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SpiroZin1: A Reversible and pH-Insensitive, Reaction-Based, Red-Fluorescent Probe for Imaging Biological Mobile Zinc

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A reversible, reaction-based sensor for biological mobile zinc was designed, prepared, and characterized. The sensing mechanism of this probe is based on the zinc-induced, ring-opening reaction of spirobenzopyran to give a cyanine fluorophore that emits in the deep-red region of the electromagnetic spectrum.

Introduction

Zinc is an essential trace element in biology.^[1,2] Although most biological zinc occurs as a structural component of proteins,^[3] there are pools of readily chelatable, or mobile, zinc in specific organs such as the brain,^[4,5] pancreas,^[6] and prostate.^[7] In the hippocampus, high concentrations of mobile zinc stored in vesicles at glutamatergic synapses^[8] have been conclusively associated with long-term potentiation.^[9] Mobile zinc also plays an important role in sensory perception.^[10–13] In the pancreas, insulin crystallization depends on the expression of the zinc transporter ZnT8,^[14] and zinc can be used as a surrogate for imaging insulin secretion.^[15] Mobile zinc accumulates in epithelial cells in the prostate,^[16] and a firm link has been established between intracellular zinc levels and prostate cancer.^[17]

The development of luminescent sensors for mobile zinc has provided important tools for investigating its many biological roles.^[18,19] Although these probes use various sensing mechanisms, most rely on the modulation of photoinduced electron transfer (PET) for their fluorescence turn-on response.^[20] PET-based probes are often sensitive to pH, however,^[21,22] and do not operate efficiently at long wavelengths (>700 nm). These limitations restrict the information that can be obtained using fluorescent sensors, especially because long-wavelength-emitting probes are preferred for studies in vivo.^[23,24]

Probes that emit a luminescent signal following a chemical reaction have been developed recently.^[25,26] A limitation of such "reaction-based" probes, however, is their irreversibility, which precludes their application to real-time monitoring of concentration changes.

In the present study, we sought to prepare a reaction-based sensor for Zn^{2+} that is reversible, pH-insensitive, and emits at

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This probe is not activated by protons and operates efficiently in aqueous solution at pH 7 and high ionic strength. The mechanism of this reaction was studied by using a combination of kinetics experiments and DFT calculations. The biocompatibility of the probe was demonstrated in live HeLa cells.

long wavelengths. We recognized that switching reactions offer an ideal platform for sensor development because of their reversibility. The equilibrium between spirobenzopyrans and their cyanine forms provides an excellent example of a thermodynamically reversible switching reaction that has been exploited extensively in materials science.^[27] This reaction is generally driven by light, but examples of chelation-induced switching have also been reported.^[28,29] Herein we report the design, synthesis, and mechanistic study of SpiroZin1, a sensor that comprises dipicolylamine as the zinc-binding unit, appended to a spirobenzopyran scaffold as well as its application in live cell imaging.

Results and Discussion

Synthesis and characterization

SpiroZin1 was synthesized in a one-pot procedure starting from commercially available dipicolylamine (DPA) and the readily accessible building blocks 1 and 2 (Scheme 1 A). The reaction involves nucleophilic attack of DPA on the benzylic chlorine of 2, followed by Knoevenagel condensation of the intermediate aldehyde with 1. SpiroZin1 was isolated as a palepink, waxy solid that forms nearly colorless solutions in common organic solvents such as CH₃OH, CH₃CN, CH₂Cl₂, and DMSO. ¹H NMR spectroscopic measurements in CD₃OD (Supporting Information Figures SI-1 and SI-2) revealed two diastereotopic singlets for the two methyl groups on the dihydroindole ring and two diastereotopic doublets for the methylene that connects the DPA arm (Scheme 1B) with the spirobenzopyran core (Figure SI-3). In addition, the two vinylic hydrogen atoms on the benzopyran ring have a coupling constant ${}^{3}J_{H-H} =$ 7.5 Hz, consistent with cis stereochemistry. These observations demonstrate that SpiroZin1 exists predominantly as the spirobenzopyran isomer (Scheme 1 B). Addition of one equivalent of ZnCl₂ turned the color of SpiroZin1 to a deep red. ¹H NMR spectra of this solution revealed only one singlet for the

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Scheme 1. A) One-pot synthesis of SpiroZin1. *Reagents and conditions*: a) K_2CO_3 , EtOH, reflux, 2 h, 40%. B) Reversible reaction of SpiroZin1 with Zn^{2+} .

methyl groups on the indolenine ring and a singlet for the methylene group that connects DPA with the rest of the molecule (Scheme 1B). The coupling constant of the vinylic hydrogen atoms also changed to ${}^{3}J_{H-H} = 16.5$ Hz, consistent with *trans* geometry of the alkene. These NMR signals prove that, in the presence of Zn²⁺, the spirobenzopyran ring is opened to produce the cyanine form.

Sensing properties

The photophysical properties of SpiroZin1 were investigated in aqueous buffer (50 mM PIPES, 100 mM KCl, pH 7). Under these conditions, SpiroZin1 absorbs only weakly in the region between 400–700 nm (Figure 1). No fluorescence could be detected for this solution upon excitation at 500 nm. Following addition of 10 equivalents of ZnCl₂, however, a strong band appeared at $\lambda_{max} = 508$ nm (1.40(3)×10⁴ m⁻¹ cm⁻¹). Excitation at



Figure 1. Electronic absorption (solid lines) and fluorescence (dashed lines) spectra of SpiroZin1 before (grey lines) and after (black lines) addition of 10 equiv of ZnCl₂. All measurements were performed with 5 μ M SpiroZin1 in aqueous buffer (50 mM PIPES, 100 mM KCl, pH 7).

508 nm produced fluorescence in the deep-red region at λ_{max} = 650 nm (φ = 0.0042(7), Figure 1). Despite the low quantum yield of the probe in the zinc-bound form, SpiroZin1 efficiently detects mobile Zn²⁺ because of the very low background in both the absorption and emission spectra of the apo form.

Titration of SpiroZin1 with ZnCl₂ revealed formation of a 1:1 complex (Figure SI-5). We propose a binding mode involving the three nitrogen atoms of the DPA arm and the oxygen atom on the cresol ring, which triggers the spirobenzopyran ring-opening reaction. This binding mode

maintains the [N₃O] coordination sphere of previously reported sensors, such as those in the ZP family,^[30,31] ZPP1,^[32] and the recently reported ZBR probes.^[33] SpiroZin1 chelates Zn²⁺ tightly with an apparent dissociation constant (K_d) of 21(1) pM (Figure SI-6). A water molecule may be bound to zinc as well.

The selectivity of SpiroZin1 for biologically relevant metal cations was investigated. Alkaline and alkali-earth metals produce no change in the fluorescence or absorbance spectra of SpiroZin1 (Figure SI-7). Paramagnetic transition metals increased the absorbance but not the fluorescence, suggesting that they cause ring opening, but their partially filled d-orbitals quench the fluorescence of the resulting cyanine. Cadmium increases both fluorescence and the absorbance, but to a lesser extent than zinc (Figure SI-7).

An advantage of SpiroZin1 is its ability to operate at low pH. The fluorescence of SpiroZin1 turns on at pH 4, 5, and 6 (Figure 2), but this increase is very small compared with the turn-on induced by Zn^{2+} at these pH values (Figure 2).

Mechanistic studies

SpiroZin1 is unique among reaction-based probes because of its reversibility. We investigated the reaction kinetics of both the turn-on event induced by Zn²⁺ and the turn-off process caused by its removal following addition of the chelating agent ethylenediaminetetraacetic acid (EDTA). The turn-on process is very rapid and was monitored by stopped-flow spectrophotometry. Similar results were obtained by stopped-flow fluorescence spectroscopy (Figure SI-8). This study revealed that turn-on occurs in two steps, each of which could be modeled by a single exponential function (Figure 3). The first process is fast with $t_{1/2} = 5.89(9)$ ms and the second step is slower, with $t_{1/2} = 1.17(2)$ s. Overall, the turn-on event occurs within seconds under physiological conditions. The turn-off process, on the other hand, can be modeled by a single-exponential decay as determined by fluorescence spectroscopy (Figure 3) and is slow, with $t_{1/2} = 8.55(1)$ min.

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Figure 2. Integrated fluorescence intensity of 5 μm SpiroZin1 at various pH values before (white bars) and after (grey bars) addition of 10 equiv of ZnCl₂. Intensities were normalized with respect to 5 μm SpiroZin1 at pH 7 before addition of zinc.

To understand the mechanism of the sensing reaction of SpiroZin1, we carried out an intrinsic reaction coordinate analysis using density functional theory (DFT) at the BP86/TZVP/LANL2DZ level of theory. We decided to model only the electronic ground state of each reaction intermediate because the experimental observations demonstrate chelation of Zn^{2+} to be the main driving force of the reaction. We note that the presence of light might influence the reaction mechanism and that our calculations are meant to provide only a qualitative representation.

The reaction was modeled starting from a zinc complex of SpiroZin1 in the spirobenzopyran form (Figure 4, structure A). The first step in the reaction is ring opening (B) to afford a *cis*-cyanine as an intermediate (C). The calculated activation energy for this process is only 2.8 kcal mol⁻¹, which agrees

qualitatively with the fast step observed by stopped-flow kinetics. The second step of the reaction is isomerization of the alkene (D) to give the final trans cyanine (E). The higher calculated activation energy of this process is also consistent with the slower kinetics measured. In the opposite direction, the slow *cis/trans* isomerization occurs first and is the rate-determining step, which agrees with the single exponential decay of fluorescence observed experimentally.

Live cell imaging

The ability of SpiroZin1 to report on the arrival of mobile zinc in live cells was assessed by using human cervical cancer cells (HeLa). HeLa cells were incubated at 37 $^{\circ}$ C with 5 μ M SpiroZin1 for 30 min before imaging. Considerable intracellular red fluorescence was detected under these conditions (Figure 5B). The reason for this high background fluorescence might be the relatively high concentration of endogenous mobile Zn²⁺ in HeLa cells $(\sim 100 \text{ pm})^{[34]}$ and the high Zn^{2+} affinity of SpiroZin1 (21(1) рм). The intracellular fluorescence, however, increased further upon incubation of the cells with 20 μM $ZnCl_2$ and 50 μ M concentrations of the Zn²⁺ ionophore 2-mercaptopyridine oxide (pyrithione) (Figure 5C). Quantification of the fluorescence intensity revealed that addition of the exogenous zinc elicited a 2.5(5)-fold turn-on. Addition of the cell-permeable chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) decreased the fluorescence, confirming that the fluorescence turn-on is induced by zinc (Figure 5D).

Summary and Conclusions

We describe the design, synthesis, and characterization of a red-emitting sensor for mobile zinc. This sensor, SpiroZin1, is based on the ring-opening reaction of spirobenzopyran to form a fluorescent cyanine. This reaction is induced by Zn²⁺, can be reversed upon treatment with a chelator, and operates even at low pH. Studies using stopped-flow kinetics, fluores-



Figure 3. A) Increase in absorbance at 508 nm of 5 μ M SpiroZin1 in aqueous buffer (50 mM PIPES, 100 mM KCl, pH 7) after addition of 100 equiv of ZnCl₂. B) Decrease in integrated fluorescence (550–800 nm, λ_{ex} =508 nm) of 5 μ M zinc-bound SpiroZin1 in aqueous buffer (50 mM PIPES, 100 mM KCl, pH 7) after addition of 100 equiv of EDTA.

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Figure 4. Intrinsic reaction coordinate analysis of the zinc-induced, ring-opening reaction of SpiroZin1: A) ringclosed starting structure; B) ring-opening transition state; C) open, cis-cyanine intermediate; D) cis/trans isomerization transition state; E) open, trans-cyanine product.

cence spectroscopy, and DFT calculations provided mechanistic details of the sensing mechanism. The biocompatibility of this sensor was assessed in cultured HeLa cells.

The modular synthesis of SpiroZin1 allows modification of the metal-binding site, which will be useful for tuning its affinity and selectivity. We envision that this design principle will inform the construction of probes that emit at even longer wavelengths.

Experimental Section

Chemistry

General: All reagents were purchased from commercial sources and used as received. Compounds 1^[35] and 2^[36] were prepared according to published methods. Solvents were purified and degassed by standard procedures. Thin-layer chromatography and flash column chromatography were performed using Brockmann 1 basic Al₂O₃. NMR spectra were acquired on a Varian Inova-300 instrument. ^1H and $^{13}\text{C}\,\text{NMR}$ chemical shifts (δ) are reported in parts per million (ppm) relative to SiMe_4 ($\delta\!=\!0\text{ ppm})$ and were referenced internally with respect to residual solvent proton peaks $({}^{1H}\delta = 3.31 \text{ ppm}$ and ${}^{13C}\delta = 49.00 \text{ ppm}$ for CH₃OH). Coupling constants (J) are reported in Hertz (Hz). Low-resolution mass spectra (LRMS) were acquired on an Agilent 1100 Series LC/MSD Trap spectrometer (LCMS), using electrospray ionization (ESI). High-resolution mass spectrometry (HR-ESI-MS) was conducted by staff at the MIT Department of Chemistry Instrumentation Facility on a Bruker Daltonics APEXIV 4.7 T FT-ICR-MS instrument. The IUPAC name of SpiroZin1 is provided and was determined using CambridgeSoft ChemBioDraw Ultra 12.0.

1-(Pyridin-2-yl)-N-(pyridin-2-ylmethyl)-N-((1',3',3',6-tetramethylspiro[chromene-2,2'-indolin]-8yl)methyl)methanamine (Spiro-Zin1): Compound 2 (150 mg, 0.728 mmol) and K₂CO₃ (302 mg, 2.18 mmol) were added to a Schlenk flask that was evacuated

and refilled with nitrogen three times. A solution of dipicolylamine (290 mg, 1.46 mmol) in EtOH (7 mL) was added via syringe. The mixture was stirred at 25 °C for 1 h. Compound 1 (219 mg, 0.728 mmol) was added as a solid in one portion, and the mixture was heated at reflux for 1 h. The suspension was filtered, and the filtrate was concentrated. The residue was purified by flash column chromatography (CH₂Cl₂, 100%) to give SpiroZin1 as a pale-pink solid (148 mg, 40%): mp: 50-51°C; $R_{\rm f} = 0.24$ (CH₂Cl₂); ¹H NMR (300 MHz, CD₃OD): $\delta = 1.13$ (s, 3 H), 1.24 (s, 3 H), 2.20 (s, 3 H), 2.66 (s, 3 H), 3.33 (d, J=18.1 Hz, 1 H), 3.52 (d, J = 18.2 Hz, 1 H), 3.59 (s, 4 H), 5.71 (d, J = 7.5 Hz, 1 H), 6.47 (d, J = 7.5 Hz, 1 H), 7.5 7.5 Hz, 1 H), 6.75-6.87 (m, 2 H), 6.81 (s, 1 H), 6.99 (s, 1 H), 7.00-7.10 (m, 2H), 7.18-7.23 (m, 2H), 7.45 (d, J=6.1 Hz, 2H), 7.67-7.70 (m, 2H), 8.34 ppm (d, J=6.0 Hz, 2H); ¹³C NMR (75 MHz, CD₃OD): $\delta =$ 19.4, 19.6, 25.2, 28.1, 51.3, 52.0, 59.7, 104.4, 106.6, 118.99, 119.01, 121.2, 122.5, 123.3, 123.4, 126.5, 127.4, 128.9, 129.7, 131.2, 136.9, 137.5, 148.0, 148.2, 150.4, 159.3 ppm; HRMS-ESI: m/z calcd for [C₃₃H₃₅N₄O]⁺: 503.2811, found: 503.2818.

Spectroscopic methods

All aqueous solutions were prepared using de-ionized water with resistivity 18.2 m Ω cm⁻¹, obtained using a Milli-Q water purification system. Solvents were procured from Aldrich and used as received. Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and 99.999% KCI



Figure 5. Microscopy images of live HeLa cells incubated with 5 µM SpiroZin1: A) differential interference contrast (DIC) image; B) red channel image before addition of zinc pyrithione; C) red channel image after incubation with 20 µM zinc pyrithione; D) red channel image after incubating for 30 min with 50 µM TPEN. Scale bar = 25 μ m.

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were purchased from Calbiochem. Stock solutions of SpiroZin1 in DMSO were prepared at a concentration of 5 mm and stored at -20°C in 1 mL aliquots and thawed immediately before each experiment. All spectroscopic measurements were conducted in aqueous buffer containing 50 mм PIPES (pH 7) and 100 mм KCl. UV/Vis spectra were acquired on a Cary 50 spectrometer using quartz cuvettes from Starna (1 cm path length). Fluorescence spectra were acquired on a Photon Technology International fluorimeter. All measurements were conducted at 25 °C maintained by circulating water baths. Extinction coefficients were determined in the 1–5 μm range in aqueous buffer. Fluorescence quantum yields were determined by using $1-5 \,\mu M$ solutions of sensor in aqueous buffer, exciting at 508 nm. Fluorescence emission spectra were integrated from 550 to 800 nm. The quantum yield calculation was standardized to resorufin, which has a reported quantum yield of 0.74 at pH 9.5, $\lambda_{ex}\!=\!572$ nm. $^{\scriptscriptstyle [37]}$

pH Titration, metal selectivity, and dissociation constant

Buffered solutions at pH 3, 4, 5, 6, and 7 were prepared by mixing different volumes of 0.1 \mbox{m} citric acid and 0.2 \mbox{m} Na₂HPO₄ in water (Table SI-1) and the pH was verified using a Mettler Toledo FE20 pH meter. The fluorescence of 5 \mbox{m} SpiroZin1 in these buffered solutions was measured before and after addition of 10 equiv of ZnCl₂ and the intensities were normalized with respect to 5 \mbox{m} SpiroZin1 at pH 7 before addition of ZnCl₂.

To determine the metal selectivity of SpiroZin1, PIPES-buffered solutions (pH 7) containing 5 μ M SpiroZin1 were prepared. To each solution, 100 equiv of NaCl, MgCl₂, CaCl₂, CdCl₂, NiCl₂, CuCl₂, MnCl₂, or CoCl₂ were added and the fluorescence intensity measured. Cu⁺ was added as a solution of [Cu(CH₃CN)₄]PF₆ in CH₃CN to 5 μ M SpiroZin1 in CH₃CN under nitrogen. To the same solutions, 100 equiv of ZnCl₂ were added and the fluorescence intensity recorded. In each case, the fluorescence intensity was normalized with respect to 5 μ M SpiroZin1 in aqueous buffer.

To determine the apparent dissociation constant (K_d), solutions containing 1 mm 1,2-diaminohydroxypropanetetraacetic acid (DHPTA) and ZnCl₂ were prepared. The total concentration of Zn²⁺ was varied from 0.1 mm to 0.9 mm, to give buffered free Zn²⁺ concentrations ranging from 1.3×10^{-12} m to 1.1×10^{-10} m.^[38] 5 µm SpiroZin1 was added to each of these solutions, which were then equilibrated in the dark for 24 h. The fluorescence of these solutions was measured and normalized with respect to 5 µm SpiroZin1 in the presence of 100 equiv of ZnCl₂. The normalized fluorescence responses (R) were plotted against the concentration of free Zn²⁺ [Zn] and the resulting data were fit to the equation $R = B[Zn]/(K_d + [Zn])$, where B = 1. Minimization of this equation gave the values B = 1.03(1), $K_d = 2.1(1) \times 10^{-11}$ m, and $R^2 = 0.995$.

Kinetic experiments

Zinc-induced ring opening of SpiroZin1 was monitored by stopped-flow UV/Vis spectroscopy configured with a single-wavelength photomultiplier and a tungsten lamp. Stopped-flow data were obtained using a Hi-Tech Scientific SF-61 DX2 stopped-flow spectrophotometer. The optical change at 508 nm was recorded to assess formation of the cyanine isomer.

Solutions containing 5 μ M SpiroZin1 and 500 μ M ZnCl₂ were prepared in aqueous buffer (50 mM PIPES, 100 mM KCl, pH 7). The reactions were maintained at 25 °C with a circulating water bath. Data points were collected on a logarithmic time scale (0.001–

10 s). A dead time determination indicated that the first usable data points occur between 1 and 2 ms and the raw data was truncated accordingly. The data used for analysis are averages of six independent measurements and were fit to a double exponential function: $A = A_0 + B_1^{[k_1t]} + B_2^{[k_2t]}$, where A is the measured absorbance, A_0 the initial absorbance, k_1 and k_2 the rate constants of the two steps, t is time, and B_1 and B_2 are constants. Minimization of this equation gave the values $k_1 = 178(3) \text{ s}^{-1}$, $k_2 = 0.59(1) \text{ s}^{-1}$, $R^2 = 0.9997$.

The ring-closing process was monitored by fluorescence spectroscopy. A solution containing 5 μ M SpiroZin1 and 5 μ M ZnCl₂ was prepared and allowed to equilibrate in the dark for 1 h. The fluorescence of this solution was measured and 100 equiv of EDTA were added. The fluorescence intensity was measured every 30 s. Fluorescence emission spectra at each time point were integrated from 550 to 800 nm and plotted against time. The data were fit to a single exponential function: $F = F_0 + B_1^{[k_1t]}$, where *F* is the integrated fluorescence, F_0 the initial integrated fluorescence, k_1 the rate constant, *t* is time, and B_1 a constant. Minimization of this equation gave the values $k_1 = 0.001351(1)$ s⁻¹, $R^2 = 0.99992$.

DFT calculations

All calculations were carried out using ORCA 2.9.1^[39] employing the BP86 functional, the TZVP basis set for C, N, O, and H, and the LANL2DZ basis set for Zn. The resolution of the identity (RI) approximation, with the appropriate auxiliary basis sets, was used in all calculations. Geometry optimizations were performed in solution by applying the implicit conductor-like screening model (COSMO) for water. The nature of the stationary points was characterized by harmonic vibrational analysis. All minima showed zero imaginary frequencies, whereas all transition states gave only one imaginary frequency along the reaction coordinate. Cartesian coordinates of all structures are provided in the Supporting Information.

Cell culture and staining procedures

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Cellgro, MediaTec Inc.), supplemented with 10% fetal bovine serum (FBS; HyClone), 1% penicillin–streptomycin, 1% sodium pyruvate, and 1% L-glutamine. The cells were grown to 90% confluence at 37°C with 5% CO₂ before being passed and plated onto poly-D-lysine-coated plates 24 h prior to imaging. Cells used were at passage number 7. A confluence level of 50% was reached at imaging. The growth medium was replaced with dyefree DMEM containing 5 μ M SpiroZin1 and 2 μ M Hoechst 33528 nuclear stain, and the cells were incubated for 30 min. Cells were rinsed with PBS buffer (2×2 mL) before addition of fresh dye-free DMEM (2 mL) and mounted on the microscope.

Fluorescence microscopy

Imaging experiments were performed using a Zeiss Axiovert 200 m inverted epifluorescence microscope equipped with an EM-CCD digital camera (Hamamatsu) and a MS200 XY Piezo Z stage (Applied Scientific Instruments). The light source was an X-Cite 120 metal-halide lamp (EXFO) and the fluorescence images were obtained using an oil-immersion objective at $63 \times$ magnification. The fluorescence filters sets used are defined as blue: excitation G 365 nm, beamsplitter FT 395 nm, emission BP 445/50 nm; red: BP

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550/25 nm, beamsplitter FT 570 nm, emission BP 605/70 nm. The microscope was operated using Volocity software (PerkinElmer).

The exposure times for acquisition of fluorescence images were kept constant for each series of images at each channel. To measure analyte-induced fluorescence changes, the cells were treated with dye-free DMEM containing 20 μ m ZnCl₂ and 50 μ m pyrithione for 10 min. To reverse the effect of zinc, the cells were exposed to dye-free DMEM containing 50 μ m TPEN for 15 min. All these experiments were carried out on the stage of the microscope. Quantification of fluorescence intensity was performed using ImageJ (version 1.45, NIH). The whole cell was selected as the region of interest. The integrated fluorescence from the background region was subtracted from the cell body region.

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Seeing red! A red-emitting and pH-insensitive probe for mobile zinc is reported. The sensing mechanism is based on a reversible ring-opening reaction that is selectively induced by Zn²⁺. The biocompatibility of this probe was validated by fluorescence microscopy in live cells. $\begin{array}{c} & & zn^{2+} \\ & & & \\ &$

P. Rivera-Fuentes, S. J. Lippard*



SpiroZin1: A Reversible and pH-Insensitive, Reaction-Based, Red-Fluorescent Probe for Imaging Biological Mobile Zinc