoplasm, intracellular hydrolases typically restore the fluorescence properties of the sensors by hydrolyzing the ester functionality appended to the fluorophore (39).

Although this approach has been widely implemented (40), it relies on endogenous enzymes, the cellular expression of which must be achieved to evade endosomal/lysosomal sequestration and enable effective mitochondrial targeting.

The diacetylated version of ZP1-TTP, designated DA-ZP1-TTP, was readily prepared by reacting ZP1-TTP with acetic anhydride overnight at room temperature. Over the course of the reaction, the mixture turned from a dark, salmon-colored solution to a light, nearly colorless liquid, which was purified by RP-HPLC. Consistent with the sensor adopting the lactone form, DA-ZP1-TTP is optically silent at λabs > 350 nm and essentially nonfluorescent (Φ < 0.001). Adding nanomolar concentrations of free zinc ions resulted in large increases in both the absorption (λabs-Zn = 510 nm; SI Appendix, Fig. S8) and fluorescence (λem-Zn = 529 nm; Fig. 3) spectral bands of DA-ZP1-TTP. These optical changes combined to yield a >140-fold increase in the fluorescence signal (Φem,Zn = 0.75 ± 0.03; Fig. 3). Analysis of the reaction products by analytical high-pressure liquid chromatography and electrospray ionization mass spectrometry confirmed that DA-ZP1-TTP was transformed back to ZP1-TTP in the presence of Zn(II) (SI Appendix, Figs. S9 and S10). Whether the mode of zinc-mediated ester hydrolysis proceeds by internal or external hydroxide attack remains to be determined (42). Of note, DA-ZP1-TTP retained a measure of reversibility, differentiating it from most reaction-based probes (30). Adding a chelator (EDTA) attenuated the fluorescence signal by approximately fivefold (Φ = 0.15 ± 0.02) in the cuvette with a concomitant redshift in the absorption spectrum (λabs = 522 nm; SI Appendix, Fig. S8).

Reversibility of the fluorescence signal is an important feature of zinc-responsive probes, enabling confirmation that the observed increase in fluorescence signal is due to Zn(II) and not the result of an artifact. By using ZP1 as the sensor scaffold, DA-ZP1-TTP retains the quenching ability of the DPA metal-binding arms, which affords a minimally fluorescent metal-free state even after hydrolysis of the acetyl groups occurs (34). Although it cannot provide complete reversal, DA-ZP1-TTP is a rare example of a reaction-based probe with even partial reversibility (28, 30).

DA-ZP1-TTP Has a Significantly Improved pH Profile. We next investigated the pH profile of DA-ZP1-TTP. The use of nitrogen atoms to coordinate zinc endows Zinpyr sensors with selectivity for zinc over calcium and magnesium, but also renders them pH-sensitive. The pKₐ values of the tertiary nitrogen atoms on the DPA units of ZP1 are 6.96 and 8.12 (34). Protonation of these amines leads to increased background fluorescence, because protons can diminish PeT in a manner similar to that of zinc ions (SI Appendix, Fig. S3) (34). In contrast to previous Zinpyr sensors, DA-ZP1-TTP displays no significant fluorescence turn-on under acidic conditions in the absence of zinc (Fig. 4); however, in the presence of excess zinc ions, the sensor shows a strong zinc-dependent fluorescence response at pH > 5. The lack of response from DA-ZP1-TTP under acidic conditions is a significant improvement over any of the current members of the Zinpyr family. We anticipate that the use of reaction-based zinc sensors will greatly improve our ability to image mobile zinc in acidic vesicles and compartments.

DA-ZP1-TTP Yields a Zinc-Selective Fluorescence Response. DA-ZP1-TTP has a zinc-induced fluorescence response and is relatively stable to uncatalyzed hydrolysis and esterase activity. Analogous to ZP1 (43), DA-ZP1-TTP responds to Zn(II) over biologically relevant cations, such as Ca(II) and Mg(II) (Fig. 5). Other late 3d-block transition metal ions, such as Co(II) and Cu(II), can coordinate to DA-ZP1-TTP but generally are not available in appreciable chelatable quantities within the cell (44). We used UV-visible spectroscopy to investigate the rate and zinc-specificity of metal-mediated ester hydrolysis. DA-ZP1-TTP deacetylation resulted in opening of the lactone ring and restoration of conjugation across the xanthene ring, as evidenced by the change in the absorption spectrum (SI Appendix, Fig. S8).

By monitoring the absorbance of the xanthene π system, we could interrogate deacetylation kinetics in a manner independent of the fluorescence response. In buffer (50 mM PIPES, 100 mM KCl, pH 7) at 37 °C, adding excess ZnCl₂ increased the absorbance at 510 nm. The kinetic trace for this process fits well with the function. The pH of the solution was increased by sequential addition of aqueous KOH. The total volume of KOH added was less than 5% of the total volume. λem = 475 nm.
Fig. 5. Metal ion selectivity of DA-ZP1-TPP. Average normalized fluorescence intensities for a 1.1 μM solution of DA-ZP1-TPP in buffer (50 mM Pipes, 100 mM KCl; pH 7) at 25 °C, after addition of 50 μM ZnCl₂ (black bars). The gray column represents the average sensor intensity after addition of 100 μM EDTA. Data were normalized to initial measured emission intensity.

a single exponential function with \( t_{1/2-Zn} = 8.1 \) s (Fig. 6 and SI Appendix, Fig. S11 and Table S2). The reaction rate, as monitored by fluorescence enhancement, is independent of zinc concentration (SI Appendix, Table S4). This result is consistent with the fast rate (4.10^6 M⁻¹ s⁻¹, 25 °C) of zinc binding for other Zinpyr derivatives (45), implying that either ester hydrolysis or lactone ring opening is the rate-determining step (SI Appendix, Fig. S12).

In contrast, in the presence of 250 μM EDTA or 1.25 U of porcine liver esterase, the rate of deacetylation for DA-ZP1-TPP is dramatically slower, with a \( t_{1/2-EDTA} \) of 8.6 h and \( t_{1/2-esterase} \) of 2.9 h (Fig. 6 and SI Appendix, Fig. S11 and Table S2).

Of note, both Cu(II) and Co(II) can mediate ester hydrolysis. These observations are consistent with the reported hydrolytic ability of both metal ions in small-molecule biomimetic and sensing systems (42, 46–48). Co(II)-mediated ester hydrolysis was fit to a single exponential function with \( t_{1/2-Co(II)} = 3.04 \) s (SI Appendix, Fig. S11 and Table S2), whereas Cu(II) had significantly slower kinetics requiring two sequential irreversible steps for a good fit. The calculated half-lives for the two irreversible steps were \( t_{1/2} = 6.08 \) min and \( t_{1/2} = 39.5 \) min, respectively (SI Appendix, Fig. S11 and Table S2). As shown in Fig. 5, however, cross-reactivity with Co(II) and Cu(II) would quench the fluorescence signal and thus would not lead to any false fluorescence response.

To determine whether ester hydrolysis depends on the metal-binding core of DA-ZP1-TPP, we measured the deacetylation kinetics of fluorescein diacetate (DA-FL) under analogous conditions. As expected, DA-FL responded rapidly to esterase, but was relatively stable to hydrolysis and zinc-mediated deacetylation (SI Appendix, Fig. S13 and Table S3). The prolonged stability of DA-ZP1-TPP with respect to hydrolytic and enzymatic deacetylation in the absence of zinc ions, coupled with the rapid zinc-induced fluorescence response of the prosensor, indicate that DA-ZP1-TPP can serve as an effective cellular imaging agent for mobile zinc.

DA-ZP1-TPP is Stable to Intracellular Hydrolases, Targets Mitochondria, and Responds to Changes in Intracellular Zinc Levels. We used HeLa cells as a model system to evaluate the ability of DA-ZP1-TPP to target mitochondria and respond to changes in intracellular zinc levels. Live HeLa cells were pretreated with medium containing 1 μM DA-ZP1-TPP and 250 nM MitoTracker Red for 30 min (37 °C, 5% CO₂) before imaging (Fig. 7). Initial images showed minimal fluorescence intensity from the construct (Fig. 7B), consistent with the low endogenous levels of mobile zinc in HeLa cells (17). Over a 2-h period, the fluorescence intensity from the sensor remained constant, implying that the acetylated form is stable against intracellular esterases (Fig. 7F). Adding 50 μM Zn/pyrithione (ZnP) resulted in an ~12-fold increase in fluorescence intensity that visually overlapped with the signal from MitoTracker Red (Fig. 7 and SI Appendix, Figs. S14 and S15). Quantitative analysis of the fluorescence signals obtained from MitoTracker Red and zinc-bound ZP1-TPP revealed that the two dyes had good colocalization (Pearson’s r = 0.64 ± 0.1). Adding 100 μM N,N,N′,N′-tetrakis-(2-pyridylmethyl)ethylenediamine (TFEN) attenuated the fluorescence signal by 2.4-fold (Fig. 7G).

Based on these fluorescence microscopy studies with HeLa cells, we conclude that (i) in contrast to the nonacylated form of the sensor (vide supra), DA-ZP1-TPP has sufficient cationic character and hydrophobicity (log P = 0.74 ± 0.18; Table 1) to target mitochondria effectively; (ii) DA-ZP1-TPP is stable against intracellular hydrolysis over a period of ≥2 h; and (iii) DA-ZP1-TPP responds to changes in intracellular mobile zinc levels, an observation corroborated by sensor shutoff on the addition of a chelator. Taken together, these data strongly support acetylation of fluorescein-based zinc sensors as an effective strategy both to improve the dynamic range of fluorescein-based zinc sensors and to facilitate cellular targeting by a localization vector.

DA-ZP1-TPP is a valuable addition to the arsenal of targetable small-molecule zinc-selective fluorescent probes. Currently, the most widely applied sensor for zinc in mitochondria is RhodZin-3, AM (40) (SI Appendix, Table S5), which requires endogenous esterases to activate and has a weak zinc affinity (Kₐ ~65 μM). This value is well above the current estimates for mitochondrial mobile zinc concentrations (17). We anticipate that this strategy will be applicable to related families of fluorophores as well.

Investigating Zinc Trafficking to Mitochondria in Prostate Cells. With a new mitochondrial-targeting sensor in hand, we used DA-ZP1-TPP to investigate the ability of prostate cell lines to accumulate zinc within their mitochondria. RWPE-1 and RWPE-2 are a pair of genetically similar cell lines that retain normal epithelial cell morphology, express cytokeratin markers for prostate epithelial cells, and are hormone-sensitive (49, 50). RWPE-2 cells are tumorigenic, however, and accumulate less zinc owing to decreased ZIP1 expression and altered cellular localization of ZIP3 (11, 50).

To explore the ability of RWPE-1 and RWPE-2 cells to sequester zinc in mitochondria, we measured the fluorescence intensity of DA-ZP1-TPP in both lines after the cells were bathed in normal or zinc-enriched (50 μM ZnCl₂) keratinocyte serum-free medium for 24 h before imaging. The fluorescence signal intensity was ~2.3-fold higher in RWPE-1 cells bathed in zinc-enriched medium compared with cells bathed in normal medium (Fig. 8 and SI Appendix, Fig. S18). This result is consistent with the ability of healthy epithelial prostate cells to accumulate high levels of zinc.
concentrations of total zinc (50, 51), but it also reveals that the observed changes correlate with increased mobile zinc within mitochondria. In contrast, RWPE-2 cells showed no statistically significant increase in fluorescence signal under analogous conditions (Fig. 8). Importantly, in both cell lines, the observed fluorescence signals from DA-ZP1-TPP are sensitive to TPEN and ZnPT and have good correlation with MitoTracker Red ($r = 0.68 \pm 0.09$ for RWPE-1 and $0.59 \pm 0.1$ for RWPE-2 (SI Appendix, Figs. S16–S18)).

To further corroborate these observations, we conducted an analogous experiment in PC-3 cells, a model cell line for human prostatic adenocarcinoma metastatic to bone (52). In PC-3 cells, DA-ZP1-TPP correlated with MitoTracker Red qualitatively but with a lower correlation coefficient ($r = 0.32 \pm 0.1$) (SI Appendix, Fig. S19). Consistent with the results for the RWPE cell line, PC-3 cells showed no statistically significant increase in sensor fluorescence intensity when bathed in zinc-enriched ($50 \mu M \text{ZnCl}_2$) RPMI 1640 medium with 10% FBS for 24 h before imaging (Fig. 8).

To account for the zinc-binding ability of FBS (53), we repeated the imaging experiments with PC-3 cells in serum-free RPMI 1640 medium supplemented with 15 $\mu M \text{ZnCl}_2$ for 3 h (SI Appendix, Fig. S20) (54). Under these conditions, no statistically significant increase in fluorescence intensity was observed.

Our fluorescence imaging results from the RWPE and PC-3 cell lines are in agreement with the results of studies using homogenized prostatic tissue, which assert a correlation between altered zinc trafficking and prostate cancer (8, 11–13). These data demonstrate the inability of tumorigenic prostate cells to accumulate mobile zinc within their mitochondria. The inability of cancer cells to accumulate mobile zinc within mitochondria is consistent with the theory that tumors need an active mitochondrial aconitase to meet the energy demands of rapidly dividing cells (6). However, the RWPE-1, RWPE-2 (SI Appendix, Fig. S21), and PC-3 cell lines all accumulate increased levels of total zinc when bathed in zinc-enriched medium (9, 11, 50).

The ability of cancer cells to accumulate zinc, along with the lack of a defined mitochondrial zinc transporter (7), raise questions about the mechanism of mobile zinc accumulation within mitochondria of prostatic epithelial cells, and how zinc trafficking is altered in tumorigenic cells. One possible explanation is differential expression of metallothionein isoforms in cancer cell lines (54); another is altered zinc trafficking pathways resulting from modified signaling cascades and transcription factor activity (13, 55, 56). Addressing these and other possibilities will require a detailed molecular understanding of the action of mobile zinc within healthy and tumorigenic prostate cells. In combination with biochemical investigations, the ability to direct zinc-responsive fluorescent probes to discrete cellular locales should provide valuable insight into the function of mobile zinc within the prostate.

**Conclusion**

DA-ZP1-TPP is a zinc-selective, reaction-based, fluorescent sensor that has an excellent dynamic range and pH profile, and it can be targeted to mitochondria in live cells. Acetylation of fluorescein-based zinc sensors simultaneously increases hydrophobicity and reduces the anionic character of the constructs, which in turn improves the cellular uptake and targetability of the probes. In contrast to the nonacetylated derivative, DA-ZP1-TPP has a vastly improved dynamic range and pH profile, and it can target mitochondria in live cells. DA-ZP1-TPP imaging of epithelial prostate cells revealed that tumorigenic lines lose the ability to accumulate mobile zinc within their mitochondria. Understanding mobile zinc trafficking in biology will require reagents such as DA-ZP1-TPP, which have the ability to visualize changes in free $Zn^{2+}$ concentrations at discrete cellular locales. By creating a mobile zinc map and tracking the accumulation and fluctuation of...
mobile zinc concentrations within the cell, we can begin to decipher the spatiotemporal mechanism behind the actions of mobile zinc and improve our understanding of its biology.

**Materials and Methods**

Materials and procedures for all experiments are supplied in the SI Appendix. Included in this appendix are the synthesis and characterization of compounds, mammalian cell culturing procedures, and details regarding fluorescence microscopy experiments. Also provided are additional figures, tables, and schemes relating to the characterization of compounds.

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