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Design and Synthesis of a Highly Sensitive Off–On Fluorescent Chemosensor for Zinc Ions Utilizing Internal Charge Transfer

Kenjiro Hanaoka,[a, b] Yasuaki Muramatsu,[a, b] Yasuteru Urano,[a] Takuya Terai,[a, b] and Tetsuo Nagano*[a, b]

Abstract: Fluorescence imaging is a powerful tool for the visualization of biological molecules in living cells, tissue slices, and whole bodies, and is important for elucidating biological phenomena. Furthermore, zinc (Zn^{2+}) is the second most abundant heavy metal ion in the human body after iron, and detection of chelatable Zn^{2+} in biological studies has attracted much attention. Herein, we present a novel, highly sensitive off–on fluorescent chemosensor for Zn^{2+} by using the internal charge transfer (ICT) mechanism. The rationale of our approach to highly sensitive sensor molecules is as follows. If fluorescence can be completely quenched in the absence of Zn^{2+}, chemosensors would offer a better signal-to-noise ratio. However, it is difficult to quench the fluorescence completely before Zn^{2+} binding, and most sensor molecules still show very weak fluorescence in the absence of Zn^{2+}. But even though the sensor shows a weak fluorescence in the absence of Zn^{2+}, this fluorescence can be further suppressed by selecting an excitation wavelength that is barely absorbed by the Zn^{2+}-free sensor molecule. Focusing on careful control of ICT within the 4-amino-1,8-naphthalimide dye platform, we designed and synthesized a new chemosensor (**1**) that shows a pronounced fluorescence enhancement with a blueshift in the absorption spectrum upon addition of Zn^{2+}. The usefulness of **1** for monitoring Zn^{2+} changes was confirmed in living HeLa cells. There have been several reports on 4-amino-1,8-naphthalimide-based fluorescent sensor molecules. However, **1** is the first Zn^{2+}-sensitive off–on fluorescent sensor molecule that employs the ICT mechanism; most off–on sensor molecules for Zn^{2+} employ the photoinduced electron transfer (PeT) mechanism.

Keywords: fluorescence · fluorescence spectroscopy · sensors · transition metals · zinc

Introduction

Zinc (Zn^{2+}) is the second most abundant heavy metal ion in the human body after iron, and it is an essential structural or catalytic cofactor of many proteins (e.g., carbonic anhydrase and zinc finger proteins).[1] In addition, there is a reservoir of Zn^{2+} that is loosely bound or chelatable in the brain,[2] pancreas,[3] and spermatozoa.[4] Although chelatable Zn^{2+} has many important roles in biological systems, its mechanisms of action are little known in comparison with those of other cations, such as Ca^{2+}, Na^{+}, and K^{+}. Therefore, there is considerable interest in detecting chelatable Zn^{2+} in biological systems.[5]

Several Zn^{2+}-selective fluorescent sensor molecules have been developed for measuring Zn^{2+} in living cells. Quinoline-based fluorescent sensor molecules (6-methoxy-8-(*p*-toluenesulfonamide)quinoline (TSQ) and its derivatives) are the most widely used fluorescent Zn^{2+} chemosensors.[6] Recently, several fluorescein-based sensors have also been reported. These chemosensors employ the photoinduced electron transfer (PeT) mechanism and have been successfully used for imaging intracellular Zn^{2+}.[7] However, the Zn^{2+}-free sensors still retain very weak fluorescence; if the fluorescence could be quenched completely in the absence of Zn^{2+}, a better signal-to-noise ratio could be obtained. Our new approach to highly sensitive off–on fluorescence detection of Zn^{2+} is based on controlling internal charge transfer

[a] Dr. K. Hanaoka, Y. Muramatsu, Dr. Y. Urano, T. Terai, Prof. Dr. T. Nagano
Graduate School of Pharmaceutical Sciences
The University of Tokyo
7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
Fax: (+81)3-5841-4855
E-mail: tlong@mol.f.u-tokyo.ac.jp

[b] Dr. K. Hanaoka, Y. Muramatsu, T. Terai, Prof. Dr. T. Nagano
CREST
Japan Science and Technology Agency
3-5 Sanbancho, Chiyoda, Tokyo 102-0075 (Japan)

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(ICT) within the dye platform to promote a change in the absorption wavelength upon Zn^{2+} binding.

On the basis of the above concept, we report herein the design and synthesis of a highly sensitive off–on fluorescent chemosensor for Zn^{2+} (**1**; Figure 1).

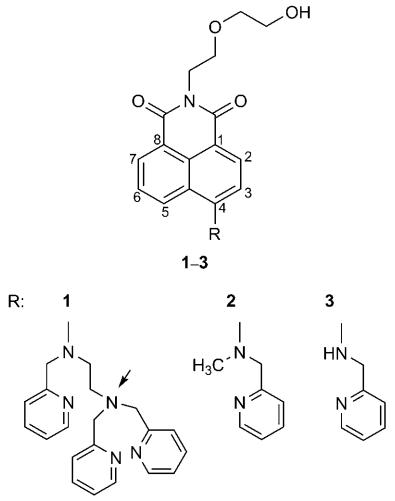

Figure 1. Chemical structures of 4-amino-1,8-naphthalimide derivatives **1**–**3**.

Results and Discussion

Design and synthesis of 1: In the design stage, we chose 4-amino-1,8-naphthalimide as the fluorophore due to its characteristic photophysical properties, such as a long emission wavelength, a large Stokes' shift, and insensitivity to pH (e.g., Lucifer Yellow).[8] Moreover, modulation of the structure of a 4-amino donor on 1,8-naphthalimide is known to affect ICT.[9] For example, making this substituent more electron-deficient results in an ICT-induced blueshift in the absorbance maxima. Therefore, we posited that modifying the 4-amino donor to form the Zn^{2+} binding moiety would provide a switch for blue-shifting the absorbance maximum. Further, the chelator *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) shows high selectivity for Zn^{2+} over other metal ions found under physiological conditions, such as Ca^{2+} and Mg^{2+}.[10] Accordingly, we introduced a TPEN-based ligand for Zn^{2+} at the 4-amino position of 1,8-naphthalimide. Water solubility was improved by incorporating 2-(2-aminoethoxy)ethanol into the fluorophore. We also examined whether the absorbance and fluorescence spectra of **1** in the absence and in the presence of 1.0 equivalents of Zn^{2+} increase linearly between 0 and 10 μM. All absorbance and fluorescence responses showed linear enhancement and it is considered, therefore, that concentration-dependent quenching does not occur in the concentration range up to 10 μM in aqueous solution (see the Supporting Information).

Fluorescence and chemical properties of 1: We assessed the spectroscopic characteristics of **1** under physiological conditions (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 100 mM, pH 7.4; Figure 2). The absorption spectrum of **1** changed upon addition of Zn^{2+} (0–1.0 equiv), with isosbestic points at $\lambda = 408$ and 324 nm, and then remained at a plateau upon further addition of Zn^{2+} (Figure 2a), in accordance with the fluorescence emission spectra (Figure 2b). The Job plot of the fluorescence emission intensity of **1** with Zn^{2+} also showed a maximum at a mole fraction ($[\mathbf{1}]/([\mathbf{1}]+[Zn^{2+}])$) of 0.5, which is indicative of the formation of a 1:1 complex. We also measured the electrospray ionization (ESI) mass spectrum of the complex between **1** and Zn^{2+}. In the mass spectrum of a solution of **1** (50 μM), $ZnSO_4$ (50 μM), dimethyl sulfoxide (DMSO; 5%), and H_2O (5%) in methanol, three major ion peaks at *m*/*z* 679, 715, and 793 were clearly observed. These peaks were assigned as $[\mathbf{1}+Zn^{2+}-H^+]^+$ (*m*/*z* 679), $[\mathbf{1}+Zn^{2+}+Cl^-]^+$ (*m*/*z* 715), and $[\mathbf{1}+Zn^{2+}+Cl^-\cdot(DMSO)]^+$ (*m*/*z* 793). These results support the idea that **1** forms a 1:1 complex with Zn^{2+}. On the other hand, in the absence of Zn^{2+} **1** displayed one major absorption band centered at $\lambda = 437$ nm ($\varepsilon = 7700\ M^{-1}cm^{-1}$) with almost no fluorescence (fluorescence quantum yield $\Phi = 0.039$). The complexation of **1** with Zn^{2+} triggered green fluorescence with characteristic absorption ($\lambda_{abs} = 380$ nm, $\varepsilon = 10100\ M^{-1}cm^{-1}$) and emission ($\lambda_{em} = 507$ nm, $\Phi = 0.942$) spectra. Thus, the fluorescence emission intensity of **1** (5 μM) increased significantly (21.7 times)

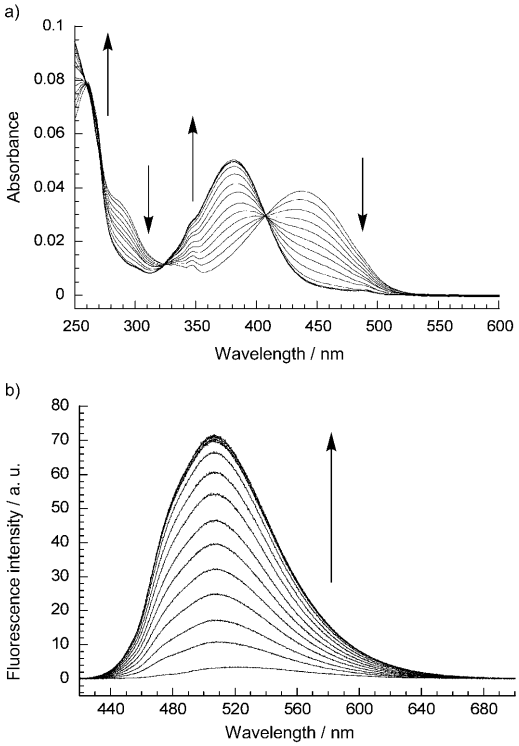

Figure 2. a) Absorbance and b) emission (excitation at $\lambda = 408$ nm) spectra of an aqueous solution of **1** (5 μM; in HEPES buffer, 100 mM, pH 7.4) upon addition of Zn^{2+} (added as $ZnSO_4$; 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, and 2.0 equiv with respect to **1**).

upon addition of 1.0 equiv of Zn^{2+}, with a large blueshift of 57 nm.

We next investigated the mechanism of fluorescence increase of **1**. The photophysical properties of **1**, **2**, and **3** (Figure 1) are summarized in Table 1. The Φ values of **2** and **3** were 0.016 and 0.397, respectively, and the Φ of **1** without Zn^{2+} was almost the same as that of **2**. From this result, it can be considered that variation of the electron-donating ability of the substituent at the 4-position of 1,8-naphthalimide changes the Φ value. The effect of pH was also examined. The fluorescence emission intensity of **1** without Zn^{2+} at $\lambda = 523$ nm was measured at various pH values, with excitation at $\lambda = 408$ nm. There was almost no effect on the emission spectrum of **1** between pH 3.0 and 9.0 in the absence of Zn^{2+} (see the Supporting Information). pH 3 is sufficiently low for the tertiary amine in the Zn^{2+} chelating moiety of **1** (marked with an arrow in Figure 1) to be protonated because TPEN has pK_a values of 7.12, 4.81, 3.30, and 2.88.[12] In fact, a tertiary amine with similar structure to **1** had a pK_a of 5.8.[13] Therefore, the finding of insensitivity to lower pH means that the fluorescence augmentation of **1** upon addition of Zn^{2+} was not due to cessation of PeT from the tertiary amine. From these results, it can be considered that the increase of the emission intensity caused by Zn^{2+} addition is due to the change in the photophysical properties of the fluorophore itself, and arises via the ICT mechanism.

Table 1. Fluorescence emission properties of **1**–**3**.[a]

Compound	λ_{abs} [nm]	λ_{em} [nm]	Φ[d]
1[b]	437	523	0.039
1+Zn^{2+}[c]	380	507	0.942
2	435	550	0.016
3	441	546	0.397

[a] All data were obtained in HEPES buffer (100 mM, pH 7.4). [b] The data were measured in the absence of Zn^{2+}. [c] The data were measured in the presence of Zn^{2+} (1.0 equiv with respect to **1**). [d] The fluorescence quantum yields were determined by using *N*-butyl-4-butylamino-1,8-naphthalimide in absolute ethanol ($\Phi = 0.81$) as the standard.[11]

The effect of adding Na^+, K^+, Ca^{2+}, Mg^{2+}, and various heavy metal ions (Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}) on the fluorescence emission intensity of **1** was also examined. Fluorescence emission enhancement of **1** (2 μM) was not observed upon addition of 100 mM Na^+ or K^+, or 5 mM Ca^{2+} or Mg^{2+} (these ions exist in high concentrations in biological systems) or 2 μM of various heavy metal ions, except for Cd^{2+} (Figure 3). Therefore, compound **1** showed high selectivity for Zn^{2+} over other cations except for Cd^{2+}, which is of little biological relevance. However, the fluorescence intensity was weakened upon addition of Co^{2+} and Cu^{2+} (1 equiv to **1**) together with Zn^{2+}, in comparison with that in the presence of Zn^{2+} alone, as shown in the Supporting Information (Figure S4). Furthermore, the binding affinity for Zn^{2+} was also assessed from the fluorescence intensity change. The affinity of **1** for Zn^{2+} was measured at pH 7.4 in a high salt environment (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)). The apparent dissociation constant, K_d, for Zn^{2+} was calculated to be 1.1 nM (see the Supporting Information), which is sufficiently small for biological applications.[14]

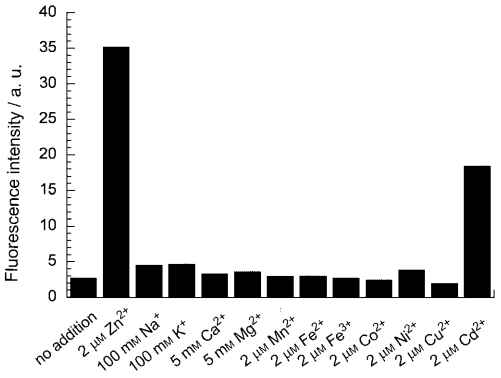

Figure 3. Fluorescence intensity change profiles of **1** (2 μM) in the presence of various cations in HEPES buffer (100 mM, pH 7.4); excitation $\lambda = 408$ nm, emission $\lambda = 507$ nm). Heavy metal ions (1.0 equiv relative to **1**) were added as $MnSO_4$, $FeCl_2$, $FeCl_3$, $CoSO_4$, $NiSO_4$, $CuSO_4$, and $CdSO_4$. Other cations were added as $ZnSO_4$ (2 μM), $NaNO_3$ (100 mM), KNO_3 (100 mM), $CaCl_2$ (5 mM), and $MgSO_4$ (5 mM).

Biological applications of 1: We also investigated the application of **1** in cultured cells (Figure 4). Cultured HeLa cells were incubated with **1** (10 μM) for 30 min at room temperature, which was sufficient for intracellular accumulation of **1** to occur as judged from the fluorescence observed in the intracellular regions (Figure 4b). The overlay of bright-field transmission and fluorescence images revealed that fluorescence signals were localized in the perinuclear region (see the Supporting Information). However, it is thought that this fluorescence is not due to Zn^{2+}, which is present in the intracellular regions, but rather is due to the solvatochromic

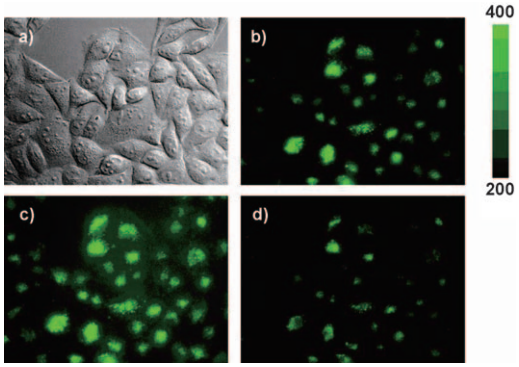

Figure 4. Bright-field transmission and fluorescence images of Zn^{2+} in HeLa cells in HBSS buffer. The fluorescence at $\lambda = 435$–565 nm, excited at $\lambda = 385$–415 nm, was measured. a) Bright-field transmission image of cells stained with **1** (10 μM) for 30 min at RT. b) Fluorescence image of cells stained with **1** (10 μM) for 30 min at RT. c) Fluorescence image of cells stained with **1** and loaded with $ZnSO_4$ (50 μM) and pyrithione (5 μM; zinc ionophore) for 2.5 min. d) Fluorescence image of the cells following addition of TPEN (100 μM) to the medium for 4.5 min.

character of **1**. When we examined the solvatochromic properties of **1**, we observed almost no fluorescence in polar protic solvents, such as water and methanol, but a small enhancement of the fluorescence intensity was seen in organic solvents, such as CH_3CN, CH_2Cl_2, and DMSO (see the Supporting Information). Moreover, 4-*N*,*N*-dimethylamino-1,8-naphthalimide (4-DMN), which has a similar structure to **1**, has been reported to be a solvatochromic fluorophore.[15] We believe these results are consistent with the idea that the majority, though not all, of compound **1** inside cells exists in relatively nonpolar aprotic environments. We then added Zn^{2+} (50 μM) and a zinc-selective ionophore, pyrithione (2-mercaptopyridine *N*-oxide, 5 μM), to the medium, inducing a prompt increase of the intracellular fluorescence (Figure 4c). This fluorescence was decreased by the extracellular addition of the cell-membrane-permeable chelator TPEN (100 μM; Figure 4d). This result demonstrates that **1** can be used to reversibly monitor changes in intracellular ionic Zn^{2+} and has potential for biological applications.

Conclusions

In conclusion, we have designed and synthesized a novel, highly sensitive fluorescent molecule (**1**) for sensing Zn^{2+} by using 4-amino-1,8-naphthalimide as a fluorophore and a TPEN derivative as a Zn^{2+} binding moiety. Compound **1** showed pronounced fluorescence enhancement, with a blue-shift in the absorption spectrum upon addition of Zn^{2+}. There have been many reports on 4-amino-1,8-naphthalimide-based ratiometric or off–on fluorescent sensor molecules for Zn^{2+}, sugars, Hg^{2+}, Pd^{2+}, pH, anions, Ca^{2+}, H_2O_2, Na^+, K^+, and Cu^{2+} by using the PeT or ICT mechanism.[13,16] However, compound **1** is the first Zn^{2+}-sensitive off–on fluorescent sensor molecule based on the ICT mechanism on a 4-amino-1,8-naphthalimide platform, though a similar ICT working principle was reported with 7-nitrobenz-2-oxa-1,3-diazole (NBD).[17] Our design strategy should be applicable to a range of off–on fluorescent sensor molecules for Zn^{2+} and other molecules of interest in biological applications.

Experimental Section

All reagents and solvents were of the highest commercial quality and were used without purification, except for ethanol, which was used after distillation. DMSO, HEPES, nitrilotriacetic acid (NTA), TPEN, 2-morpholinoethanesulfonic acid (MES), and *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) were purchased from Dojindo Laboratories (Japan). 4-(*n*-Butylamino)naphthalic-1,8-*N*-butylimide was prepared according to a literature procedure.[18] All other reagents and solvents were purchased from Tokyo Chemical Industry Co. (Japan), Wako Pure Chemical Industries (Japan), or Aldrich Chemical Co. (USA). Silica gel column chromatography was performed by using Chromatorex-NH (Fuji Silysia Chemical, Kasugai, Japan), Silica Gel 60N (spherical, neutral), or Silica Gel 60 (spherical) (Kanto Chemical Co., Tokyo, Japan).

Instruments: ^1H and ^{13}C NMR spectra were recorded by using a JNM-LA300 or a JNM-LA400 (JEOL) spectrometer. Mass spectra were measured by using a JMS-T100 LC AccuTOF spectrometer (ESI^+; JEOL). HPLC purification was performed by using a Jasco PU-1587 system fitted with a reversed-phase column (GL Sciences (Tokyo, Japan), Inertsil Prep-ODS 30×250 mm). UV/Vis spectra were obtained by using a V-550 UV/VIS spectrophotometer (Jasco). The fluorescence emission or excitation spectra were recorded by using a FP-6500 spectrofluorometer (Jasco). The slit width was 3 nm for both excitation and emission.

UV/Vis absorption spectrum measurements: The absorption spectra of **1** (5 μM) were measured at RT in an aqueous solution buffered to pH 7.4 (100 mM HEPES buffer containing 0.25% DMSO as a cosolvent). Zn^{2+} was added as $ZnSO_4$ (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, and 2.0 equiv of Zn^{2+} with respect to **1**).

Fluorescence emission and excitation spectral measurements: The fluorescence emission spectra of **1** (5 μM) were measured in HEPES buffer (100 mM, pH 7.4) at RT, following excitation at $\lambda = 408$ nm. The fluorescence excitation spectra of **1** (5 μM) were also measured in HEPES buffer (100 mM, pH 7.4) at RT (fixed emission at $\lambda = 507$ nm). The amounts of Zn^{2+} added were 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, and 2.0 equiv with respect to **1** in both emission and excitation spectral measurements.

Quantum yield measurements: The fluorescence spectra were measured by using a FP-6500 spectrofluometer (Jasco). The slit width was 3.0 nm for both excitation and emission. The photomultiplier voltage was 400 V. The fluorescence spectra of **1**, **2**, and **3** were measured in HEPES buffer (100 mM, pH 7.4) at RT, with irradiation at $\lambda = 408$ nm. The quantum yields of compounds were evaluated by using a relative method with reference to a fluorescence standard, *N*-butyl-4-butylamino-1,8-naphthalimide ($\Phi = 0.81$ in absolute ethanol).[11] The quantum yields of compounds can be expressed by Equation (1),[19] in which Φ is the quantum yield ("st" denotes the reference and "x" denotes the sample), A is the absorbance at the excitation wavelength, n is the refractive index, and D is the area (on an energy scale) of the fluorescence spectra. The sample and the reference were excited at the same wavelength ($\lambda = 408$ nm). The sample absorbance at the excitation wavelength was kept as low as possible to avoid fluorescence errors ($A_{exc} < 0.02$).

$$\Phi_x/\Phi_{st} = [A_{st}/A_x][n_x^2/n_{st}^2][D_x/D_{st}] \quad (1)$$

Apparent dissociation constant (K_d) measurements: Upon addition of various concentrations of Zn^{2+}, the fluorescence intensity and the absorbance of **1** linearly changed up to a 1:1 $[Zn^{2+}]/[\mathbf{1}]$ molar ratio, and the fluorescence and absorption spectra remained at a plateau with further addition of Zn^{2+}. Furthermore, the Job plot analysis revealed that maximum fluorescence intensity was obtained at a 1:1 ratio. The ESI mass spectrum also showed the ion peaks of a 1:1 complex between Zn^{2+} and **1**. These data suggested that **1** forms a 1:1 complex with Zn^{2+}. So, the apparent dissociation constant, K_d, was determined from the fluorescence intensity in HEPES buffer (100 mM, pH 7.4, $I = 0.1$ ($NaNO_3$)) at RT ($\lambda_{exc} = 408$ nm). $[Zn^{2+}]$ was controlled by using $ZnSO_4$/NTA (0–10 mM:10 mM) systems.[20] The fluorescence intensity data were fitted to Equation (2), in which F is the fluorescence intensity, F_{max} is the maximum fluorescence intensity, F_0 is the fluorescence intensity with no addition of Zn^{2+}, and $[Zn^{2+}]_f$ is the free Zn^{2+} concentration. The value of K_d was determined from the fittings for the fluorescence intensity.

$$F = F_0 + (F_{max} - F_0)([Zn^{2+}]_f)/(K_d + [Zn^{2+}]_f) \quad (2)$$

Effect of pH on the fluorescence intensity: The following buffers were used: $ClCH_2COOH$–$ClCH_2COONa$ buffer (100 mM, pH 3.0 and 3.6), AcOH–AcONa buffer (100 mM, pH 4.2, 4.8 and 5.4), MES buffer (100 mM, pH 5.7, 6.1 and 6.5), HEPES buffer (100 mM, pH 7.0, 7.4 and 8.0), and CHES buffer (100 mM, pH 8.5 and 9.0). The fluorescence intensity (excitation $\lambda = 408$ nm, emission $\lambda = 523$ or 507 nm in the absence or presence of Zn^{2+} (2 μM), respectively) of each sample of **1** (2 μM) was plotted.

Metal ion selectivity measurements: The fluorescence emission enhancement of **1** was measured in HEPES buffer (100 mM, pH 7.4) at RT (excitation $\lambda = 408$ nm, emission $\lambda = 507$ nm). Heavy metal ions (2 μM) were

added as $MnSO_4$, $FeCl_2$, $FeCl_3$, $CoSO_4$, $NiSO_4$, $CuSO_4$, and $CdSO_4$. Other cations were added as $ZnSO_4$ (2 μM), $NaNO_3$ (100 mM), KNO_3 (100 mM), $CaCl_2$ (5 mM), and $MgSO_4$ (5 mM).

Preparation of cells: HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin, and 1% streptomycin (Invitrogen) at 37°C in a CO_2/air (5:95) incubator. The cells were grown on an uncoated 35 mm diameter glass-bottomed dish (MatTek, Ashland, USA), and washed twice with Hanks' balanced salt solution (HBSS) buffer (Invitrogen), then the medium was replaced with HBSS buffer before imaging.

Microscopy and imaging methods: The imaging system was comprised of an inverted microscope (IX71; Olympus) and a cooled CCD camera (Cool Snap HQ; Roper Scientific, Tucson, USA). The microscope was equipped with a xenon lamp (AH2-RX; Olympus), a ×40 objective lens (Uapo/340, N.A. 1.35; Olympus), a dichroic mirror (t2003 DM; Olympus), an excitation filter (XF1076 400AF30; OMEGA), and an emission filter (XF3088 435ALP; OMEGA). The whole system was controlled using MetaFluor 7.1 software (Molecular Devices, Meta Imaging Software, PA, USA). HeLa cells were grown on an uncoated 35 nm diameter glass-bottomed dish (MatTek, Ashland, USA) and washed twice with HBSS buffer; then the cells were incubated with **1** (10 μM) in HBSS buffer for dye loading for 30 min at RT, which was long enough for intracellular accumulation of **1** to occur, as judged from the fluorescence seen in the intracellular regions (Figure 4). The stained cells were washed twice with HBSS buffer, and the medium was replaced with HBSS buffer before imaging (0 min). We then added zinc sulfate (50 μM) and pyrithione (5 μM) to the medium on the microscope stage, inducing a prompt increase of intracellular fluorescence at 4.5 min. This fluorescence decreased upon extracellular addition of the cell-membrane-permeable chelator TPEN (100 μM) to cell samples on the microscope stage at 9.5 min. The fluorescence images were measured every 30 s.

Dye loading conditions: The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37°C in a CO_2/air (5:95) incubator. The cells were washed with HBSS buffer, and then incubated with **1** (10 μM) in HBSS buffer for dye loading for 30 min at RT. The stained cells were washed twice with HBSS buffer, and the medium was replaced with HBSS buffer before imaging.

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[1] a) B. L. Vallee, K. H. Falchuk, *Physiol. Rev.* **1993**, *73*, 79–118; b) J. M. Berg, Y. Shi, *Science* **1996**, *271*, 1081–1085.
[2] C. J. Frederickson, *Int. Rev. Neurobiol.* **1989**, *31*, 145–238.
[3] P. D. Zalewski, S. H. Millard, I. J. Forbes, O. Kapaniris, A. Slavotinek, W. H. Betts, A. D. Ward, S. F. Lincoln and I. Mahadevan, *J. Histochem. Cytochem.* **1994**, *42*, 877–884.
[4] P. D. Zalewski, X. Jian, L. L. L. Soon, W. G. Breed, R. F. Seamark, S. F. Lincoln, A. D. Ward, F. Z. Sun, *Reprod. Fertil. Dev.* **1996**, *8*, 1097–1105.
[5] a) K. Kikuchi, K. Komatsu, T. Nagano, *Curr. Opin. Chem. Biol.* **2004**, *8*, 182–191; b) E. L. Que, D. W. Domaille, C. J. Chang, *Chem. Rev.* **2008**, *108*, 1517–1549.
[6] a) C. J. Frederickson, E. J. Kasarskis, D. Ringo, R. E. Frederickson, *J. Neurosci. Methods* **1987**, *20*, 91–103; b) D. D. Savage, C. Y. Montano, E. J. Kasarskis, *Brain Res.* **1989**, *496*, 257–267; c) P. D. Zalewski, I. J. Forbes, W. H. Betts, *Biochem. J.* **1993**, *296*, 403–408.
[7] a) T. Hirano, K. Kikuchi, Y. Urano, T. Higuchi, T. Nagano, *J. Am. Chem. Soc.* **2000**, *122*, 12399–12400; b) T. Hirano, K. Kikuchi, Y. Urano, T. Nagano, *J. Am. Chem. Soc.* **2002**, *124*, 6555–6562; c) G. K. Walkup, S. C. Burdette, S. J. Lippard, R. Y. Tsien, *J. Am. Chem. Soc.* **2000**, *122*, 5644–5645; d) S. C. Burdette, C. J. Frederickson, W. Bu, S. J. Lippard, *J. Am. Chem. Soc.* **2003**, *125*, 1778–1787.
[8] W. W. Stewart, *J. Am. Chem. Soc.* **1981**, *103*, 7615–7620.
[9] a) A. P. de Silva, H. Q. N. Gunaratne, J. L. Habib-Jiwan, C. P. McCoy, T. E. Rice, J. P. Soumillion, *Angew. Chem.* **1995**, *107*, 1889–1891; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1728–1731; b) N. A. O'Connor, S. T. Sakata, H. Zhu, K. J. Shea, *Org. Lett.* **2006**, *8*, 1581–1584.
[10] L. G. Sillén, A. E. Martell, *Stability Constants of Metal-Ion Complexes*, RCS, London, **1964**.
[11] M. S. Alexiou, V. Tychopoulos, S. Ghorbanian, J. H. P. Tyman, R. G. Brown, P. I. Brittain, *J. Chem. Soc. Perkin Trans. 2* **1990**, 837–842.
[12] "NIST Critically Selected Stability Constants of Metal Complexes": NIST Standard Reference Database 46, Version 6.0, A. E. Martell, R. M. Smith, **2001**.
[13] J. Fan, Y. Wu, X. Peng, *Chem. Lett.* **2004**, *33*, 1392–1393.
[14] K. Komatsu, K. Kikuchi, H. Kojima, Y. Urano, T. Nagano, *J. Am. Chem. Soc.* **2005**, *127*, 10197–10204.
[15] G. Loving, B. Imperiali, *J. Am. Chem. Soc.* **2008**, *130*, 13630–13638.
[16] a) J. Wang, Y. Xiao, Z. Zhang, X. Qian, Y. Yang, Q. Xu, *J. Mater. Chem.* **2005**, *15*, 2836–2839; b) Z. Xu, X. Qian, J. Cui, R. Zhang, *Tetrahedron* **2006**, *62*, 10117–10122; c) R. Parkesh, T. C. Lee, T. Gunnlaugsson, *Org. Biomol. Chem.* **2007**, *5*, 310–317; d) S. Jin, J. Wang, M. Li, B. Wang, *Chem. Eur. J.* **2008**, *14*, 2795–2804; e) S. Trupp, A. Schweitzer, G. J. Mohr, *Org. Biomol. Chem.* **2006**, *4*, 2965–2968; f) B. Liu, H. Tian, *Chem. Commun.* **2005**, 3156–3158; g) X. Guo, X. Qian, L. Jia, *J. Am. Chem. Soc.* **2004**, *126*, 2272–2273; h) L. Duan, Y. Xu, X. Qian, *Chem. Commun.* **2008**, 6339–6341; i) A. P. de Silva, T. E. Rice, *Chem. Commun.* **1999**, 163–164; j) T. Gunnlaugsson, P. E. Kruger, T. C. Lee, R. Parkesh, F. M. Pfeffer, G. M. Hussey, *Tetrahedron Lett.* **2003**, *44*, 6575–6578; k) H. He, K. Jenkins, C. Lin, *Anal. Chim. Acta* **2008**, *611*, 197–204; l) D. Srikun, E. W. Miller, D. W. Domaille, C. J. Chang, *J. Am. Chem. Soc.* **2008**, *130*, 4596–4597; m) H. He, M. A. Mortellaro, M. J. P. Leiner, S. T. Young, R. J. Fraatz, J. K. Tusa, *Anal. Chem.* **2003**, *75*, 549–555; n) H. He, M. A. Mortellaro, M. J. P. Leiner, R. J. Fraatz, J. K. Tusa, *J. Am. Chem. Soc.* **2003**, *125*, 1468–1469; o) Z. Xu, X. Qian, J. Cui, *Org. Lett.* **2005**, *7*, 3029–3032; p) C. Lu, Z. Xu, J. Cui, R. Zhang, X. Qian, *J. Org. Chem.* **2007**, *72*, 3554–3557; q) M. Taki, Y. Watanabe, Y. Yamamoto, *Tetrahedron Lett.* **2009**, *50*, 1345–1347; r) Y. Mei, P. A. Bentley, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3131–3134.
[17] a) W. Jiang, Q. Fu, H. Fan, W. Wang, *Chem. Commun.* **2008**, 259–261; b) F. Qian, C. Zhang, Y. Zhang, W. He, X. Gao, P. Hu, Z. Guo, *J. Am. Chem. Soc.* **2009**, *131*, 1460–1468.
[18] S. Ghorbanian, J. H. P. Tyman and V. Tychopoulos, *J. Chem. Technol. Biotechnol.* **2000**, *75*, 1127–1134.
[19] Q. Y. Chen, C. J. Feng, Q. H. Luo, C. Y. Duan, X. S. Yu, D. J. Liu, *Eur. J. Inorg. Chem.* **2001**, 1063-.1069.
[20] a) K. Hanaoka, K. Kikuchi, H. Kojima, Y. Urano, T. Nagano, *Angew. Chem.* **2003**, *115*, 3104–3107; *Angew. Chem. Int. Ed.* **2003**, *42*, 2996–2999; b) T. Hirano, K. Kikuchi, Y. Urano, T. Higuchi and T. Nagano, *J. Am. Chem. Soc.* **2000**, *122*, 12399–12400.

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