A Fluorescent Heteroditopic Ligand Responding to Free Zinc Ion over Six Orders of Magnitude Concentration Range

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Supporting Information

ACRONYMS OF CHEMICALS

Boc_2O	di-*tert*-butyl dicarbonate
Bu_4NI	tetrabutylammonium iodide
CaH_2	calcium hydride
$CDCl_3$	chloroform-*d*
DCM	dichloromethane
DIPEA	diisopropylethylamine
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol tetraacetic acid
Et_3N	triethylamine
EtOAc	ethyl acetate
EtOH	ethanol
H_2SO_4	sulfuric acid
HCl	hydrochloric acid
HEDTA	N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid
HEPES	4-(2-hydroxyethyl)piperazineethanesulfonic acid
K_2CO_3	potassium carbonate
KNO_3	potassium nitrate
MeCN	acetonitrile
MeOH	methanol
Na_2CO_3	sodium carbonate
Na_2SO_4	sodium sulfate
$NaBH(OAc)_3$	sodium triacetoxyborohydride
NaH	sodium hydride

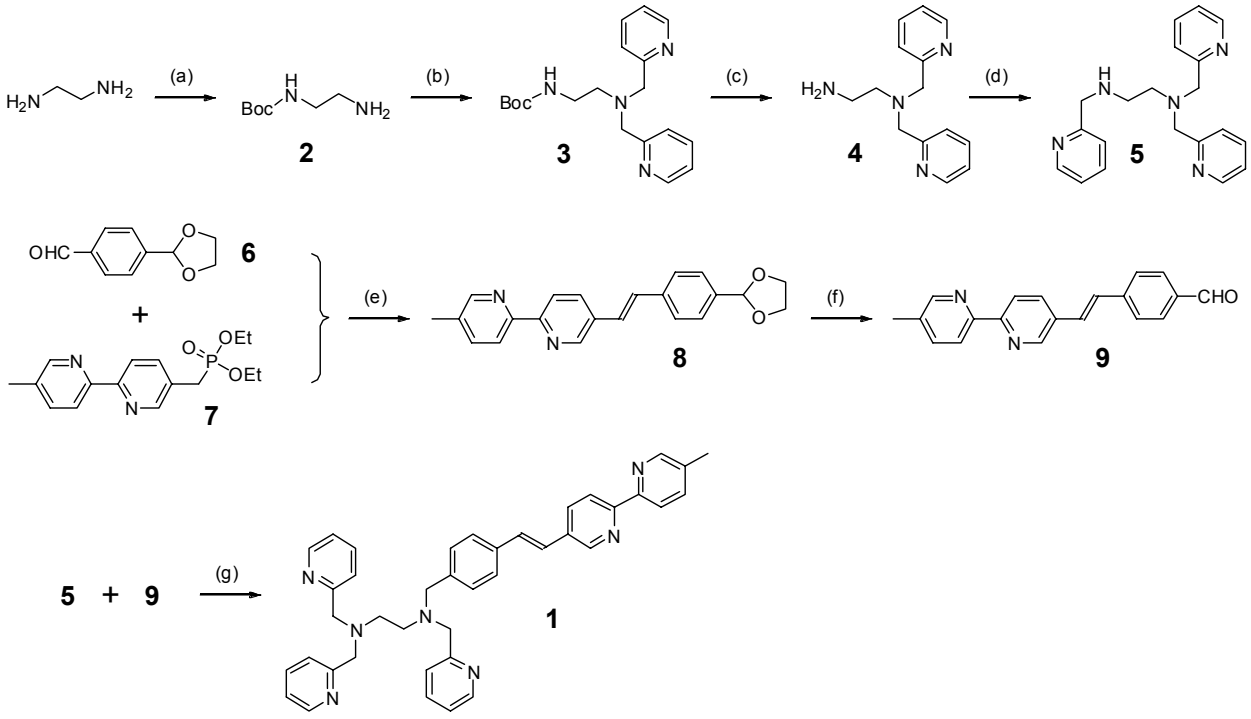
NaOH	sodium hydroxide
NTA	nitrilotriacetic acid
TBAP	tetrabutylammonium perchlorate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
$ZnCl_2$	zinc chloride
$Zn(ClO_4)_2$	zinc perchlorate

MATERIALS AND GENERAL METHODS

Reagents and solvents were purchased from various commercial sources and used without further purification unless otherwise stated. Water used in titration experiments were deionized using Barnstead NANOpure Diamond water system. MeCN (OmniSolv, EMD) and DMSO (ACS Reagent, > 99.9%, Sigma-Aldrich) were directly used in titration experiments without purification. All reactions were carried out in oven- or flame-dried glassware in an inert atmosphere of argon. Analytical thin-layer chromatography (TLC) was performed using pre-coated TLC plates with silica gel 60 F254 or with aluminum oxide 60 F254 neutral. Flash column chromatography was performed using 40-63 μm (230-400 mesh) silica gel or alumina (80-200 mesh, pH 9-10) as the stationary phases. THF and DCM were dried by distilling from sodium-benzophenone and CaH_2, respectively, in continuous stills under argon protection. ^1H and ^{13}C NMR spectra were recorded at 300 MHz and 75 MHz, respectively. All chemical shifts were reported in δ units relative to tetramethylsilane. $CDCl_3$ was treated with alumina gel prior to use. High resolution mass spectra were obtained at the Mass Spectrometry Laboratory at FSU.

Spectrophotometric and fluorimetric titration experiments were conducted on a Varian Cary 100 Bio UV-Visible Spectrophotometer and a Varian Cary Eclipse Fluorescence Spectrophotometer, respectively.

SYNTHESIS



Scheme S1. (a) Boc_2O, Et_3N, EtOH, 80%; (b) 2-picolyl chloride, Na_2CO_3, Bu_4NI (cat.), EtOH, reflux, 60%; (c) (1) TFA, DCM, (2) 2 M NaOH, 90%; (d) 2-pyridinecarboxaldehyde, $NaBH(OAc)_3$, rt, 64%; (e) NaH, 1,2-dimethoxyethane, rt, 72%; (f) HCl/H_2O/THF, rt, 100%; (g) $NaBH(OAc)_3$, rt, 44%.

Compound 2. Boc_2O (5.0 g, 23 mmol) was added to a mixture of ethylenediamine (5.0 mL, 75 mmol) and Et_3N (1.0 mL) in EtOH (12 mL) at 0 °C. The mixture was stirred at rt for 2 h. After solvent removal, the residue was dissolved in DCM (20 mL) and extracted with 1 M acetic acid (3 × 20 mL). The aqueous layer was then alkalized by addition of a NaOH solution (2.0 M) and extracted using DCM. The organic layer was dried over Na_2SO_4 and evaporated to afford **2** (2.3 g, 82%). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 5.16 (s, 1H), 3.11-3.06 (m, 2H), 2.71 (t, J = 6.0 Hz, 2H), 1.37 (s, 9 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ/ppm 156.4, 79.1, 43.5, 41.9, 28.5.

Compound 3. A mixture of **2** (228 mg, 1.42 mmol) and 2-picolyl chloride (488 mg, 2.98 mmol) was dissolved in dry EtOH (20 mL). Na_2CO_3 (631 mg, 5.95 mmol) and Bu_4NI (catalytic amount) were added sequentially. The mixture was heated to reflux for 6 h under argon protection. After cooling down, the solvent was removed under vacuum and the residue was partitioned between an aqueous solution of NaOH (0.1 M) and DCM. The organic layer was dried over Na_2SO_4 and evaporated. The residue was chromatographed on alumina gel to afford **3** (293 mg, 60%). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 8.54 (d, J = 4.2 Hz, 2H), 7.63 (t, J = 9.0 Hz, 2H), 7.41 (d, J = 7.8 Hz, 2H), 7.17-7.13 (m, 2H), 5.81 (s, br, 1H), 3.86 (s, 4H), 3.23-3.21 (m, 2H), 2.70 (t, J = 5.4 Hz, 2H), 1.44 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ/ppm 159.5, 156.4, 149.3, 136.6, 123.3, 122.2, 70.8, 60.4, 53.7, 38.7, 26.7.

Compound 4 (*J. Am. Chem. Soc.* **2005**, *127*, 818). A solution of **3** (293 mg, 0.85 mmol) in DCM (3 mL) was added dropwise TFA (7.5 mL) at 0 °C. The mixture was stirred for 1 h before the removal of the solvent. The residue was partitioned between DCM and aqueous NaOH (0.1 M). The organic layer was dried over Na_2SO_4 before solvent was evaporated to afford **4** (186 mg, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ/ppm 8.48 (d, J = 4.2 Hz, 2H), 7.59 (t, J = 9.0 Hz, 2H), 7.42 (d, J = 7.8 Hz, 2H), 7.12-7.07 (m, 2H), 3.80 (s, 4H), 2.76 (t, J = 6.0 Hz, 2H), 2.63 (t, J = 6.0 Hz, 2H), 2.18 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ/ppm 159.6, 149.1, 136.5, 123.1, 122.1, 60.7, 57.2, 39.6.

Compound 5 (*J. Am. Chem. Soc.* **2005**, *127*, 818). Compound **4** (509 mg, 2.10 mmol) was added to an anhydrous 1,2-dichloroethane (20 mL) solution of 2-pyridinecarboxaldehyde (224 mg, 2.09 mmol) under argon protection. The mixture was stirred for 2 days at rt before $NaBH(OAc)_3$ (1.11 g, 5.24 mmol) was added. The mixture was stirred for another 4 h before the solvent was removed under vacuum. The residue was washed with basic brine (pH = 11) and extracted with DCM (3 × 50 mL). The organic portions were dried over Na_2SO_4 before concentrated under vacuum. The residue was chromatographed on alumina gel by using MeOH/DCM from 0/100 to 3/100 to afford pure compound **5** (447 mg, 64 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 8.42 (s, 3H), 7.57-7.45 (m, 5H), 7.18 (d, J = 8.4 Hz, 1H), 7.06-7.02 (m, 3H), 3.75 (s, 6H), 2.70 (s,

4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ/ppm 160.0, 159.7, 149.2, 149.0, 136.4, 136.3, 123.0, 122.1, 122.0, 121.8, 60.7, 55.0, 54.1, 46.8.

Compound 8. NaH (26 mg, 60% in mineral oil, 0.66 mmol) was added to a solution of **6** (35 mg, 0.2 mmol, *Chem. Eur. J.* **2008**, *14*, 2894) in anhydrous dimethoxyethane (0.5 mL). The suspension was stirred for 8 min. The flask was cooled in an ice bath (0 °C) and was added dropwise the solution of **7** (63 mg, 0.2 mmol, *J. Org. Chem.* **2008**, *73*, 8321) in anhydrous dimethoxyethane (0.5 mL). The reaction was stirred overnight before icy brine was added to quench the reaction. The reaction mixture was partitioned between DCM and basic brine (pH = 11). The organic layer was dried over Na_2SO_4, followed by solvent removal under vacuum. The residue was chromatographed on silica gel using 10% EtOAc in DCM to afford compound **8** (49 mg, 72%). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 8.76 (d, J = 1.8 Hz, 1H), 8.52 (s, 1H), 8.37 (d, J = 8.4 Hz, 1H), 8.30 (d, J = 8.4 Hz, 1H), 7.98 (dd, J = 2.4, 8.4 Hz, 1H), 7.65-7.49 (m, 5H), 7.41 (d, J = 8.4 Hz, 1H), 7.15 (d, J = 8.4 Hz, 1H), 5.84 (s, 1H), 4.19-4.04 (m, 4H), 2.41(s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ/ppm 155.8, 153.5, 149.9, 148.3, 137.8, 137.6, 133.6, 132.7, 130.4, 127.1, 126.9, 126.6, 125.6, 120.8, 120.8, 103.7, 65.5, 29.9, 18.6; HRMS (ESI+): calcd. ($M+Na^+$) 367.1423, found 367.1418.

Compound 9. Compound **8** (49 mg, 0.14 mmol) was dissolved in a mixed solvent (7 mL) of 37% HCl/H_2O/THF = 1/6/7. The solution was stirred overnight before partitioned using basic brine (pH = 11) and DCM (3 × 25 mL). The organic portions were dried over K_2CO_3 before concentrated under vacuum. The residue was chromatographed (alumina, DCM/EtOAc from 10/1 to 2/1) to afford **9** (31 mg, 73%). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 10.02 (s, 1H), 8.79 (d, J = 1.8 Hz, 1H), 8.53 (s, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.32 (d, J = 8.4 Hz, 1H), 8.03 (dd, J = 1.6, 8.4 Hz, 1H), 7.91 (d, J = 8.4 Hz, 2H), 7.72-7.67 (m, 3H), 7.29-7.26 (m, 2H), 2.42 (s,3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ/ppm 191.7, 156.0, 153.3, 149.9, 148.6, 142.9, 137.7, 135.9, 133.9, 132.1, 130.5, 129.4, 128.5, 127.3, 120.9, 121.0, 18.6; HRMS (ESI+): calcd. ($M+H^+$) 301.1341, found 301.1333.

Compound 1. Compound **5** (53 mg, 0.16 mmol) was added to an anhydrous 1,2-dichloroethane (2 mL) solution of compound **9** (50 mg, 0.17 mmol) under argon

protection. The mixture was stirred overnight at rt before $NaBH(OAc)_3$ (50 mg, 0.23 mmol) was added. The mixture was stirred for another 4 h before the solvent was removed under vacuum. The residue was washed with basic brine (pH = 11) and extracted with DCM (3 × 50 mL). The organic portions were dried over Na_2SO_4 before concentrated under vacuum. The residue was chromatographed on alumina gel using MeOH/DCM from 0/100 to 2/100. The isolated product (43 mg, 44%) was precipitated from a DCM solution by addition of hexanes to afford pure *trans*-**1** (26 mg, 27%). ^{1}H NMR (300 MHz, $CDCl_3$): δ/ppm 8.74 (d, J = 1.8 Hz, 1H), 8.50 (d, J = 1.8 Hz, 1H), 8.36 (d, J = 4.8 Hz, 1H), 8.30 (d, J = 4.8 Hz, 1H), 7.97 (dd, J = 1.8, 8.4 Hz, 1H), 7.62-7.55 (m, 4H), 7.48-7.43 (m, 5H), 7.31 (d, J = 7.8 Hz, 2H), 7.23-7.06 (m, 5H), 3.78 (s, 4H), 3.73 (s, 2H), 3.60 (s, 2H), 2.75-2.72 (m, 4H), 2.40 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ/ppm 160.3, 160.0, 155.3, 153.6, 149.9, 149.2, 149.0, 148.2, 139.8, 137.6, 136.5, 135.7, 133.5, 133.0, 130.8,129.4, 126.8, 124.6, 123.0, 122.0, 120.9, 120.7, 61.0, 60.9, 59.0, 52.5, 52.3, 18.6; HRMS (ESI+): calcd. ($M+H^+$) 640.3165, found 640.3148.

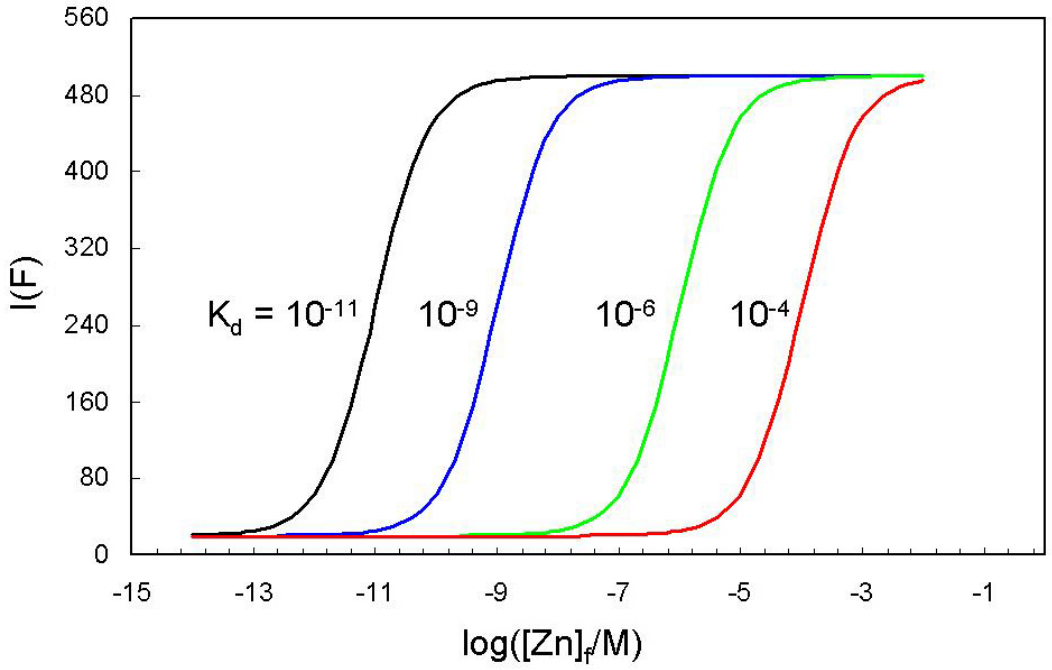


Figure S1. Simulated fluorescence intensity dependence on free zinc concentration ($[Zn]_f$) of four monotopic zinc probes with different affinities.

$$[Zn]_f = K_d \frac{I - I_{min}}{I_{max} - I}$$ Equation S1 (*J. Cell Biol.* **1982**, *94*, 325)

I – fluorescence intensity. I_{min} (minimum intensity) and I_{max} (maximum intensity) were set at 20 and 500, respectively, to generate curves in Figure S1.

PROCEDURES FOR TITRATION EXPERIMENTS

(1) Zinc titration in MeCN (Figures S2-4)

An MeCN solution of **1** (2.0 μM), $Zn(ClO_4)_2$ (16 μM), DIPEA (2 μM), and TBAP (5 mM) was titrated into a semi-micro quartz spectrophotometer (or fluorimeter) cuvette (Starna®) containing an MeCN solution of **1** (840 μL, 2.0 μM), DIPEA (2 μM), and TBAP (5 mM) at rt. The spectra (absorption, fluorescence, and excitation) were collected and overlaid.

(2) Zinc titration in metal ion buffers (Figures 2, S5)

An aqueous solution of **1** (2.0 μM) containing 10% DMSO, $Zn(ClO_4)_2$ (40 mM), EDTA (5 mM), EGTA (5 mM), HEDTA (5 mM), NTA (5 mM), HEPES (50 mM) at pH 7, and KNO_3 (100 mM) was titrated into a semi-micro quartz spectrophotometer cuvette (Starna®) containing an aqueous solution of **1** (840 μL, 2.0 μM) with 10% DMSO, EDTA (5 mM), EGTA (5 mM), HEDTA (5 mM), NTA (5 mM), HEPES (50 mM) at pH 7, and KNO_3 (100 mM) (Figure 2). $[Zn]_f$ was calculated using "Webmaxc Standard" (http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm).

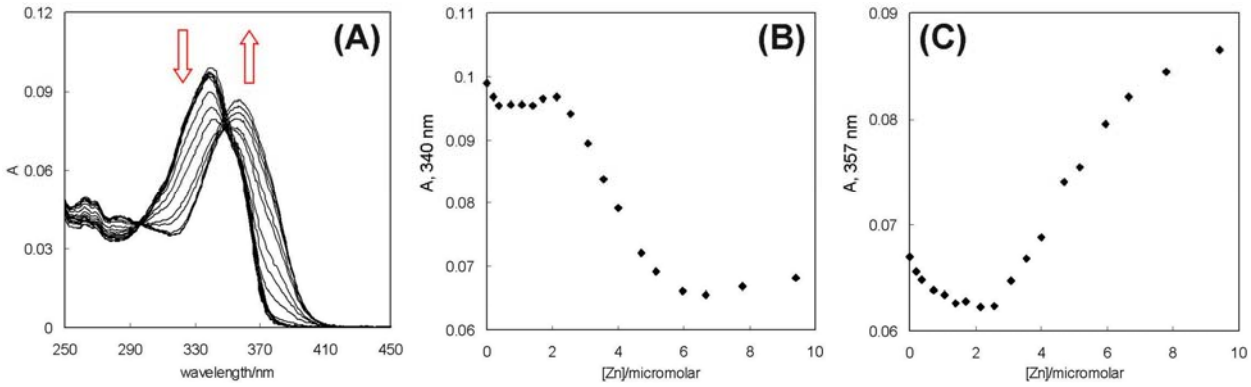


Figure S2. (A) Spectrophotometric titration of **1** (2.0 μM) with $Zn(ClO_4)_2$ (0 – 9.4 μM) in MeCN (TBAP: 5.0 mM; DIPEA: 2.0 μM). Arrows represent the direction of spectral changes upon addition of zinc. (B) Absorbance values at 340 nm and at (C) 357 nm vs. the concentration of $Zn(ClO_4)_2$.

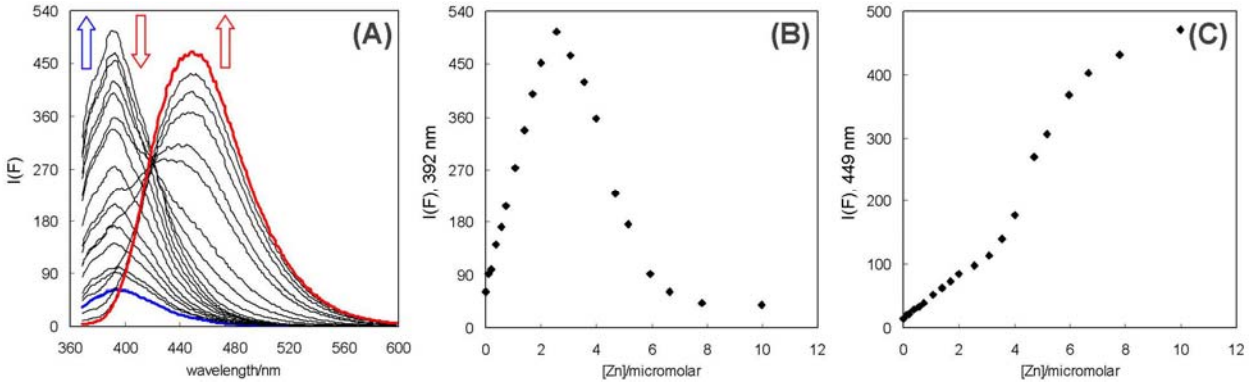


Figure S3. (A) Fluorimetric titration of **1** (2.0 μM, λ_{ex} = 357 nm) with $Zn(ClO_4)_2$ (0 – 10.0 μM) in MeCN (TBAP: 5.0 mM; DIPEA: 2.0 μM). The first and last spectra are coded blue and red, respectively. The blue and red arrows represent spectral changes when [zinc] is low and high, respectively. (B) Fluorescence intensity values at 392 nm and at (C) 449 nm versus the concentration of $Zn(ClO_4)_2$.

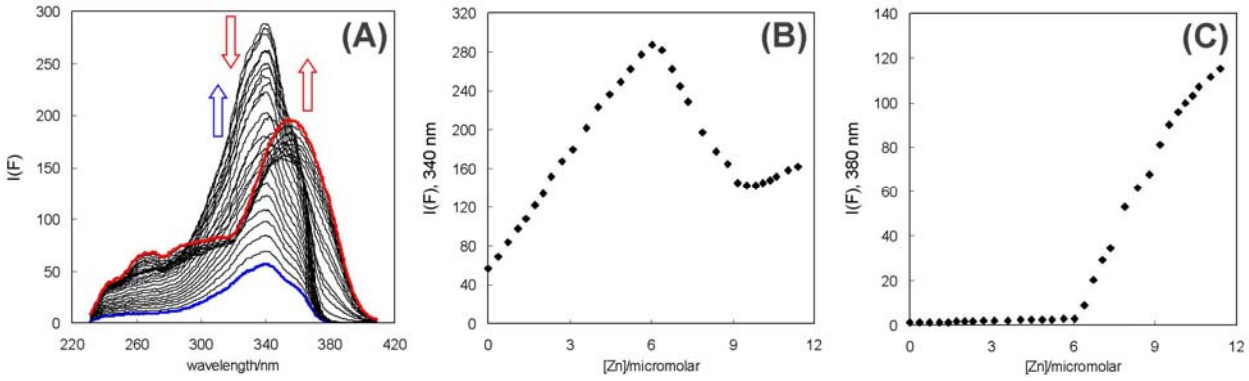


Figure S4. Excitation spectra (λ_{em} = 420 nm) of **1** (2.0 μM) in the presence of $Zn(ClO_4)_2$ (0 – 11.4 μM) in MeCN (TBAP: 5 mM; DIPEA 2.0 μM). The first and last spectra are coded blue and red, respectively. The blue and red arrows represent spectral changes when [zinc] is low and high, respectively. Fluorescence intensity (420 nm) values excited at (B) 340 nm and (C) 380 nm vs. the concentration of $Zn(ClO_4)_2$.

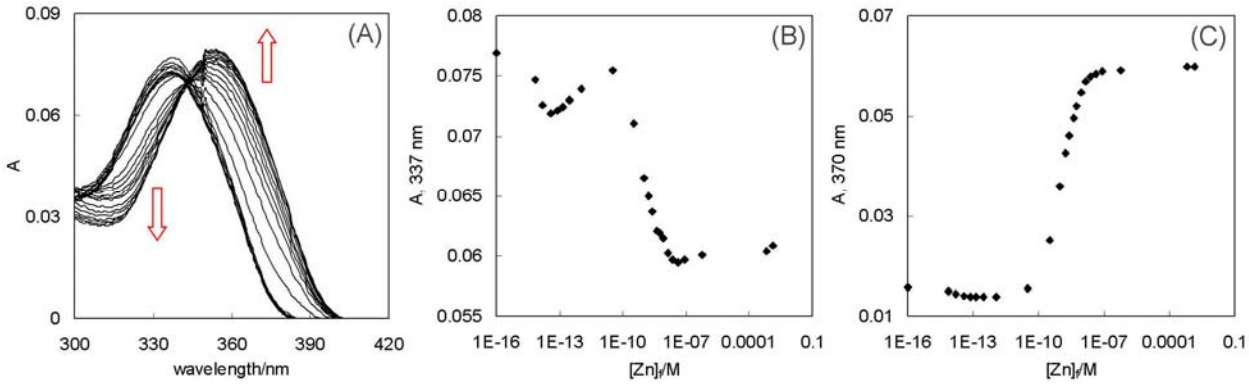


Figure S5. (A) Absorption spectra of **1** (2.0 μM) in the presence of $Zn(ClO_4)_2$ (0 – 15.6 mM) in 10% DMSO aqueous solution (HEPES: 50 mM, pH = 7.0, KNO_3: 100 mM; EDTA: 5 mM; EGTA: 5 mM; NTA: 5 mM). Arrows represent the direction of spectral changes upon addition of zinc. Absorbance values of **1** at (B) 337 nm and at (C) 370 nm vs. the concentration of free zinc ion.

FLUORESCENCE QUANTUM YIELD DETERMINATION

Fluorescence quantum yields were determined in 10% DMSO metal ion buffer (EDTA: 5 mM, EGTA: 5 mM, HEDTA: 5 mM, NTA: 5 mM, HEPES: 50 mM at pH 7, KNO_3: 100 mM) by a relative method using solutions of 2-aminopyridine (ϕ_f = 0.66, 1 N H_2SO_4), and quinine sulfate (ϕ_f = 0.546, 1 N H_2SO_4) as references. The excitation wavelengths were the λ_{max} of the respective species. The fluorescence quantum yields were calculated using Equation S2.

$$\phi_u = [(A_s F_u n^2)/(A_u F_s n_0^2)]\phi_s \qquad \text{Equation S2}$$

Where A_s and A_u are the absorbance values of the samples and reference solutions at their respective excitation wavelengths, F_s and F_u are the corresponding integrated fluorescence intensity, and n is the refractive index of the solvent of the sample (n) or of the reference (n_0). Absorbance of samples and references at their respective excitation wavelengths was kept below 0.1.

The dizinc samples were prepared using 2 μM of the respective ligands and 20 mM of $Zn(ClO_4)_2$, under which conditions the metal ion buffer is expected to be saturated.

Monozinc complex data were obtained using a recently reported method (*J. Org. Chem.* **2008**, *73*, 8321).

LIVE-CELL IMAGING EXPERIMENTS

(1) Cell culture

HeLa (ATCC, CCL2 line) cells were cultured in a 50:50 mixture Dulbecco's Modified Eagle Medium and Ham's F12 (Sigma) supplemented with antibiotics and 12% Cosmic Calf Serum (Hyclone) in a 37 °C incubator with 5% CO_2. The cells were grown to approx. 60-80% confluency and transferred to Bioptechs Delta-T imaging chambers for further analysis. The stock solution of compound **1** (6 μL, 1.7 mM in DMSO) was diluted in the culture media (1 mL) to 10 μM. The cells were incubated with the dye-containing media at 37 °C for 30 min (longer incubation, e.g. 22 h, does not appear to affect the fluorescent properties, or show toxicity to the cells) after which fresh media (1 mL × 2) was used to wash the stained cells. Fresh media was added in the dish, which was then ready for imaging. The zinc ion was added using media solutions containing pyrithione (20 μM, diluted from a 10 mM stock solution in DMSO using the media) and $ZnCl_2$ (0 – 100 μM, diluted from a 100 mM $ZnCl_2$ stock solution in water using the media) and the dishes were incubated for 10 min before imaging.

(2) Imaging procedure

Delta-T culture chambers were transferred to a Nikon TE2000 microscope equipped with (a) Exfo Excite light source, (b) Nikon Plan-Fluoro 40x/.75 NA dry objective, (c) Omega Q-Max Blue filter set with excitation from 355 – 405 nm and emission from 420 – 480 nm, (d) Chroma FITC HYQ filter set with excitation from 460 - 500 nm and emission from 510 - 560 nm, (e) Omega Q-max red filter with excitation from 530 - 570 nm and emission from 600 - 650 nm, (f) 410 nm Long Pass dichroic mirror, and (g) Q-Imaging Retiga camera. The temperature was controlled at 37 °C with the dish controller and a heated lid. A mixture of 5% CO_2 in air was passed through a humidity

chamber and then directly into the culture chamber. All the images were acquired with a 500 ms exposure time. The background fluorescence of all images was subtracted at input levels “50”, which corresponded to the readings of cell-free regions. Because **1** and its complex were localizing in mitochondria (see text), the cells were co-stained with MitoTracker Red whose emission was used for focusing purposes prior to image acquisition.

(3) Co-staining experiments

Mitotracker Red CMXRos. Cells were plated the day before staining at approx 60% confluency. The appropriate concentrations of MitoTracker Red (0.3 μM) and **1** (10 μM) were applied to the delta T dish for 30 min. The dish was washed twice with clean media, then $ZnCl_2$ (100 μM) was added with pyrithione (20 μM) for 10 minutes, after which the media was again replaced prior to imaging. An Omega Q-max red filter was used for collecting emission from Mitotracker Red.

For co-localization of **1** with targeted fluorescent protein fusions, cells were transfected with Effectene (QIAGEN) using a DNA concentration of 1 μg/μL and were allowed to incubate for 16-24 h before imaging in Delta-T chambers. Prior to imaging, the media was replaced, the cells washed with fresh media and incubated in the presence of **1** (10 μM) for 30 min. The media was replaced and $ZnCl_2$ (100 μM) was added with pyrithione (20 μM). After 10-min incubation, the cells were washed with fresh media and imaged.

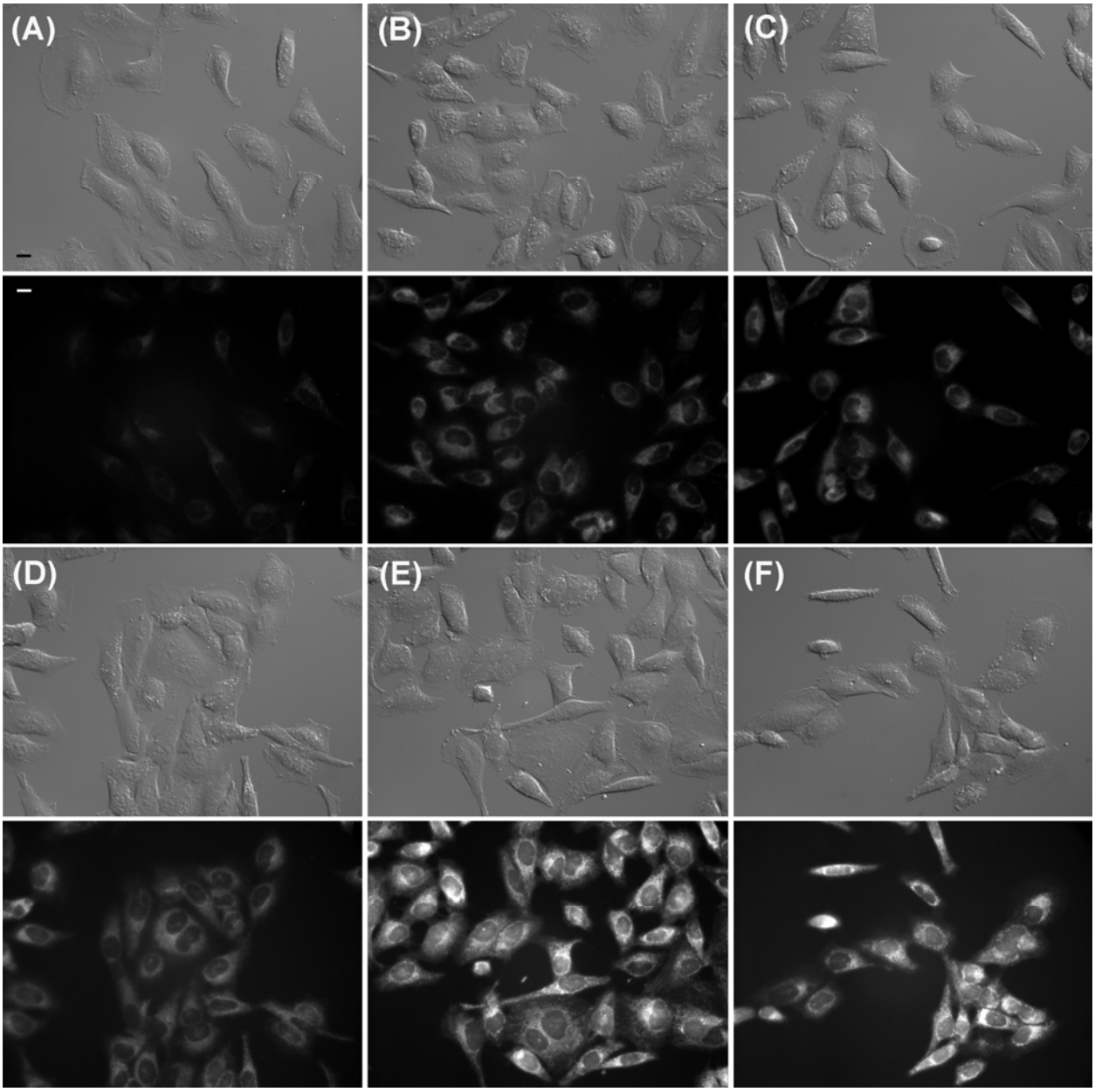


Figure S6. Differential interference contrast (DIC) images are combined with fluorescence images shown in Figure 3. Scale bar: 10 μm.

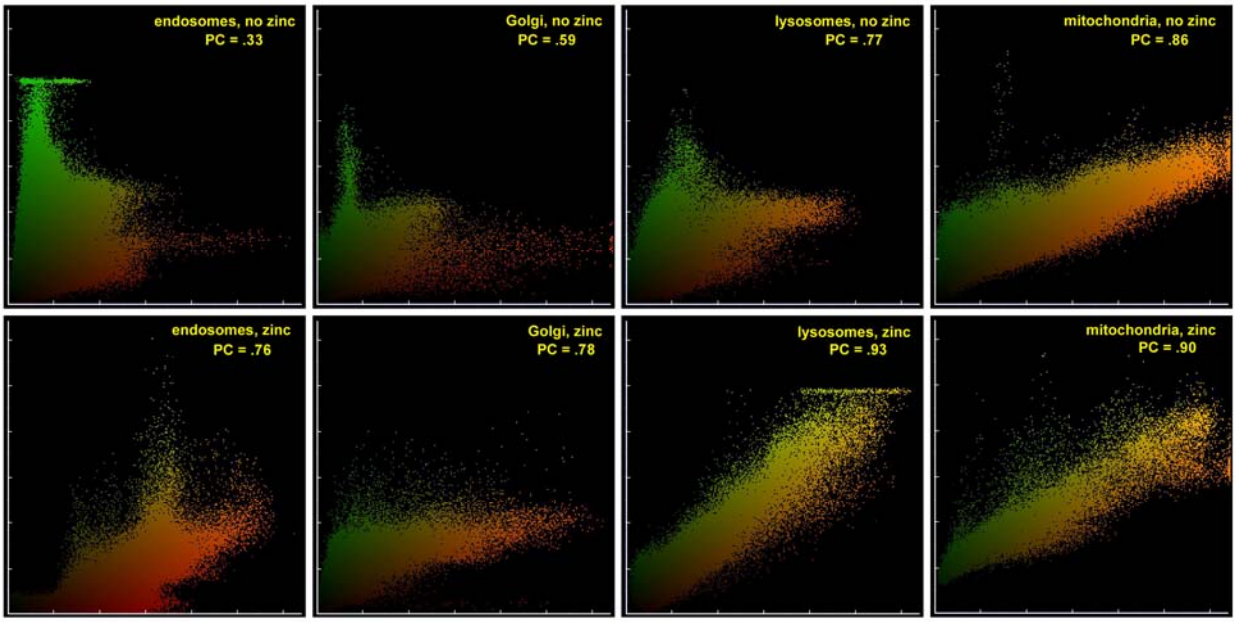


Figure S7. Scatterplots of compound **1** used in determining the Pearson's coefficients.

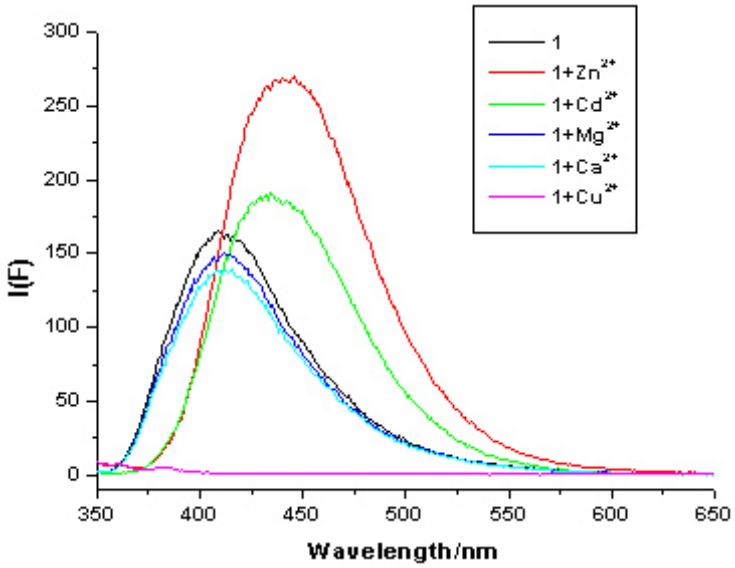


Figure S8. Fluorescence spectra of compound **1** (1.6 μM, λ_{ex} = 340 nm) in an aqueous solution (20 mM HEPES, pH = 7.4, 125 mM NaCl, 1.2 mM $MgCl_2$, 5.9 mM KCl, 2.4 mM $CaCl_2$, 0.5 mM EDTA, and 0.5 mM EGTA) in the presence of 2.6 mM of Zn^{2+} (red), Cd^{2+} (green), Mg^{2+} (blue), Ca^{2+} (cyan), and Cu^{2+} (pink).