A Turn-On Fluorescent Sensor For Detecting Nickel in Living Cells

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Synthetic Materials and Methods. All reactions were carried out under a dry nitrogen atmosphere. Silica gel P60 (SiliCycle) was used for column chromatography. Analytical thin layer chromatography was performed using SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick). Compound **1** was prepared according to previously reported procedures.[1—3] *N*-phenyldiethanolamine and *p*-toluenesulfonyl chloride were purchased from Acros Organics (Morris Plains, NJ) and were used as received. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. Pluronic F-127 and Hoescht-3342 were purchased from Invitrogen (Carlsbad, CA). ^{1}H and ^{13}C NMR spectra were collected in $CDCl_3$ or CD_3OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C on a Bruker AVB-400 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard notation of parts per million using the peak of residual proton signals of $CDCl_3$ as an internal reference. Low-resolution mass spectral analyses were carried out using a 6130 quadrupole LC/MS 1200 Series (Aglient Technologies, Santa Clara, CA). High-resolution mass spectral analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Dimethyl 2,2'-(2,2'-(phenylazanediyl)bis(ethane-2,1-diyl)bis(sulfanediyl))diacetate (2). A solution of **1** (5.03 g, 10.3 mmol) in anhydrous DMF (60 mL) was added dropwise over 1 h to a mixture of methyl thioglycolate (2.0 mL, 22.4 mmol) and Cs_2CO_3 (7.34 g, 22.5 mmol) in anhydrous DMF (40 mL) at 60 °C. The reaction mixture continued to stir for 2 d. The reaction was filtered, and the salts were washed with DMF (50 mL). Water (200 mL) and brine (50 mL) were added to the filtrate, and the reaction was extracted with EtOAc (1 x 200 mL). The organic phase was washed with water (2 x 150 mL) and brine (2 x 50 mL), dried over Na_2SO_4, filtered, and concentrated to dryness. The crude reaction mixture was purified by flash column chromatography (silica gel, CH_2Cl_2 to 1% MeOH in CH_2Cl_2) to furnish **2** as a yellow oil (1.51 g, 41%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.88 (t, 4H, J = 7.6 Hz), 3.32 (s, 4H), 3.61 (t, 4H, J = 7.6 Hz), 3.78 (s, 6H), 6.73-6.78 (m, 3H), 7.25-7.30 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.7, 33.4, 50.8, 52.5, 112.0, 116.9, 129.6, 146.6, 170.8. LRESI-MS calculated for [MH^+] 358.1, found 358.1.

Dimethyl 2,2'-(2,2'-(4-formylphenylazanediyl)bis(ethane-2,1-diyl)bis(sulfanediyl)) diacetate (3). $POCl_3$ (0.75 mL, 8.0 mmol) was added dropwise to a stirring solution of **2** (1.45 g, 4.5 mmol) in anhydrous DMF (10 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. The crude mustard yellow reaction mixture was poured into water (150 mL), cooled to 0 °C, and the pH was adjusted to pH 7-8 with solid K_2CO_3. The reaction mixture was stirred at room temperature for 10 min and extracted with CH_2Cl_2 (3 x 100 mL). The organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated to dryness. The crude reaction mixture was purified by flash column chromatography two times (1st column: silica, CH_2Cl_2; 2nd column: silica, $CHCl_3$) to provide **3** as a yellow oil (0.929 g, 60%). ^{1}H

NMR (400 MHz, $CDCl_3$): δ 2.72 (t, 4H, *J* = 7.4 Hz), 3.16 (s, 4H), 3.52 (t, 4H, *J* = 7.4 Hz), 3.58 (s, 6H), 6.60 (d, 2H, *J* = 8.8 Hz), 7.56 (d, 2H, *J* = 8.4 Hz), 9.55 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.9, 32.8, 50.2, 52.1, 53.4, 110.3, 110.7, 125.4, 131.8, 151.0, 170.3, 189.6. LRESI-MS calculated for [MH^+] 386.1, found 386.1.

10-(4-(Bis(2-(2-methoxy-2-oxoethylthio)ethyl)amino)phenyl)-5,5-difluoro-1,3,7,9-tetramethyl-5H-dipyrrolo[1,2-c:1',2'-f][1,3,2]diazaborinin-4-ium-5-uide (4). Five drops of TFA were added dropwise to a vigorously stirring solution of **3** (449 mg, 1.17 mmol) and 2,4-dimethylpyrrole (245 mg, 2.58 mmol) in anhydrous CH_2Cl_2 (150 mL). The resulting red solution was stirred at room temperature in the dark for 90 min. DDQ (293 mg, 1.29 mmol) was then added in one portion and reaction was stirred for an additional hour. DIEA (3.0 mL, 17.2 mmol) was then added dropwise to this mixture over a period of 10 min, and the resulting orange-brown solution was allowed to stir for an additional 20 min. BF_3•OEt_2 (3.0 mL, 23.7 mmol) was then added dropwise over a period of 5 min, and the resulting purple solution was allowed to stir overnight. A second BODIPY condensation reaction of the same scale was performed (450 mg, 1.17 mmol of **3**) in parallel and both reaction mixtures were combined for a single workup procedure. The combined reactions were stirred with water (100 mL) for 10 min, and then water (50 mL) and brine (50 mL) were added. The resulting slurry was allowed to sit for 30 min, during which time the mixture separated into two phases. The aqueous layer was decanted, and the organic phase was washed with water (2 x 100 mL). The aqueous layers were then re-extracted with CH_2Cl_2 (3 x 100 mL), and the combined organic layers were washed with water (1 x 100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness. The crude purple residue was purified by flash column chromatography (silica, 1% EtOAc in CH_2Cl_2) to afford **4** as an orange solid with a green luster (535 mg, 38%). ^1H NMR (400 MHz, 10% CD_3OD in $CDCl_3$): δ 1.42 (s, 6H), 2.47 (s, 6H), 2.83 (t, 4H, *J* = 7.4 Hz), 3.24 (s, 4H), 3.58 (t, 4H, *J* = 7.4 Hz), 3.69 (s, 6H), 5.91 (s, 2H), 6.71 (d, 2H, *J* = 8.4 Hz), 6.98 (d, 2H, *J* = 8.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.4, 14.6, 29.3, 32.2, 50.6, 52.4, 112.0, 120.8, 122.5, 129.1, 131.9, 142.6, 143.0,147.0, 154.6, 170.6. LRESI-MS calculated for [MH^+] 604.2, found 604.3.

Nickelsensor-1 (NS1, 5). LiOH (85 mg, 3.54 mmol) was added to a solution of diester **4** (515 mg, 0.89 mmol) in anhydrous THF (5 mL) and anhydrous MeOH (7 mL). The resulting red-orange solution continued to stir overnight. The reaction mixture was concentrated to dryness, and the remaining residue was dissolved in water (75 mL) and $CHCl_3$ (50 mL). The pH of the aqueous layer was adjusted to pH 5-6 by 0.5 M HCl and extracted with $CHCl_3$ (2 x 50 mL). The combined organics were washed with water (75 mL), dried over Na_2SO_4, filtered, concentrated to dryness, and purified by flash column chromatography (silica, 3% MeOH in CH_2Cl_2) to furnish NS1 as an orange solid (362 mg, 71%). ^1H NMR (400 MHz, $CDCl_3$): δ 1.40 (s, 6H), 2.45 (s, 6H), 2.81 (t, 4H, *J* = 7.6 Hz), 3.21 (s, 4H), 3.57 (t, 4H, *J* = 7.6 Hz), 5.90 (s, 2H), 6.70 (d, 2H, *J* = 8.4 Hz), 6.98 (d, 2H, *J* = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.4, 14.6, 29.4, 33.6, 50.7, 112.1, 120.9, 122.6, 129.2, 132.1, 142.9, 143.3, 147.2, 154.8, 172.8. HRESI-MS calculated for [MH^+] 576.1968, found 576.1990.

Nickelsensor-1-acetoxymethyl ester (NS1-AM, 6). Bromomethyl acetate (110 mg, 0.72 mmol) in anhydrous DMF (1 mL) was added dropwise to a solution of NS1 (101 mg, 0.18 mmol) and DIEA (185 mg, 1.43 mmol) in anhydrous DMF (2 mL). The reaction

mixture was allowed to stir overnight. Water (10 mL) was added to the reaction mixture and extracted with EtOAc (3 x 10 mL). The combined organics were washed with water (5 x 20 mL), dried over Na_2SO_4, filtered, concentrated to dryness, and purified by flash column chromatography (silica, 1.5% EtOAc in CH_2Cl_2) to furnish NS1-AM as a red-orange oil (99 mg, 78% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.45 (s, 6H), 2.09 (s, 6H), 2.51 (s, 6H), 2.87 (t, 4H, J = 7.4 Hz), 3.30 (t, 4H, J = 7.4 Hz), 3.30 (s, 4H), 3.61 (t, 4H, J = 7.6 Hz), 5.76 (s, 2H), 5.95 (s, 2H), 6.74 (d, 2H, J = 8.4 Hz), 7.04 (d, 2H, J = 8.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.6, 14.7, 20.7, 29.5, 33.2, 50.7, 79.7, 112.2, 121.0, 123.0, 129.3, 132.1, 142.7, 143.1, 147.1, 154.9, 169.1, 169.5. HRESI-MS calculated for [MH^+] 720.2391, found 720.2400.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in aqueous 20 mM HEPES, pH 7.1. Absorption spectra were recorded on a Varian Cary 50 spectrophotometer (Walnut Creek, CA) and fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ) equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog multiplier detection unit, and MD5020 motor driver. Samples for absorption and emission measurements were contained in 1-cm x 1-cm quartz cuvettes (1.4-mL volume, Starna, Atascadero, CA). Fluorescence quantum yields were determined by reference to fluorescein in 0.1 M (Φ = 0.95).[4] Excitation was provided at 488 nm, and collected emission was integrated from 498 to 700 nm. The apparent dissociation constant (K_d) was determined from a plot of normalized fluorescence response versus [Ni^{2+}]. The data were fitted to the following equation: $F = (F_{max}[Ni^{2+}] + F_{min}K_d)/(K_d + [Ni^{2+}])$, where F is the observed fluorescence, F_{max} is the fluoresence for the Ni^{2+}:NS1 complex, and F_{min} is the fluorescence for the free NS1 dye. The Hill coefficient was determined from the slope of the linear least squares fit of $\log[(F_{min}-F_1)/(F_1-F_{max})]$ vs. $\log[Ni^{2+}]$.[5] Ni^{2+} was delivered in the form of $NiCl_2$ x $2H_2O$ from an aqueous stock solution (1, 5, 10, 25, 100, 500 mM). All other metal ions tested for metal ion selectivity studies with the exception of Pb^{2+} and Fe^{2+} were from their chloride salts as aqueous solutions. Pb^{2+} was delivered in the form of aqueous $Pb(NO_3)_2$. Ammonium iron(II) sulfate hexahydrate was used as a source of Fe^{2+}. This salt was dissolved in degassed water, and then aliquots of the stock Fe^{2+} solution were added to a degassed aqueous solution of NS1. The solution was degassed once again before the addition of Ni^{2+}.

Preparation and Staining of Cell Cultures. Cells were grown in the Tissue Culture Facility at the University of California, Berkeley with expert technical assistance from Ann Fischer and Michelle Yasukawa. A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, Carlsbad, CA) and glutamine (2 mM). Two days before imaging, cells were passed and plated on 12-mm glass coverslips coated with poly-L-lysine (50 mg/mL, Sigma, St. Louis, MO). For all experiments, solutions of dyes (from 5 mM stocks in DMSO) were made in HBSS without calcium chloride, magnesium chloride, magnesium sulfate, sodium bicarbonate, or phenol red (HBSS, pH 7.1, Invitrogen, Carlsbad, CA). For nickel treatment, A549 cells were cultured as described above. One day prior to imaging, 1 mM $NiCl_2$ was added to cells from a 0.1 M aqueous

stock solution. Cells were then incubated at 37 °C, 5% CO_2. After 18 hours, the media was exchanged for HBSS with 10 μM dye and incubated for 35 min.

Fluorescence Imaging Experiments. Confocal fluorescence images were acquired at the Molecular Imaging Center at the University of California, Berkeley. Imaging experiments were performed with a Zeiss LSM510 META/NLO Axioplan 2 laser scanning microscope and a 40x water-immersion objective lens. Excitation of NS1-loaded cells at 488 nm was carried out with an argon ion laser (37% laser power), and emission was collected in a window from 494 nm—634 nm using a META detection system. Excitation of Hoechst-3342 was carried out using a MaiTai two-photon laser at 780-nm pulses (36% laser power, mode-locked Ti:sapphire laser, Tsunami Spectra Physics) and emission was collected between 452—538 nm. A 1:1 (v/v) mixture of NS1-AM and Pluronic F-127 (10 μM) and Hoescht-3342 (5 μM) was incubated with live cell samples for 35 min at 37 °C, 5% CO_2. Addition of TPEN (1 mM from a 0.1 M stock in DMSO) was performed directly on the microscope stage. Image analysis was performed in ImageJ.

References

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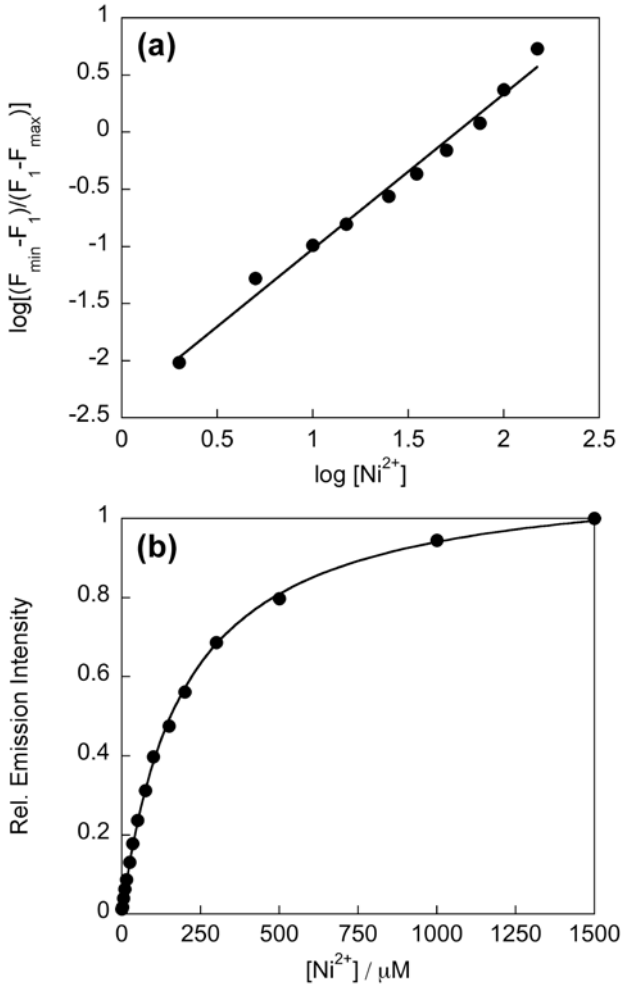

Figure S1. (a) Hill plot of the normalized fluoresence responses of 2 μM NS1 to various [Ni^{2+}]. The data points shown are for added Ni^{2+} concentrations of 2, 5, 10, 15, 25, 35, 50, 75, 100, 150 μM, with a slope of 1.36 ± 0.06. Spectra were acquired in 20 mM HEPES, pH 7.1. Excitation was provided at 488 nm and the collected emission was integrated over 498—700 nm. (b) Normalized fluoresence responses of 2 μM NS1 to Ni^{2+} solutions for K_d value determination. Excitation was provided at 488 nm and the collected emission was integrated over 498—700 nm. Spectra were acquired in HEPES, pH 7.1. The data points shown are for Ni^{2+} added at 0, 2, 5, 10, 15, 25, 35, 50, 75, 100, 150, 200, 300, 500, 1000, and 1500 μM. The apparent K_d value is 193 ± 3 μM.

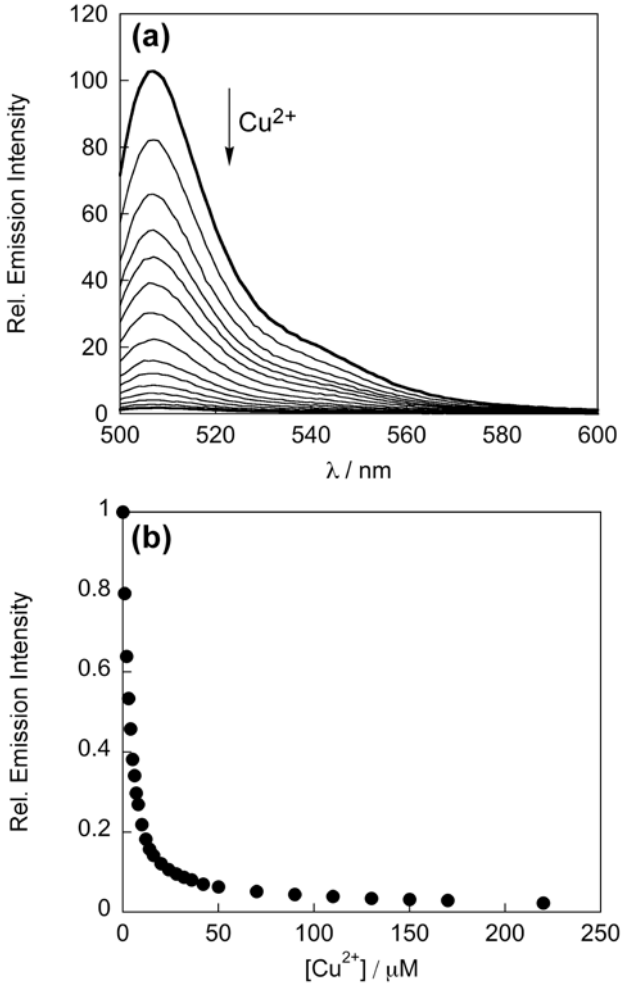

Figure S2. (a) Fluorescence response of 2 μM NS1 with 100 μM Ni^{2+} to Cu^{2+}. Spectra are shown for Cu^{2+} concentrations of 0, 1, 2, 3, 4, 5, 7, 10, 14, 20, 32, 50, 90, 150, 220 μM. Spectra were acquired in 20 mM HEPES, pH 7.1. Excitation was provided at 488 nm and the collected emission was integrated over 498—700 nm. (b) Normalized fluoresence responses of 2 μM NS1 with 100 μM Ni^{2+} to Cu^{2+} for relative K_d value determination. Excitation was provided at 488 nm and the collected emission was integrated over 498—700 nm. Spectra were acquired in 20 mM HEPES, pH 7.1. The data points shown are for Cu^{2+} added at 0, 1, 2, 3, 5, 6, 7, 8, 10, 12, 14, 16, 20, 24, 28, 32, 36, 42, 50, 70, 90, 110, 130, 150, 170 and 220 μM. The apparent K_d value is 0.58 μM.

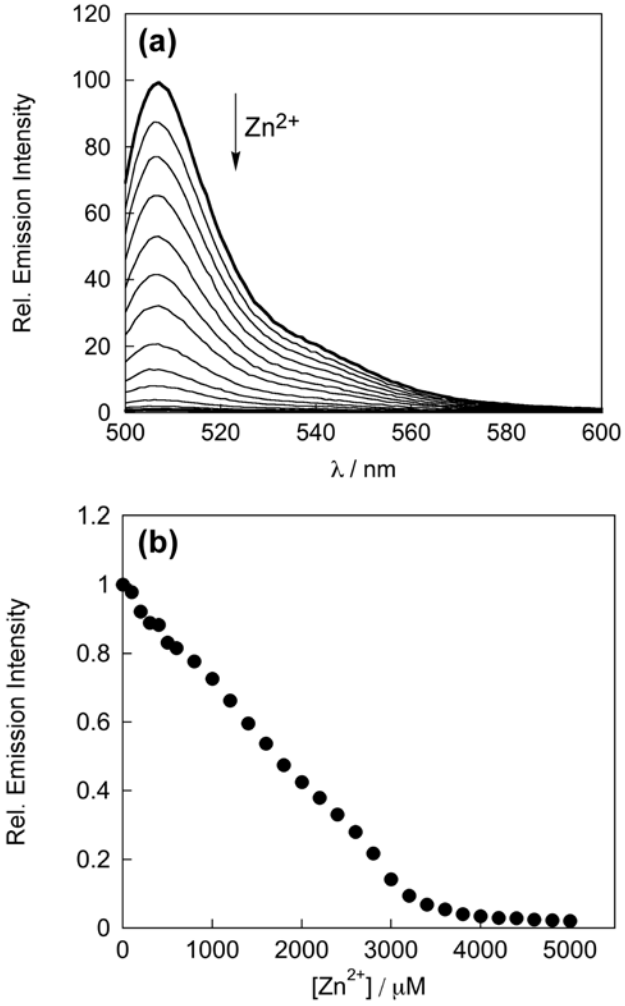

Figure S3. (a) Fluorescence response of 2 μM NS1 with 100 μM Ni^{2+} to Zn^{2+}. Spectra are shown for Zn^{2+} concentrations of 0, 400, 800, 1200, 1600, 2000, 2400, 2800, 3000, 3200, 3600, 4000, 4400, and 5000 μM Zn^{2+}. Spectra were acquired in 20 mM HEPES, pH 7.1. Excitation was provided at 488 nm and the collected emission was integrated over 498—700 nm. (b) Normalized fluoresence responses of 2 μM NS1 with 100 μM Ni^{2+} to Zn^{2+} for relative K_d value determination. Excitation was provided at 488 nm and the collected emission was integrated over 498—700 nm. Spectra were acquired in 20 mM HEPES, pH 7.1. The data points shown are for Zn^{2+} added at 0, 100, 200, 300, 400, 500, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3400, 3600, 3800, 4000, 4200, 4400, 4600, 4800, and 5000 μM. The apparent K_d value is 1640 μM.

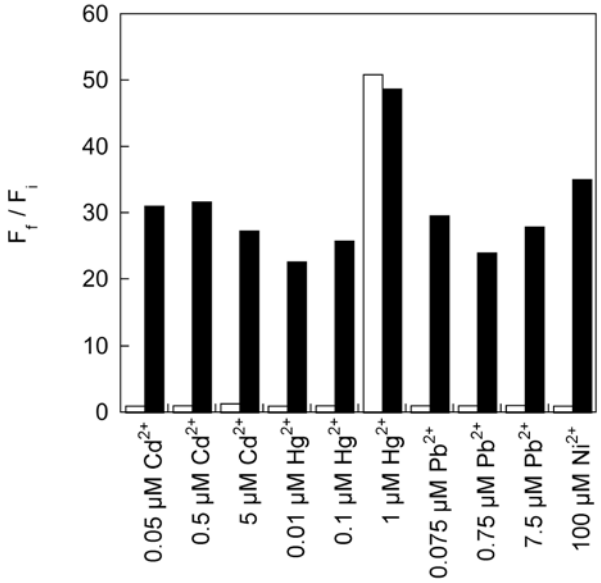

Figure S4. Fluorescence responses of 2 μM NS1 to various heavy metal ions. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Spectra were acquired in 20 mM HEPES, pH 7.1. White bars represent the addition of the maximum allowable EPA level, 10 times the maximum allowable EPA level, and 100 times the maximum allowable EPA level of the competing heavy metal ion to a 2 μM solution of NS1. Black bars represent the addition of 100 μM Ni^{2+} to the solution. Excitation was provided at 488 nm, and the emission was integrated over 498—700 nm.

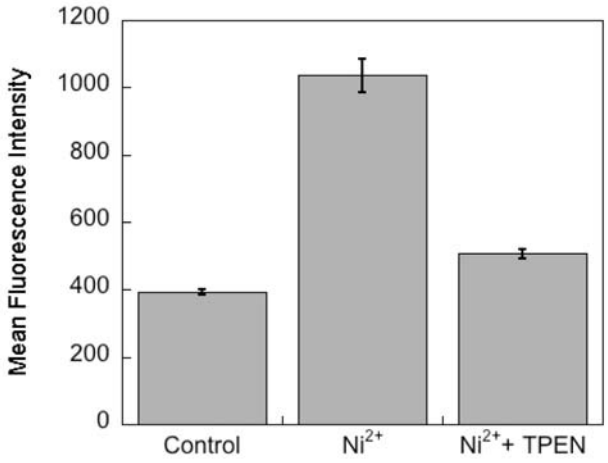

Figure S5. Plot of the mean fluoresence intensity of control A549 cells, cells supplemented with 1 mM $NiCl_2$, and 1 mM Ni^{2+}-supplemented cells treated with 1 mM of the divalent metal chelator TPEN stained with a 1:1 (v/v) mixture of NS1-AM and Pluronic F-127 (10 μM) and Hoescht-3342 (5 μM). These data represent the mean fluorescence intensity of at least three images with standard error.