Supporting information

A Highly Selective Turn-on Fluorescence Probe for Iron(II) to Visualize Labile Iron in Living Cells

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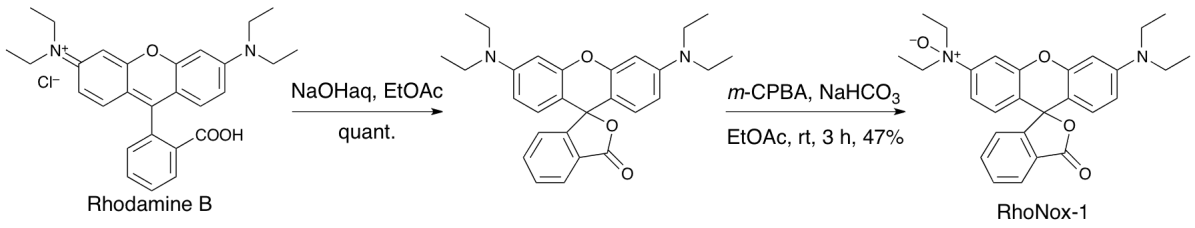
Contents

General: All chemicals used in this study were commercial products of the highest available purity and were further purified by the standard methods, if necessary. ^{1}H-NMR spectra were obtained on a JEOL ECA-500 spectrometer at 500 MHz. ^{13}C-NMR spectra were obtained on a JEOL ECA-500 spectrometer at 125 MHz. Spectra were obtained in $CDCl_3$ unless otherwise specified. Chemical shifts of ^{1}H-NMR are referenced to TMS. Chemical shifts of ^{13}C-NMR are referenced to $CDCl_3$ (77.0), unless otherwise specified. Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. ESI-mass spectra were measured on a JEOL JMS-T100TD mass spectrometer. High-resolution mass spectra (HRMS) were measured on a JEOL JMS-T100TD by using PEG as an internal standard. Reagents and solvents were purchased from commercial sources. Anhydrous organic solvents were prepared by distillation with drying reagents before every use. Reactions were monitored by silica gel TLC (Merck Silica gel 60 PF_{254}) with visualization of components by UV light (254 nm) or with visual observation of the dye spots, and UV-Vis. Products were purified on a silica gel column chromatography (Taiko-shoji AP-300S).

1. Synthesis of RhoNox-1

Scheme S1. Synthesis of RhoNox-1



Rhodamine B base was prepared from commercially available Rhodamine B as described previously.[1] To a mixture of Rhodamine B base (100 mg, 0.21 mmol) and sodium bicarbonate (19 mg, 0.23 mmol) in ethyl acetate (10 mL) was slowly added *m*-chloroperbenzoic acid (mCPBA) (40 mg, 0.23 mmol) at 0 °C. The mixture was warmed to room temperature and then stirred for 3 h. Insoluble materials were removed by filtration with a pad of celite, and then the filtrate was evaporated. The residue was purified with silica gel column chromatography ($CHCl_3$: MeOH = 10: 1) to afford **RhoNox-1** as pale purple solid (40 mg, 42%). TLC R_f = 0.47 ($CHCl_3$: MeOH = 9: 1); ^{1}H NMR (500 MHz, $CDCl_3$) δ : 1.18-1.13 (m, 12H), 3.36 (q, 4H, *J* = 6.9 Hz), 3.70 (brs, 4H), 6.38 (dd, 1H, *J* = 9.1, 2.3 Hz), 6.47 (d, 1H, *J* = 2.3 Hz), 6.58 (d, 1H, *J* = 8.6 Hz), 6.84 (d, 1H, *J* = 8.6 Hz), 7.21 (d, 1H, *J* = 7.4 Hz), 7.26 (br, 1H), 7.65 (t, 1H, *J* = 7.5 Hz), 7.69 (t, 1H, *J* = 7.4 Hz), 7.97 (d, 1H, *J* = 2.3 Hz), 8.04 (d, 1H, *J* = 7.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ : 8.5, 12.6, 44.6, 67.1, 83.3, 97.7, 104.5, 108.9, 112.4, 115.9, 120.1, 124.1, 125.2, 127.0, 128.9, 129.0, 129.9, 135.1, 149.9, 152.2, 152.8, 169.5; HRMS (ESI+): *m/z* calculated for $C_{28}H_{31}N_2O_4^+$: 459.2278, found 459.2264.

2. Steady-state absorption and fluorescence spectroscopy

The UV-vis absorption spectra were recorded on a Shimadzu UV-3100s. Fluorescence spectra were recorded using a JASCO FP6600 with a slit width of 5 nm and 6 nm for excitation and emission, respectively. To reduce fluctuation in the excitation intensity during measurement, the lamp was kept on for 30 min prior to the experiment. The path length was 1 cm with a cell volume of 3.0 mL. For determination of pK_{a1} and pK_{a2}, absorption spectra were measured in 200 mM sodium phosphate buffer at various pH values. A pH profile of absorbance at 492 nm was fitted to the following equation (eq. 1).

$$A = \frac{A_0 + A_1 \times 10^{(\mathrm{pH}-\mathrm{p}K_{a1})} + A_2 \times 10^{(\mathrm{pH}-\mathrm{p}K_{a1}-\mathrm{p}K_{a2})}}{1 + 10^{(\mathrm{pH}-\mathrm{p}K_{a1})} + 10^{(\mathrm{pH}-\mathrm{p}K_{a1}-\mathrm{p}K_{a2})}} \quad \text{(eq.1)}$$

where pK_{a1} and pK_{a2} are the acid dissociation constants of RhoNox-1; A_0 is the initial absorbance at 492 nm at pH 1.60; and A_1 and A_2 are the maximum absorbance at 492 nm associated with the corresponding pK_a values.

For all the fluorescence measurements, RhoNox-1 was used at a final concentration of 2 μM (from 1 mM stock solution in DMSO) in 50 mM HEPES (3 mL, pH 7.4), and excitation wavelength was 540 nm. For measurements with various concentrations of Fe^{2+}, each concentration of $FeSO_4$ (from 10 mM or 1 mM stock solution in water) was added to 2 μM RhoNox-1 solution in HEPES buffer. After incubation at 25 °C for 1 h, fluorescence spectra were measured.

Fluorescence responses of RhoNox-1 to various metal ions were measured as follows. An aqueous solution of transition metal ion species (stock solutions: 10 mM for $MnSO_4$, $CoSO_4$, $NiSO_4$, $FeSO_4$, $FeCl_3$, $CuSO_4$, and $ZnSO_4$; 1 M for NaCl, KCl, $MgCl_2$, and $CaCl_2$) or $[Cu(CH_3CN)_4]PF_4$ (from 10 mM stock solution in MeCN) was added to give the final concentrations of 1 mM for Na^+, K^+, Mg^{2+}, and Ca^{2+} and 20 μM for other metal ion species. The mixtures were kept at room temperature for 1 h and then applied to fluorescence measurement. For the inhibition test, RhoNox-1 was mixed with each metal ion in 50 mM HEPES in the presence of $FeSO_4$ (20 μM). The mixtures were incubated for 1 h at room temperature, and then fluorescence spectra were measured. The relative quantum yield of RhoNox-1 was obtained by comparing the area under the emission spectrum of the test sample exited at 540 nm with that of a solution of rhodamine B in EtOH, which has a quantum yield of 0.97.[2]

Selectivity for reductants and reactive oxygen species, effect of chelator, and effect of glutathione were tested under the conditions as follows.

Reductants and reactive nitrogen species

$Na_2S_2O_3$: 100 μM from 100 mM stock solution in water

Sodium ascorbate : 1 mM from 100 mM stock solution in water

Cysteine : 1 mM from 100 mM stock solution in water

Glutathione : 1 mM from 100 mM stock solution in HEPES buffer (pH was adjusted to 7.4)

$NaNO_2$: 100 μM from 100 mM stock solution in water

Reactive oxygen species

$O_2^{\bullet-}$: 100 μM from saturated KO_2 solution in DMSO (ca. 1 mM)[3]

H_2O_2 : 100 μM from 100 mM stock solution in water

•OH : 200 μM H_2O_2 and 20 μM $FeSO_4$ (1 mM stock solution of RhoNox-1 in MeOH was used instead of DMSO because DMSO is a quencher of hydroxyl radical)[4]

NaOCl : 100 μM from 100 mM stock solution in water

NO : 100 μM NOC-5 from 10 mM stock solution in 0.1 M NaOHaq.

Metal ion with reductant

Glutathione+Cu^+: 1 mM glutathione from 100 mM stock solution in HEPES buffer (pH was adjusted to 7.4) and 20 μM $[Cu(MeCN)_4]PF_6$ from10 mM stock solution in MeCN.

Glutathione+Cu^{2+}: 1 mM glutathione from 100 mM stock solution in HEPES buffer (pH was adjusted to 7.4) and 20 μM $CuSO_4$ from10 mM stock solution in water.

Chelator

2,2'-Bipyridyl : 100 μM 2,2'-bipyridyl from 100 mM in stock solution in DMSO and 20 μM $FeSO_4$.

RhoNox-1 (2 μM) was incubated under the each condition in 50 mM HEPES buffer (pH 7.4) for 1 h, and then fluorescence spectra were measured.

3. Product analysis

To a 50 μM solution of RhoNox-1 in 50 mM HEPES buffer (pH 7.4) was added a solution of $FeSO_4$ (final, 500 μM). The mixture was kept for 1 h under ambient conditions. The products were analyzed with a LC-MS (HP 1100 LC system equipped with Agilent 1946B ESI-mass system, Waters symmetry C_{18} column, 3.5 μm, 4.6 × 100 mm) eluted with H_2O/ CH_3CN (30:70 to 5:95 over 15 min) containing 0.05% formic acid. The retention time was compared with that of an authentic sample, rhodamine B in 50 mM HEPES buffer (pH 7.4). The consumption rate of RhoNox-1 was calculated from the integrated areas of the chromatogram of absorbance at 540 nm. We also calculated the yield from absorption at 555 nm by using a UV-vis spectrometer.

4. NMR analysis

Samples for ^{1}H-NMR analysis of RhoNox-1 were prepared by mixing 80 μL of 50 mM RhoNox-1 CD_3OD solution and 720 μL of 200 mM sodium phosphate buffer (pD = 1.8, 7.3, and 13.0, pD = pH + 0.40 [5]). The final concentration of RhoNox-1 was 5 mM, and the measurements were performed at room temperature.

5. Computational study

Density functional theory (DFT) calculations with Becke's three parametrized Lee-Yang-Par (B3LYP) exchange functional with 6-31G* basis sets were carried out for the geometry optimizations and the molecular orbital energy calculations of RhoNox-1 using Gaussian 03W program.

6. Cyclic volutammetry

Cyclic volutammetry was performed on a 600A electrochemical analyzer (ALS). A three-electrode arrangement in a single cell was used for the measurements: a Pt wire as the auxiliary electrode, a glassy carbon electrode as the working electrode, and an Ag/Ag+ electrode as the reference electrode. The sample solution contained 0.50×10^{-3} M sample and 0.1 M tetrabutylammonium parchlorate (TBAP) as a supporting electrolyte in MeCN, and argon was bubbled for 10 min before each measurement. The obtained potentials were converted to those vs (Fc/Fc^{+}) by using ferrocene as an internal standard. The scan rate was 0.2 V s^{-1}. All the measurements were performed under Ar atmosphere.

The ΔG_{et} value was calculated based on Rhem-Weller equation,

$$\Delta G_{et} = E(D^{+}/D) - E(A/A^{-}) - \Delta E_{00} + w_p$$

where $E(D^{+}/D)$ = 0.69 V (the obtained oxidation potential of the donor (*N*-oxide domain)), $E(A^{-}/A))$ = –1.70 V (the obtained half wave reduction potential of the acceptor (xanthene)), ΔE_{00} (zero-zero transition energy) = 2.31 eV and w_p (work term) = –0.72 eV. The ΔE_{00} value was obtained on the basis of $\Delta E_{00} = (E_{abs}(max) + E_{em}(max)) / 2$. The w_p value was calculated by using the contact radical ion pair model with the calculated distance between the acceptor and the donor[6] (= 5 Å).

7. Cell culture experiments

Human hepatocellular carcinoma (HepG2) cells or Human adenocarcinoma (MCF-7) cells were cultured in modified essential medium (MEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 1% Antibiotic-Antimyocotic (Gibco), and 2 mM glutamine at 37 °C in a 5% CO_2/ 95% air incubator. Two days before use, cells (1.0×10^5) were seeded on Advanced TC glass-bottomed dishes (CELLview^{TM} Cell Culture Dish, Greiner).

8. Confocal fluorescence imaging experiments

Confocal fluorescence images were acquired with a Zeiss LSM 700 laser-scanning microscope system. Experiments were performed with a 40x oil-immersion objective lens.

For all imaging experiments, Hank's Balanced Salt Solution (HBSS, Gibco) containing calcium and magnesium without phenol red was used. For Fe^{2+} uptake experiments, cells were treated with 100 μM

ferrous ammonium sulfate (FAS, $Fe(NH_4)_2(SO_4)_2$ from 10 mM stock solution in water)[7] or 100 μM $FeSO_4$ (from 10 mM stock solution in water) in MEM without FBS at 37 °C for 30 min. For all imaging experiments, after washing the cells with HBSS (×3), RhoNox-1 (5 μM, from 1 mM stock solution in DMSO) was added. After incubation for 1 h (for HepG2 cells) or 15 min (for MCF-7 cells) at 37 °C, the cells were washed with HBSS (×3), and then imaged. For chelation experiments, 1 mM 2,2'-bipyridyl (Bpy, from 1 M stock solution in DMSO) was added to the cells simultaneously with RhoNox-1. For nuclear staining, Hoechst 33342 (1.0 μg/mL, from 0.1 mg/mL stock solution in water) was added to the cells 15 min before imaging. For co-staining experiments, cells were treated with 100 nM Mitotracker Green FM (mitochondria, 1 h at 37 °C), 50 nM Lysotracker Green DND-26 (lysosome, 1 h at 37 °C), 500 nM ERtracker Green (endoplasmic reticulum, 30 min at 37 °C), or 1 μM BODIPY-FL ceramide-BSA complex (golgi, 30 min at 4 °C) in HBSS prior to treatment with RhoNox-1 (10 μM) in HBSS for 30 min at 37 °C. For all control experiments, the corresponding amounts of vehicles (FAS, $FeSO_4$: water, Bpy: DMSO) were added to the cells instead. Image analysis was performed with ImageJ.

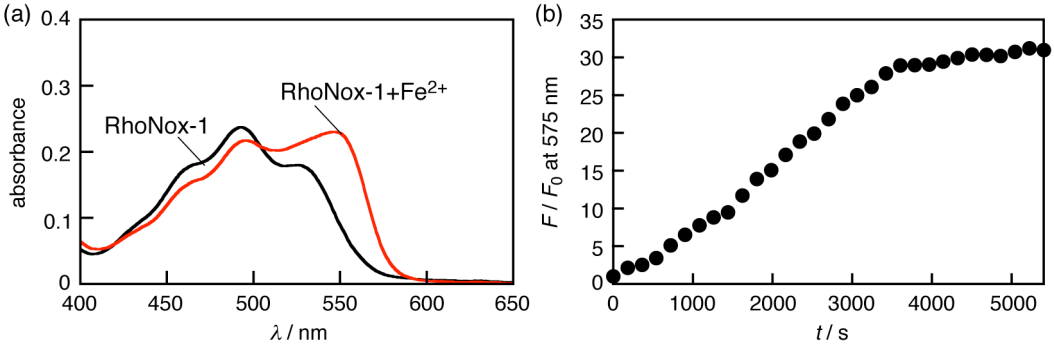


Figure S1. (a) UV-vis spectra before (black line) and after (red line) reaction of RhoNox-1 (5 μM) with 20 μM Fe^{2+} in 50 mM HEPES buffer (pH 7.4). (b) Time course plot of relative fluorescence intensity at 575 nm for the reaction of RhoNox-1 and 20 μM $FeSO_4$.

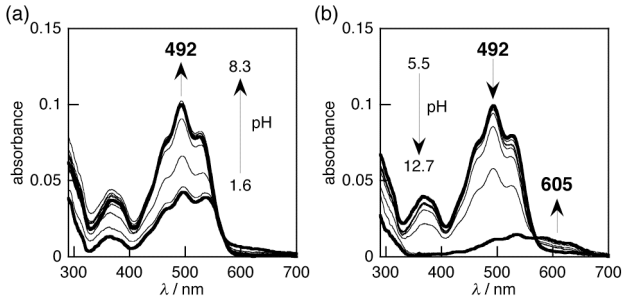


Figure S2. (a) and (b) Absorbance spectra of RhoNox-1 (2 μM) at various pH (1.60, 2.05, 3.22, 4.30, 4.62, 5.53, 6.10, 6.60, 6.90, 7.32, 7.91, 8.33, 8.90, 10.4, 11.2, 12.1, 12.7).

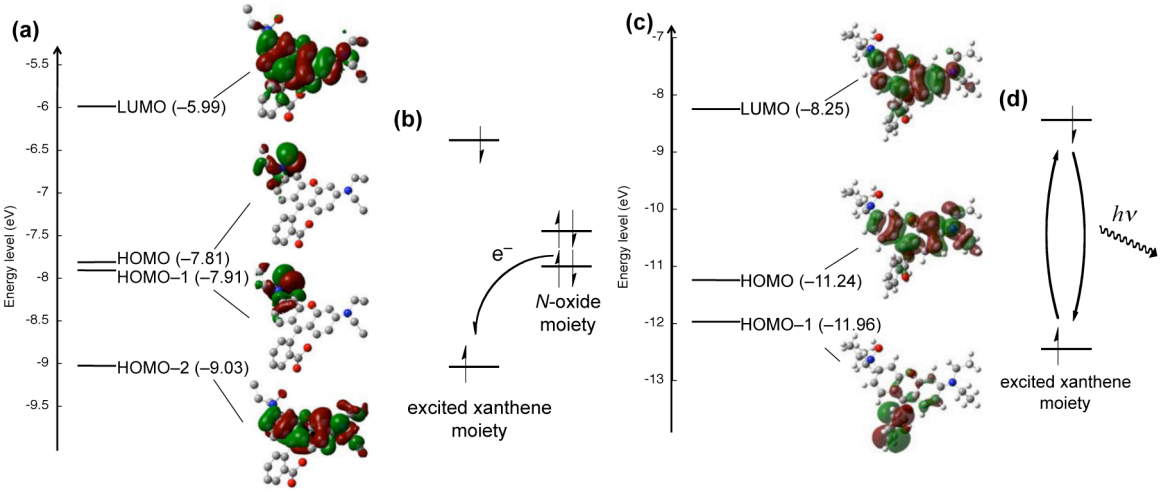


Figure S3. The calculated frontier molecular orbitals of the deprotonated (a) and the protonated (c) forms of RhoNox-1 and their energy levels. The numbers in parentheses indicate the calculated energy levels (eV). (b), (d) Schematic representation of photo-induced electron transfer (PET) from *N*-oxide to excited xanthene moiety.

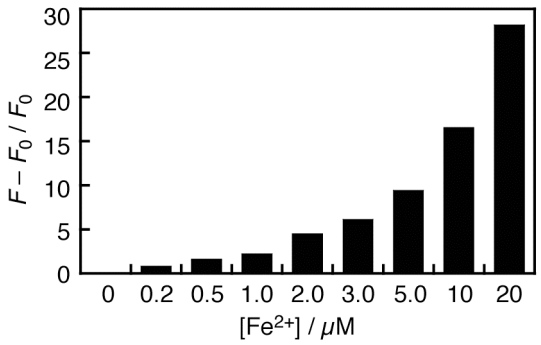


Figure S4. (a) Plot of fluorescence intensity changes of 2 μM RhoNox-1 after reaction with various concentrations of Fe^{2+}. Fluorescence intensities of saturation points were plotted against initial Fe^{2+} concentration.

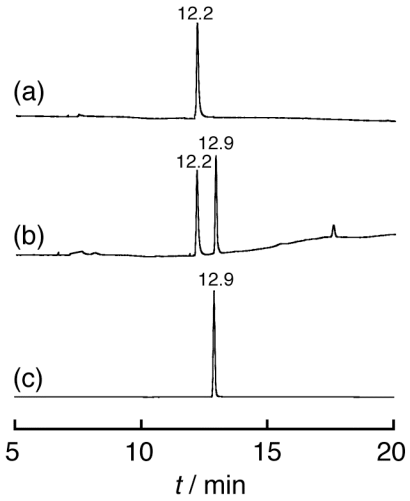


Figure S5. HPLC chromatograms of the reaction mixture of 50 μM RhoNox-1 and 500 μM $FeSO_4$ acquired by LC-MS system. (a) Before addition of $FeSO_4$. (b) Reaction mixture after incubation for 1 h at room temperature. (c) Rhodamine B prepared as the authentic sample. The reaction was carried out in 50 mM HEPES buffer (pH 7.4). The HPLC gradient was as follows: solvent A (0.05% formic acid in water), solvent B (0.05% formic acid in CH_3CN); 5-20 min, 30-95% B. The absorbance at 540 nm was monitored. Total ion mass spectra were simultaneously measured to give mass peak of 459.1 (calcd for $[RhoNox\text{-}1+H]^+ = 459.2$) at 12.2 min and 443.2 (calcd for $[Rhodamine\ B + H]^+ = 443.2$) at 12.9 min. The yield of rhodamine B was evaluated to be around 15%.

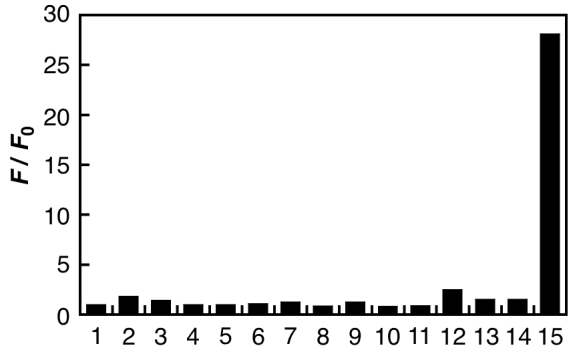


Figure S6. Fluorescence response of RhoNox-1 against various reductants, reactive oxygen species, and chelator. Bars represent relative fluorescence intensities at 575 nm with excitation wavelength at 540 nm. **1**: apo, **2**: 100 μM $Na_2S_2O_3$, **3**: 1 mM sodium ascorbate, **4**: 1 mM cysteine, **5**: 1 mM glutathione, **6**: 100 μM $NaNO_2$, **7**: 100 μM $O_2^{\bullet-}$, **8**: 100 μM H_2O_2, **9**: •OH, **10**: 100 μM NaOCl, **11**: 100 μM NOC-5**, 12**: 20 μM Cu^+ and 1 mM glutathione, **13**: 20 μM Cu^{2+} and 1 mM glutathione, **14**: 100 μM 2,2'-bipyridyl and 20 μM $FeSO_4$, **15**: 20 μM $FeSO_4$. All spectra were acquired with 2 μM RhoNox-1 after 1 h incubation in 50 mM HEPES buffer (pH 7.4) at room temperature.

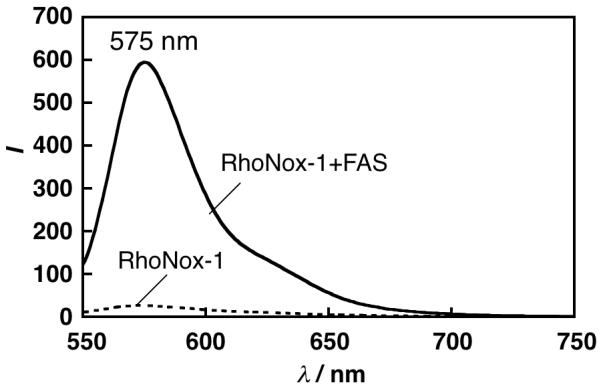


Figure S7. Fluorescence response of RhoNox-1 before (dashed line) and after (solid line) reaction with 20 μM Ferrous Ammonium Sulfate (FAS) at 25 °C for 1 h. Data were acquired at 25 °C in 50 mM HEPES buffer (pH 7.4) with excitation at 540 nm.

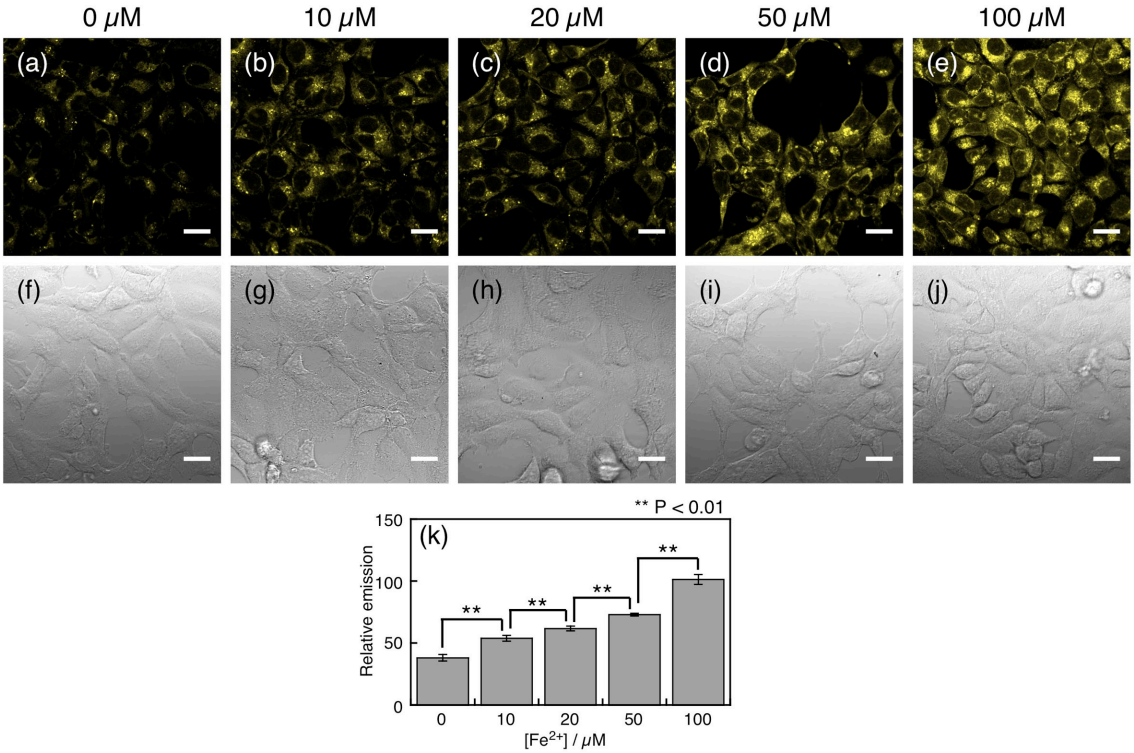


Figure S8. (a)-(e) Fluorescence microscopic images of HepG2 cells supplemented with various concentrations of Fe^{2+} and stained with RhoNox-1. Cells were treated with 0, 10, 20, 50, and 100 μM $Fe(NH_4)_2(SO_4)_2$ for 30 min in MEM(–). After washing the cells, 5 μM RhoNox-1 was added and the cells were incubated for 1 h. Excitation was provided with 555 nm laser. (f)-(j) Bright field images of the same slices of (a)-(e) respectively. (k) Quantification of data in (a)-(e). Statistical analyses were performed with a Student's *t*-test. $^{**}P < 0.01$ (n = 4). Error bars in (k) show ± s.e.m. Scale bars indicate 20 μm.

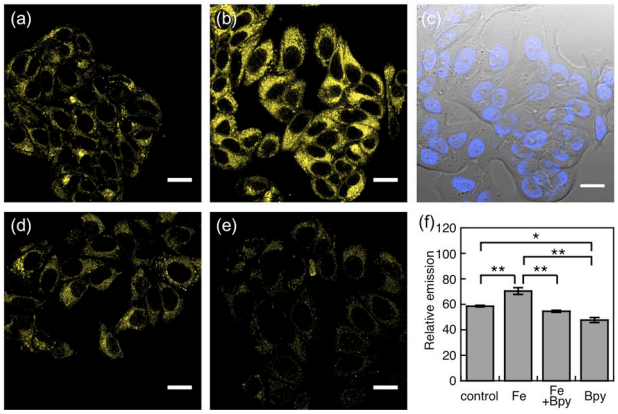


Figure S9. Confocal fluorescence microscopy images for Fe^{2+} detection in MCF-7 cells using RhoNox-1. Ferrous Ammonium Sulfate, $(NH_4)_2Fe(SO_4)_2$ was used for Fe^{2+} source. (a) Image of MCF-7 cells incubated with 5 μM RhoNox-1 at 37 °C for 15 min. (b) Image of MCF-7 cells supplemented with 100 μM Fe^{2+} at 37 °C for 30 min and then treated with 5 μM RhoNox-1 at 37 °C for 15 min. (c) Bright field image of the same slice of (b) overlaid by nuclear staining (Hoechst 33352). (d) Image of MCF-7 cells treated with 100 μM FAS at 37 °C for 30 min, and then 1 mM 2,2'-bipyridyl (Bpy) at 37 °C for 15 min and 5 μM RhoNox-1 at 37 °C for 15 min. (e) Image of MCF-7 cells treated with 1 mM Bpy at 37 °C for 15 min and 5 μM RhoNox-1 at 37 °C for 15 min. (f) Quantification of data in (a), (b), (d), and (e). Statistical analyses were performed with a Student's *t*-test. $^{**}P < 0.01$, and $^{*}P < 0.05$ (n = 4). Error bars in (f) show ± s. e. m. Scale bars indicate 20 μm.

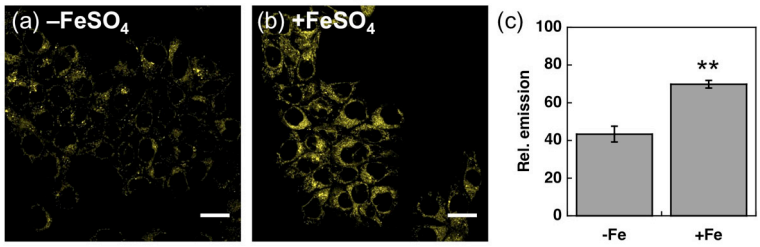


Figure S10. Confocal fluorescence microscopy images for Fe^{2+} detection in HepG2 cells using RhoNox-1. Ferrous Sulfate, $FeSO_4$, was used for the Fe^{2+} source. (a) Image of HepG2 cells incubated with 5 μM RhoNox-1 at 37 °C for 1 h. (b) Image of HepG2 cells supplemented with 100 μM Fe^{2+} at 37 °C for 30 min and then treated with 5 μM RhoNox-1 at 37 °C for 1 h. (c) Quantification of data in (a) and (b). Statistical analyses were performed with a Student's *t*-test. $^{**}P < 0.001$ (n = 4). Error bars in (c) show ± s.e.m. Scale bars indicate 20 μm.

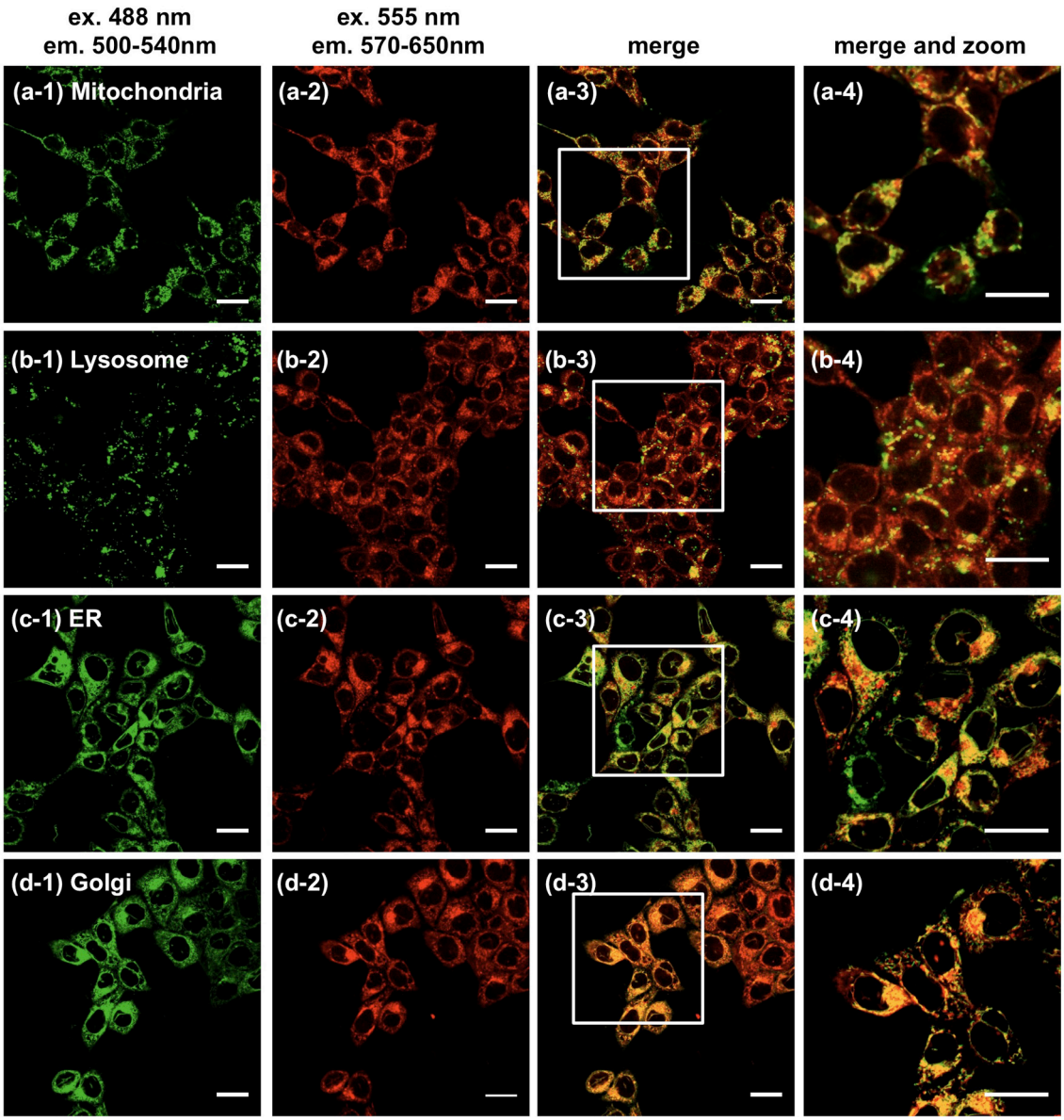


Figure S11. Fluorescence microscopic images of RhoNox-1 (10 μM) treated HepG2 cells co-stained with Mitotracker Green (a), Lysotracker Green (b), ERtracker Green (c), or BODIPY-FL-ceramide BSA complex (golgi, d). (a-1)-(d-1) Images obtained through a band path filter of 500-540 nm with 488 nm excitation (signals from organelle staining dye). (a-2)-(d-2) Images obtained through a band path filter of 570-650 nm with 555 nm excitation (signal from RhoNox-1). (a-3)-(d-3) Merged images of 500-540 nm channels and 570-650 nm channels. The white boxes indicate magnified regions for (a-4)-(d-4). (a-4)-(d-4) Magnified images of 3. Scale bars indicate 20 μm.

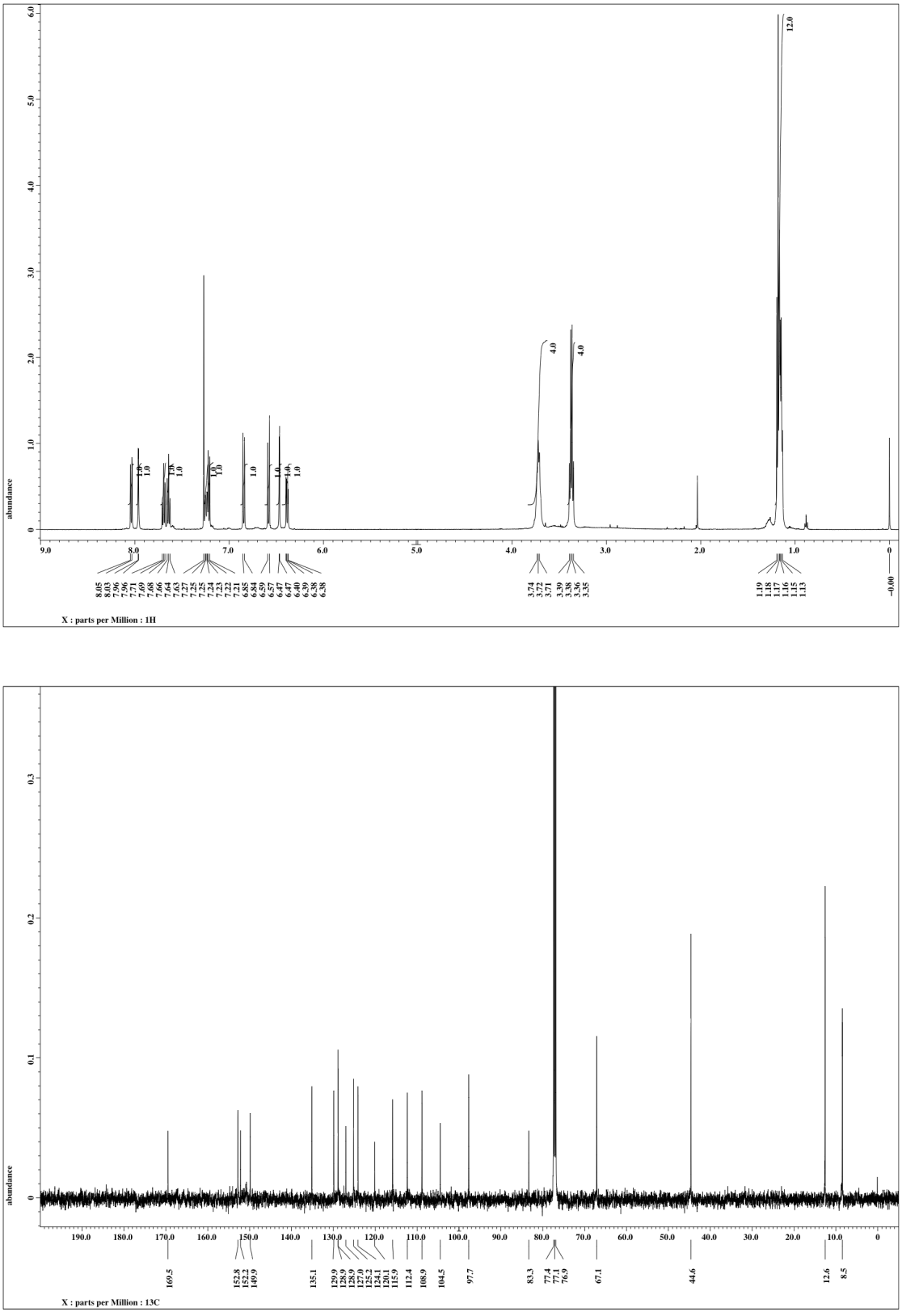


Figure S12. ^{1}H-NMR (upper) and ^{13}C-NMR (lower) spectra of RhoNox-1 in $CDCl_3$.

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