Supporting Information

For

Iron Complex-Based Fluorescent Probes for Intracellular Hydrogen Peroxide Detection

Yutaka Hitomi*, Toshiyuki Takeyasu, and Masahito Kodera

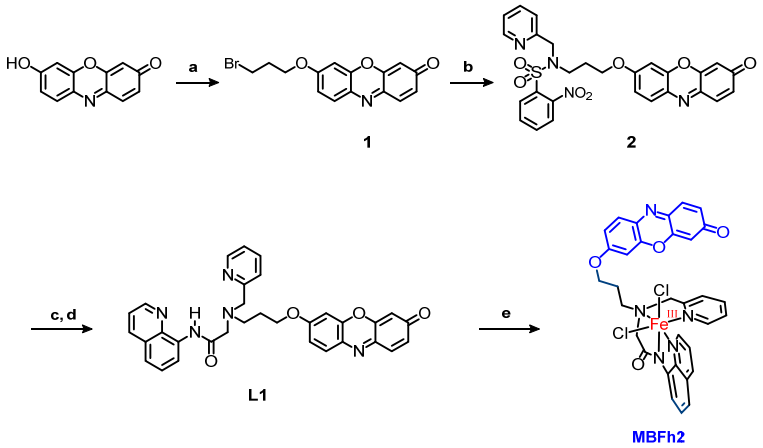
yhitomi@mail.doshisha.ac.p

EXPERIMENTAL SECTION

Materials. The reagents and the solvents used in this study, except the ligand and the iron complex, were commercial products of the highest available purity and were further purified by the standard methods, if necessary. Catalase (Sigma # 038K7005) and epidermal growth factor (Sigma #072M4096V) was purchased from Aldrich, and glucose oxidase (Wako #074-02401) and β-D-glucose (Wako #041-00595) were purchased from Wako Pure Chemicals. Resorufin was purchased from Tokyo Chemical Industry. PF1 was synthesized according to the literature.[S1] HeLa cells and A431 cells were purchased from the RIKEN cell bank.

Characterization. FT-IR spectra were recorded on a Shimadzu IRAffinity-1 spectrometer with a Pike MIRacle10 ATR system (ZnSe). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JMN-A 500 spectrometer. Electrospray ionization mass spectroscopy (ESI-MS) was performed on a JEOL JMS-T100CS spectrometer. UV–visible spectra were taken on an Agilent 8543 UV–visible spectrometer. Fluorescence spectra were taken with λ_{ex} = 570 nm on a Hitachi F-7000 spectrophotometer. HPLC analyses were performed with Shimadzu LC-10AT high-performance liquid chromatography equipped with a reverse phase column (Wako Sil-II 5C18, 4.6 mm × 250 mm) and a photodiode array detector Shimadzu SPD-M10AVP. Elemental analyses were recorded on a Perkin-Elmer Elemental Analyzer (2400 Series II). Microplate assays were carried out with an OPTImax (Molecular Devices). Fluorescence images of cells were taken by an Olympus IX81 fluorescence microscope.

Synthesis.



Scheme S1. Synthetic scheme of MBFh2. Reagents: (a) 1,3-dibromopropane, K_2CO_3, DMF; (b) 2-nitro-*N*-(2-pyridinylmethyl)-benzenesulfonamide, K_2CO_3, MeCN; (c) PhSH, K_2CO_3, DMF; (d) 2-bromo-*N*-8-quinolinylacatamide, K_2CO_3, CH_3CN; (e) $FeCl_3 \cdot 6H_2O$, Et_3N, MeOH/MeCN.

7-(3-bromopropoxy)phenoxazin-3-one (1): To a solution of resorufin (1.00 g, 4.68 mmol) and K_2CO_3 (0.97 g, 7.04 mmol) in DMF (30 mL) was added 1,3-dibromopropane (2.84 g, 14.1 mmol) in one portion under an atmosphere of N_2. The reaction mixture was stirred at 60 °C overnight. After evaporation of the solvent, the resulting brown material was dissolved in CH_2Cl_2 (100 mL). After removal of insoluble materials by Celite filtration, the filtrate was concentrated to give the product as a reddish brown powder, from which desired product was isolated as an reddish orange solid by silica-gel column chromatography (eluent: hexane:AcOEt = 1:1) in 61% yield (0.95 g). 1H NMR ($CDCl_3$, 500 MHz): δ (ppm) = 2.39 (2H, m, $BrCH_2C\mathit{H}_2CH_2O$-), 3.63 (2 H, t, J = 6.3 Hz, $BrC\mathit{H}_2CH_2CH_2O$-), 4.23 (2 H, t, J = 5.7 Hz, $BrCH_2CH_2C\mathit{H}_2O$-), 6.33 (1 H, d, J = 1.8 Hz, $Resf_{H\text{-}6}$), 6.82-6.86 (2 H, m, $Resf_{H\text{-}4}$ and $Resf_{H\text{-}8}$), 6.95 (1 H, dd, J = 9.1, 1.8 Hz, $Resf_{H\text{-}2}$), 7.43 (1 H, d, J = 9.7 Hz, $Resf_{H\text{-}9}$), 7.72 (1 H, d, J = 9.1 Hz, $Resf_{H\text{-}1}$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm) = 29.6, 32.1, 66.5, 100.9, 107.0, 114.1, 128.7, 131.8, 134.5, 134.9, 145.8, 145.9, 150.0, 162.8, 186.5; FT-IR (ATR) 1606.7 cm^{-1} (C=O); ; MS (ESI, pos) *m/z* calcd for $C_{15}H_{12}BrNNaO_3$ $[M + Na]^+$: 356.0. Found: 356.1.

7-[3-{*N*-(2-nitrobenzenesulfonyl)-*N*-(2-pyridyl-methyl)}aminopropoxy]phenoxazin-3-one (2): To an acetonitrile solution (40 ml) containing 7-(3-bromopropoxy)phenoxazin-3-one (421 mg, 1.2 mmol), 2-nitro-*N*-(2-pyridinylmethyl)-benzenesulfonamide (421 mg, 1.4 mmol) and K_2CO_3 (215 mg, 1.6 mmol) was refluxed overnight. After removal of K_2CO_3 by Celite filtration, the filtrate was concentrated to give the product as a reddish brown powder, from which desired product was isolated as an orange solid by silica-gel column chromatography (eluent: hexane:AcOEt = 1:1-0:1) in 70% yield (460 mg); ^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm) = 2.03 (2H, m, $-NCH_2C\mathit{H}_2CH_2O$-),

3.60 (2 H, t, J = 7.4 Hz, -NCH_2CH$_2$CH$_2$O-), 3.98 (2 H, t, J = 5.6 Hz, -NCH$_2$CH$_2$CH_2O-), 4.70 (2 H, s, -CH_2Py), 6.33 (1 H, d, J = 1.9 Hz, Resf$_{H-6}$), 6.65 (1 H, d, J = 2.9 Hz, Resf$_{H-4}$), 6.80 (1 H, dd, J = 9.1, 2.9 Hz, Resf$_{H-2}$), 6.84 (1 H, dd, J = 9.7, 1.9 Hz, Resf$_{H-8}$), 7.20 (1 H, dd, J = 7.2, 4.0 Hz, Py$_{H-5}$), 7.42 (1 H, d, J = 9.7 Hz, Resf$_{H-9}$), 7.45 (1 H, d, J = 7.2 Hz, Py$_{H-4}$), 7.57-7.62 (3 H, m, Ns), 7.65-7.69 (2 H, m, Resf$_{H-1}$ and Py$_{H-3}$), 8.02 (1 H, m, Ns), 8.49 (1 H, d, J = 4.0 Hz, Py$_{H-6}$); ^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm) = 27.5, 45.2, 53.3, 65.9, 100.7, 106.9, 107.0, 114.0, 122.9, 123.1, 124.4, 128.5, 131.2, 131.6, 131.9, 133.4, 133.7, 134.5, 134.9, 137.2, 145.8, 148.2, 148.2, 149.5, 150.0, 156.2, 162.7, 186.5; FT-IR (ATR) 1339 cm^{-1} (sym NO_2), 1537 cm^{-1} (asym NO_2), 1611 cm^{-1} (C=O); MS (ESI, pos) m/z calcd for $C_{27}H_{22}N_4NaO_7S$ $[M + Na]^+$: 569.2. Found: 569.1.

Ligand (**L1**): To a DMF solution (3 ml) containing 2 (80 mg, 0.14 mmol) and K_2CO_3 (30 mg, 0.22 mmol) was added thiophenol (30 μL, 0.28 mmol) under an atmosphere of N_2. The reaction mixture was stirred at room temperature overnight. After removal of insoluble materials by Celite filtration, the filtrate was distilled off under reduced pressure. Reddish oil containing 7-{3-*N*-(2-pyridyl-methyl)aminopropoxy}phenoxazin-3-one was obtained without further purification. The solid was added to an acetonitrile solution (30 mL) containing 2-bromo-*N*-quinolin-8-yl-acetamide 2 (84 mg, 0.32 mmol) and K_2CO_3 (60 mg, 0.43 mmol), and the mixture was stirred for 48 h under an atmosphere of N_2. After removal of K_2CO_3 by Celite filtration, the filtrate was concentrated to give the product as a reddish brown oil, from which desired product was isolated as a reddish brown solid by silica-gel column chromatography (eluent: $CHCl_3$:MeOH:NH_4OH = 50:1:0.1) in 13% yield, 2 steps (10 mg); ^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm) = 2.12 (2H, m, -NCH$_2$CH_2CH$_2$O-), 2.92 (2 H, t, J = 6.3 Hz, -NCH_2CH$_2$CH$_2$O-), 3.47 (2 H, s, -CH_2CO-), 4.00 (2 H, s, -CH_2Py), 4.26 (2 H, t, J = 5.7 Hz, -NCH$_2$CH$_2$CH_2O-), 6.26 (1 H, d, J = 2.1 Hz, Resf$_{H-6}$), 6.45 (1 H, d, J = 2.3 Hz, Resf$_{H-4}$), 6.67 (1 H, dd, J = 9.7, 2.3 Hz, Resf$_{H-2}$), 6.67 (1 H, dd, J = 9.7, 2.1 Hz, Resf$_{H-8}$), 7.14 (1 H, dd, J = 6.9, 5.2 Hz, Py$_{H-5}$), 7.35-7.44 (4 H, m, Resf $_{H-1}$, Resf$_{H-9}$, Qu$_{H-3}$, and Qu$_{H-5}$), 7.48 (1 H, dd, J = 8.3, 8.0 Hz, Qu$_{H-6}$), 7.57 (1 H, dd, J = 8.0, 7.4 Hz, Py$_{H-4}$), 7.96 (1 H, d, J = 8.0 Hz, Py$_{H-3}$), 8.04 (1 H, d, J = 8.0 Hz, Qu$_{H-4}$), 8.53 (1 H, d, J = 5.2 Hz, Py$_{H-6}$), 8.73 (2 H, m, Qu$_{H-2}$ and Qu$_{H-7}$), 11.4 (1 H, s, -N*H*CO); ^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm) = 27.1, 51.4, 60.4, 61.8, 66.2, 100.1, 106.7, 106.8, 114.5, 116.8, 121.7, 121.8, 122.7, 123.6, 127.7, 128.1, 128.2, 131.2, 134.4, 134.9, 136.4, 136.8, 138.8, 145.3, 148.3, 149.4, 150.0, 158.5, 163.0, 170.0, 186.5; FT-IR (ATR) 1593 cm^{-1} (C=O), 1682 cm^{-1} (C=O), 3297 cm^{-1} (N–H); HRMS (ESI, pos) m/z calcd for $C_{32}H_{27}N_5NaO_4$ $[L1 + Na]^+$: 568.1961. Found: 568.1917 (-4.39 mmu).

Synthesis of Iron Complexes $Fe^{III}(L1)Cl_2$ (MBFh2): $FeCl_3 \cdot 6H_2O$ (8.4 mg, 31.2 μmol) was placed in a glass vial, and methanol (500 μL) was added to form a yellow solution. This solution was then added to a second vial containing **L1** (14 mg, 25.6 μmol) in acetonitrile (1 mL). To the mixture was

added triethylamine (4.6 μL, 33.2 μmol). The mixture turned to greenish brown, and was allowed to stand overnight. The resulting brown precipitate was collected, and after vacuum-drying a brown powder was isolated weighting 12 mg (69%); UV–vis (MeOH): 631 nm (1042 M^{-1} cm^{-1}), 461 nm (29255 M^{-1} cm^{-1}), 375 nm (17842 M^{-1} cm^{-1}); HRMS (ESI, pos) *m*/*z* calcd for $C_{33}H_{29}FeN_5O_5$ $[Fe^{III}(\mathbf{L1}) + OMe]^+$: 631.1518. Found: 631.1492 (-2.64 mmu).

Cell culture

The cells cultured in Dulbecco's modified Eagle's medium (DMEM, Wako 048-30275) supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest, S05831S1820), penicillin (100 units/mL), and streptomycin (100 μg/mL) in a humidified incubator under 5% CO_2 in 95% air.

MTT Cell Viability Assay

One or two days before experiment, the cells (5×10^5 cells/mL) were passaged and plated in phenol red-free medium in 96-well plate (Iwaki) and allowed to grow to ~80% confluence. Solutions of MBFh2 in HEPES containing 0.1 g/L $CaCl_2$ and 0.1 g/L $MgCl_2$ were prepared from a 500 μM stock solution in DMSO. The cells were covered in HEPES buffer containing MBFh2 at various concentrations (1-10 μM) or a vehicle control. The cells were then incubated for 3 h at 37 °C at which point 10 μL of the MTT reagent solution (Dojindo) was added to all wells. The cells were incubated at 37 °C and the absorbance intensities at 570 nm were measured using a plate reader after 3 h.

Fluorescence imaging of H_2O_2 in HeLa cells and EGF-induced H_2O_2 in A431 cells.

HeLa or A431 cells were seeded (5×10^5 cells/mL) onto a glass base dish (Iwaki, 3910-035) one or two days before imaging and allowed to grow to ~80% confluence. For EGF stimulus experiment, A431 cells were cultured in DMEM without FBS for a day before the imaging experiment. The medium replaced with HEPES containing 0.1 g/L $CaCl_2$ and 0.1 g/L $MgCl_2$, and then 5 μM MBFh2 was loaded into the cells at 37°C for 10 min, washed three times with HEPES. HeLa cells were exposed to 1 to 50 μM H_2O_2. A431 cells were stimulated with 500 ng/mL EGF. Fluorescence images were taken after 40 min incubation at 37 °C.

Standard curves for quantification of MBFh2 in cells.

HeLa or A431 cells were seeded (5×10^5 cells/mL) in each microplate well of a 96-well plate (Thermo Scientific, 96F non-treated black mirowell) one day before imaging and allowed to grow to ~80% confluence. Cells were incubated with an acetoxymethyl ether variant of resorufin whose concentrations were varied from 0.078 to 5 μM at 37°C for 30 min, washed three times with HEPES. The fluorescent intensity was quantified using a FilerMax F5 (Molecular Devices) with an excitation

filter of 550 nm (BW 20 nm) and an emission filter of 595 nm (BW 35 nm).

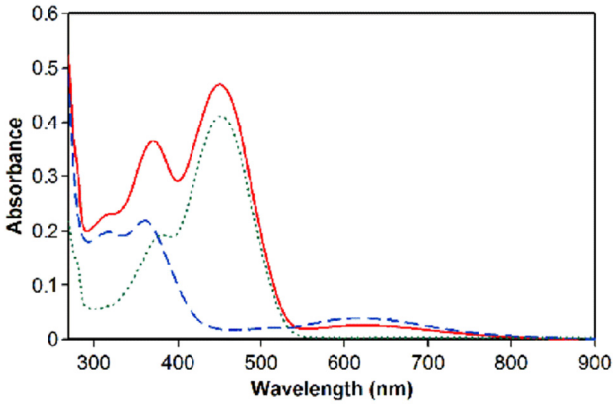


Fig. S1 UV–vis spectra of MBFh2 (red solid), *O*-alkyl resorufin **1** (green dotted line), and $Fe^{III}(mpaq)Cl_2$ (blue dashed line) in MeCN.

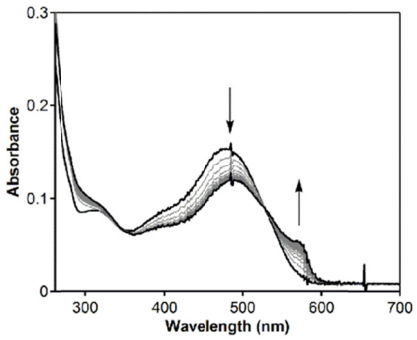


Fig. S2 Absorption spectral changes during the reaction of MBFh2 (10 μM) with 100 equiv. of H_2O_2 in 20 mM HEPES buffer containing 2.5% DMSO (pH 7.2) at 37°C.

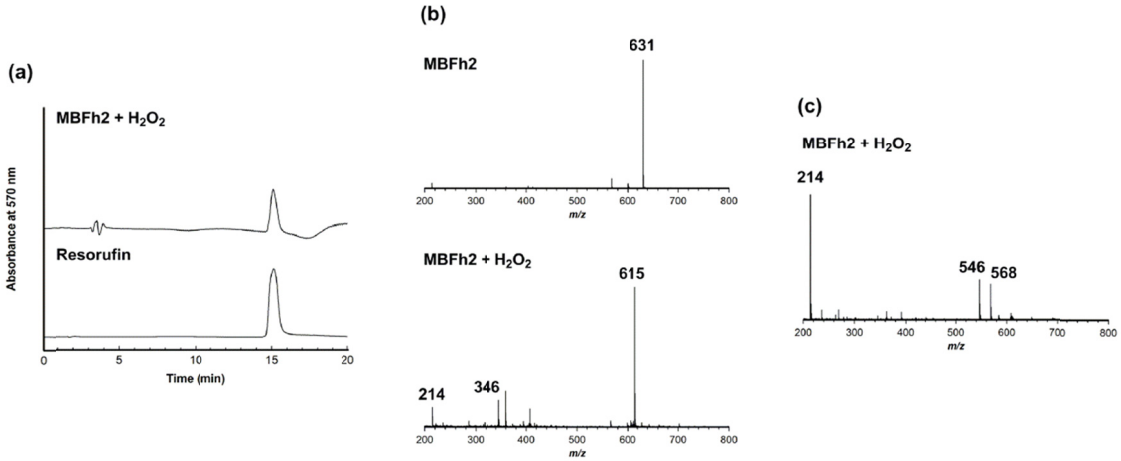


Fig. S3 (a) HPLC chromatograms of the reaction mixture of MBFh2 (50 μM) with 10 equiv. H_2O_2 in

20 mM HEPES buffer containing 2.5% DMSO (pH 7.2) after 30 min (upper) and of resorufin as an authentic sample (lower). A linear gradient (A/B = 90/10 to 10/90 over 7 min) was used (eluent A: water, eluent B: MeOH, flow rate = 1.0 mL/min, monitored at 570 nm). (b) MBFh2 (25 μM) with 1 equiv. H_2O_2 in MeOH containing 150 μM Et_3N after 30 min (lower), and of MBFh2 in MeOH before reaction with H_2O_2 (upper). The signal at *m/z* 631 in the upper panel corresponds to $[Fe^{III}(L1)(OMe)]^+$. The signals at *m/z* 214, 346, and 615 in the lower panel match with monoprotonated resoufin, $\{[Fe^{III}(L)] - H\}^+$, and $\{[Fe^{III}(L1)] + O - H\}^+$, respectively. (c) ESI-TOF MS spectra of the reaction mixture of MBFh2 (25 μM) with 10 equiv. H_2O_2 in 20 mM ammonium acetate solution containing 5% MeOH after 30 min. The signals at *m/z* 214, 546, and 568 in the panel match with monoprotonated resoufin, $[(L1) + H]^+$ and $[(L1) + Na]^+$, respectively.

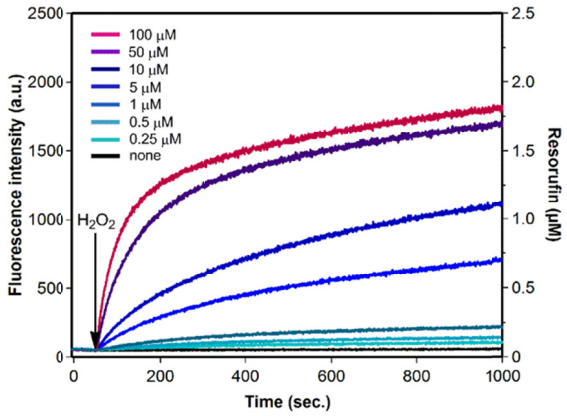


Fig. S4 Fluorescence response curve of MBFh2 upon addition of H_2O_2. Conditions: 20 mM HEPES buffer (pH 7.2, 2.5% DMSO) at 37°C, [MBFh2] = 5 μM. λ_{ex} = 570 nm. λ_{em} = 590 nm.

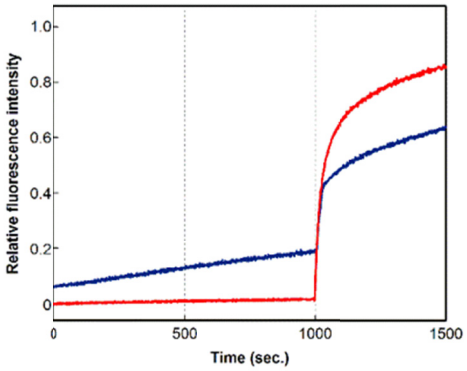


Fig. S5 Time-course of the fluorescence response of MBFh1 (blue) and MBFh2 (red) to H_2O_2 (20 mM HEPES, pH 7.2, at 25 °C). Collected emission was 590 nm (Excitation at 570 nm). H_2O_2 was added at 1000 sec.

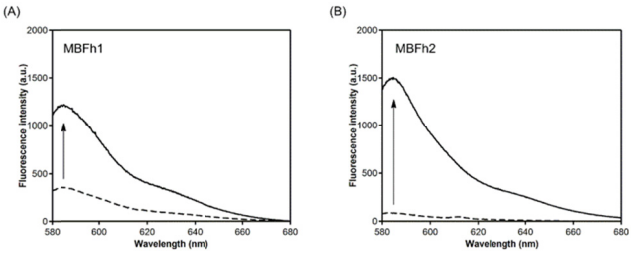


Fig. S6 Changes in fluorescence spectra of MBFh1 (5 μM) (A) and MBFh2 (5 μM) (B) with H_2O_2 (500 μM) in 20 mM HEPES buffer containing 2.5% DMSO (pH 7.2) at 37°C (λ_{ex} = 530 nm).

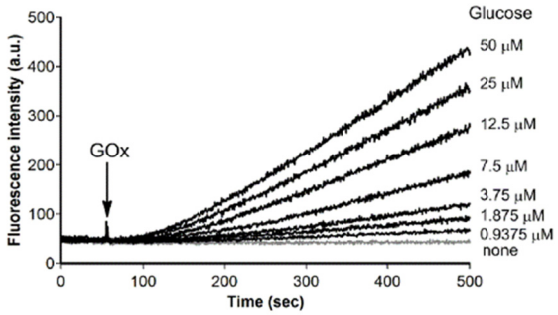


Fig. S7 Fluorogenic detection of enzymatically generated H_2O_2 by MBFh2. Conditions: 20 mM HEPES buffer (pH 7.2, 2.5% DMSO) at 37°C, [MBFh2] = 5 μM, 0-50 μM D-glucose, 8 μg/mL glucose oxidase. Fluorescence intensity (λ_{ex} = 570 nm) was followed at 590 nm.

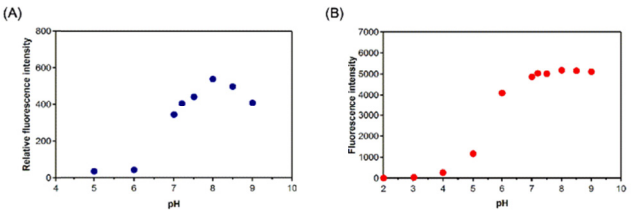


Fig. S8 (A) pH Dependence of the fluorescence intensity caused by the reaction of MBFh2 (5 μM) with H_2O_2 (100 μM). (B) pH Dependence of the fluorescence intensity of resorufin.

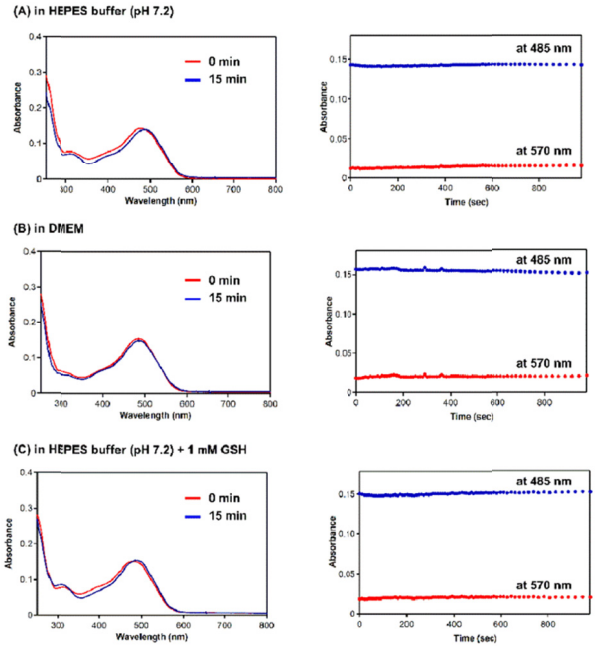


Fig. S9 Absorption spectra of MBFh2 in (A) 20 mM HEPES, pH 7.2, (B) DMEM without phenol red, and (C) 20 mM HEPES (pH 7.2) containing 1 mM GSH at 37°C. Right panels show the time course of absorption intensity at 485 and 570 nm for 15 min.

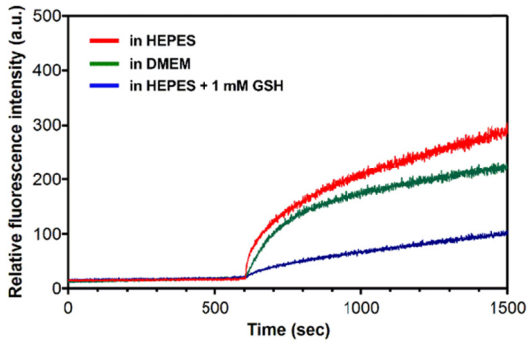


Fig. S10 Stability and reactivity of MBFh2. Time-course of the fluorescence intensity of MBFh2 (5 μM) upon addition of H_2O_2 (500 μM) in 20 mM HEPES, pH 7.2 (red), DMEM without phenol red (green), and 20 mM HEPES (pH 7.2) containing 1 mM GSH (blue) at 37°C. Collected emission was 590 nm (Excitation at 570 nm). H_2O_2 was added at 600 sec.

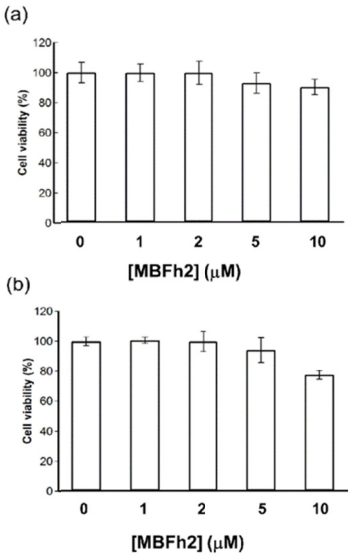


Fig. S11 Metabolic viability of HeLa cells (a) and A431 cells (b) treated with the solution of MBFh2 at varied concentrations. Each bar represents a normalized average of at least three and up to six wells of a 96-well plate.

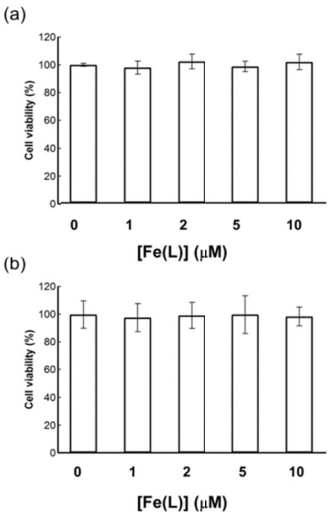


Fig. S12 Metabolic viability of HeLa cells (a) and A431 cells (b) treated with the solution of Fe(L) at varied concentrations. Each bar represents a normalized average of at least three and up to six wells of a 96-well plate.

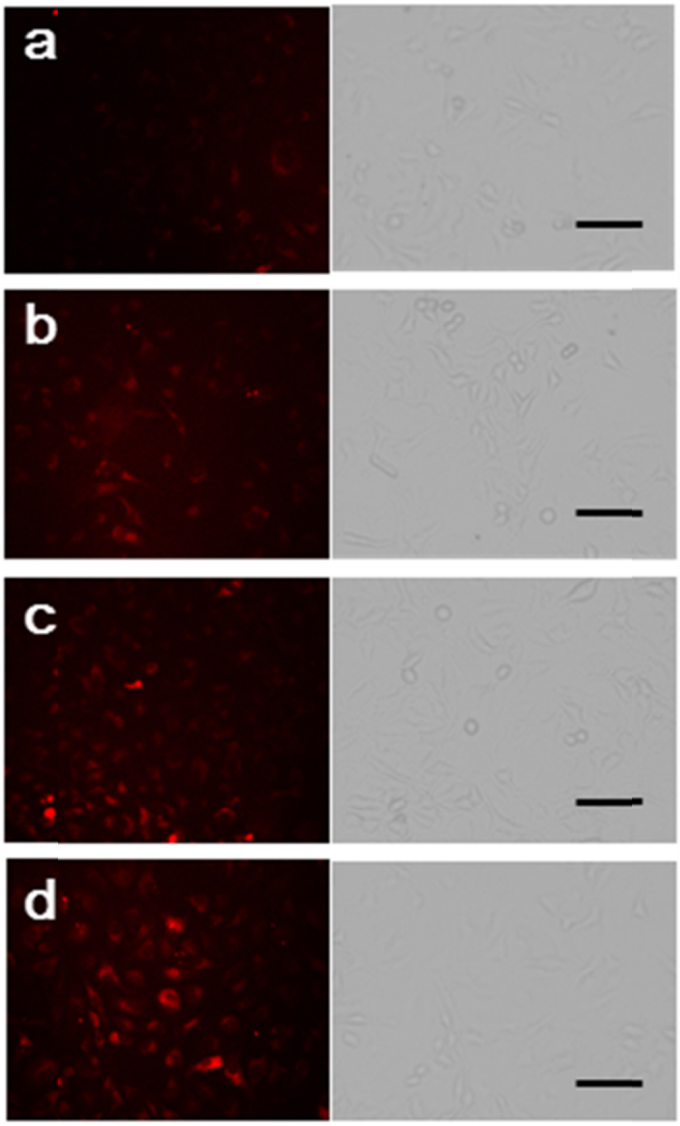


Fig. S13 Live-cell imaging with MBFh2. MBFh2-loaded HeLa cells treated with (a) vehicle control, (b) 1, (c) 10 and (d) 50 μM H_2O_2 for 40 min at 37 °C. Bright-field transmission image of the same cells were shown in right (Scale bar, 100 μm).

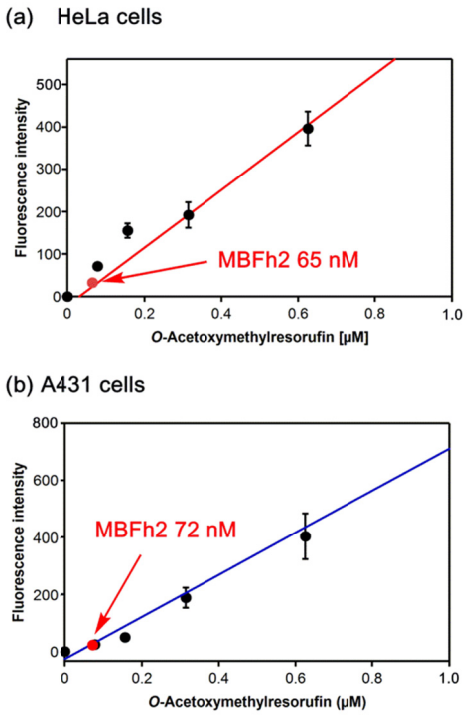


Fig. S14 Quantification of MBFh2 uptaken by cells. Lines indicate standard curves constructed using cell-permeable *O*-acetoxymethylresorufin.

Reference

S1. Chang, M. C. Y.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2004**, *126*, 15392.