

Supporting Information
for
**Development of Highly Sensitive Fluorescent Probes for
Detection of Intracellular Copper(I) in Living Systems**

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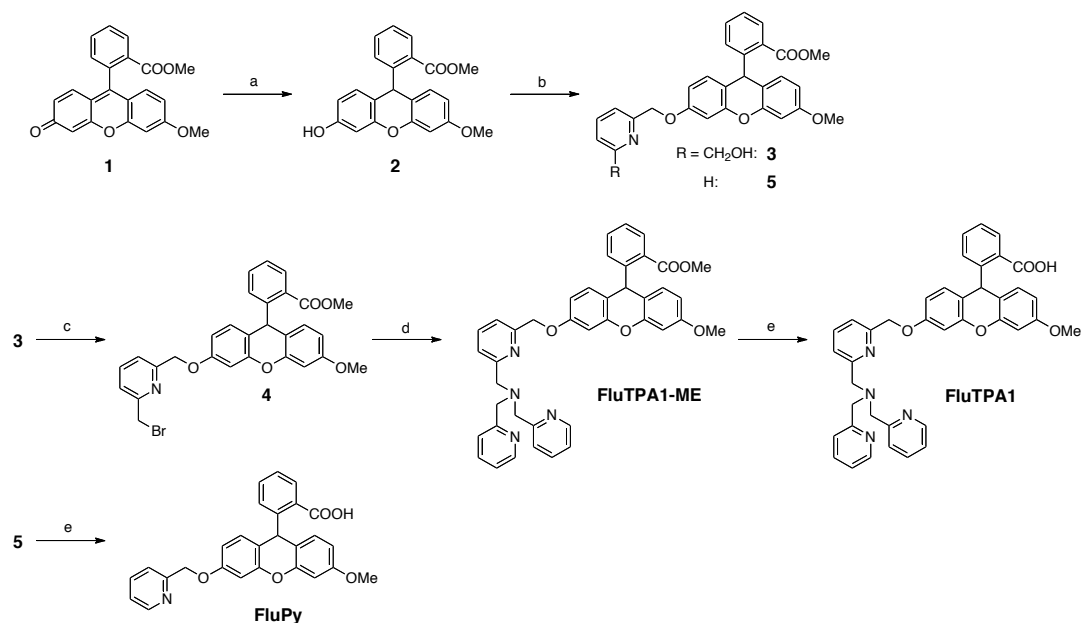
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1. Synthesis

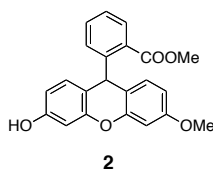
General. All chemicals used in this study were commercial products of the highest available purity and were further purified by the standard methods, if necessary.¹ 2-(6-methoxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid methyl ester² **1**, 6-hydroxy-9-(2-tolyl)-3*H*-xanthen-3-one³ **6**, and bis(2-picolyl)(6-chloromethyl-2-picolyl)amine⁴ **6CITMPA** were prepared according to the published procedures. NMR spectra were recorded on a JEOL JNM-EX-270 (at 270 MHz to ¹H, 68 MHz to ¹³C) or a JEOL alpha-500 (at 500 MHz to ¹H, 126 MHz to ¹³C). Chemical shifts are given in ppm relative to tetramethylsilane (TMS). Electrospray ionization mass spectra (ESI-MS) were recorded with a micrOTOF II focus (Bruker). High-resolution mass spectra (HRMS) were measured with a JMS-DX-300 (JEOL). TLC analyses were performed on Silica gel 60-F₂₅₄ (Merck). Flash chromatography was performed on silica gel (Merck Silica Gel 60). Reverse phase HPLC was performed on Waters Delta 600 system (10 × 250 mm XBridgeTMC₁₈).

Scheme S1. Syntheses of FluTPA1 and FluPy



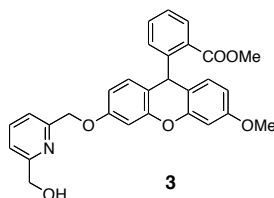
(a) NaBH₄/MeOH, 99 % (b) for **3**: 6-bromomethyl-2-pyridinemethanol, K₂CO₃/MeCN, 94 %; for **5**: 2-(chloromethyl)pyridine hydrochloride, K₂CO₃/MeCN, 72 % (c) PBr₃/CHCl₃, 98 % (d) di(2-picolyl)amine, KI, K₂CO₃/CH₃CN, 93 % (e) NaOH/MeOH-H₂O, for **FluTPA1** 76 %; for **FluPy** 91 %.

2-(3-hydroxy-6-methoxy-9H-xanthen-9-yl)benzoic acid methyl ester, 2.



To a solution of **1** (2.46 g, 6.83 mmol) in MeOH (200 mL) was added NaBH₄ (3.00 g, 8.0 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min, and at room temperature for 2 h. After removal of the solvent, the residue was dissolved in ether (150 mL). The organic layer was then washed with water (100 mL × 3), dried over MgSO₄, and concentrated in vacuo to yield the air-sensitive product **2** as a yellow powder (2.46 g, 99 %). The purity was confirmed by ¹H-NMR and the product was used without further purification for the next step. ¹H-NMR (270 MHz, CDCl₃) δ = 3.77 (s, 3H), 3.94 (s, 3H), 5.63 (s, 1H), 6.14 (s, 1H), 6.42 (dd, 1H, *J* = 8.6, 2.7 Hz), 6.51 (dd, 1H, *J* = 8.6, 2.7 Hz), 6.59 (d, 1H, *J* = 2.7 Hz), 6.63 (d, 1H, *J* = 2.7 Hz), 6.87 (d, 1H, *J* = 8.6 Hz), 6.92 (d, 1H, *J* = 8.6 Hz), 7.09 (dd, 1H, *J* = 7.6, 1.4 Hz), 7.19 (td, 1H, *J* = 7.6, 1.4 Hz), 7.32 (td, 1H, *J* = 7.6, 1.4 Hz), 7.77 (dd, 1H, *J* = 7.6, 1.4 Hz); ¹³C-NMR (126 MHz, CDCl₃) δ = 37.8, 52.4, 55.4, 101.1, 103.0, 110.1, 111.0, 116.8, 116.9, 126.1, 129.3, 129.5, 130.6, 130.8, 131.8, 132.4, 148.2, 151.3, 151.4, 155.3, 159.1, 169.1; ESI-MS *m/z* calcd for C₂₂H₁₉O₅ [M+H]⁺: 362.1; found: 362.1.

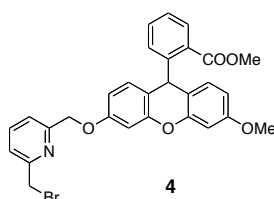
2-[3-(2-hydroxymethyl-6-pyridylmethoxy)-6-methoxy-9H-xanthen-9-yl]benzoic acid methyl ester, 3.



To a solution of **2** (1.96 g, 5.42 mmol) in degassed CH₃CN (70 mL) were added 6-bromomethyl-2-pyridinemethanol (1.09 g, 5.42 mmol) and K₂CO₃ (4.15 g, 30 mmol). The reaction mixture was refluxed under Ar overnight. After removal of the insoluble materials by filtration, the filtrate was evaporated. The residue was dissolved in AcOEt (100 mL), and the organic layer was washed with water (100 mL × 2) and brine (100 mL), dried over MgSO₄, and concentrated. The crude residue was purified by SiO₂ chromatography (AcOEt:hexane = 3:2) to give alcohol **3** as a white solid (2.47 g, 94 %). ¹H-NMR (500 MHz, CDCl₃) δ = 3.77 (s, 3H), 3.80 (brs, 1H), 3.94 (s, 3H), 4.77 (s, 2H), 5.16 (s, 2H), 6.20 (s, 1H), 6.51 (dd, 1H, *J* = 8.7, 2.5 Hz), 6.60 (dd, 1H, *J* = 8.7, 2.5 Hz), 6.64 (d, 1H, *J* = 2.5 Hz), 6.71 (d, 1H, *J* = 2.5 Hz), 6.92 (d, 1H, *J* = 8.7 Hz), 6.95 (d, 1H, *J* = 8.7 Hz), 7.10 (dd, 1H, *J* =

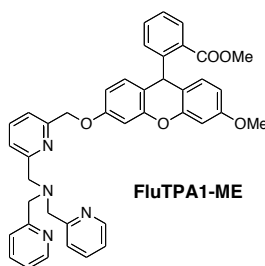
7.8, 1.0 Hz), 7.16 (d, 1H, $J = 7.5$ Hz), 7.18 (td, 1H, $J = 7.8, 1.0$ Hz), 7.31 (td, 1H, $J = 7.8, 1.0$ Hz), 7.39 (d, 1H, $J = 7.5$ Hz), 7.68 (t, 1H, $J = 7.5$ Hz), 7.79 (dd, 1H, $J = 7.8, 1.0$ Hz); $^{13}\text{C-NMR}$ (126 MHz, CDCl_3) $\delta = 37.7, 52.3, 55.3, 64.0, 70.5, 101.0, 102.2, 110.2, 110.6, 116.8, 117.5, 119.3, 119.7, 126.0, 129.3, 129.5, 130.5, 130.7, 131.7, 132.4, 137.5, 148.2, 151.3, 151.4, 156.0, 157.9, 158.5, 159.2, 168.7$; ESI-MS m/z calcd for $\text{C}_{29}\text{H}_{26}\text{N}_1\text{O}_6$ $[\text{M}+\text{H}]^+$: 483.2; found: 483.2.

2-[3-(2-bromomethyl-6-pyridylmethoxy)-6-methoxy-9H-xanthen-9-yl]benzoic acid methyl ester, **4**.



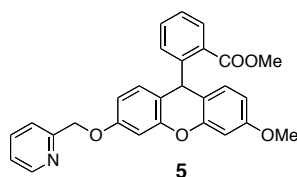
A solution of PBr_3 (325 mg, 1.2 mmol) in CHCl_3 (10 mL) was added dropwise to a solution of alcohol **3** (967 mg, 2.0 mmol) in CHCl_3 (30 mL) at 0°C over a period of 15 min. The mixture was stirred at 0°C for 10 min, then at room temperature for 2 h. Saturated NaHCO_3 aqueous solution (50 mL) was added, and the organic layer was washed with water (100 mL \times 2) and brine (100 mL), dried over MgSO_4 , and concentrated. The crude residue was purified by SiO_2 chromatography (CHCl_3) to give bromide **4** as a pale yellow oil (1.07 g, 98 %). $^1\text{H-NMR}$ (270 MHz, CDCl_3) $\delta = 3.79$ (s, 3H), 3.95 (s, 3H), 4.56 (s, 2H), 5.17 (s, 2H), 6.20 (s, 1H), 6.52 (dd, 1H, $J = 8.6, 2.6$ Hz), 6.60 (dd, 1H, $J = 8.6, 2.6$ Hz), 6.63 (d, 1H, $J = 2.6$ Hz), 6.72 (d, 1H, $J = 2.6$ Hz), 6.91-6.97 (m, 2H), 7.10 (dd, 1H, $J = 7.6, 1.4$ Hz), 7.19 (td, 1H, $J = 7.6, 1.4$ Hz), 7.33 (td, 1H, $J = 7.6, 1.4$ Hz), 7.36 (d, 1H, $J = 7.8$ Hz), 7.45 (d, 1H, $J = 7.8$ Hz), 7.72 (t, 1H, $J = 7.8$ Hz), 7.79 (dd, 1H, $J = 7.6, 1.4$ Hz); $^{13}\text{C-NMR}$ (68 MHz, CDCl_3) $\delta = 32.1, 37.8, 52.4, 55.4, 70.5, 101.1, 103.0, 110.1, 111.0, 116.8, 117.5, 119.4, 119.9, 126.0, 129.2, 129.4, 130.5, 130.7, 131.5, 132.3, 137.2, 148.2, 149.9, 151.4, 156.1, 158.0, 158.5, 159.1, 168.5$; ESI-MS m/z calcd for $\text{C}_{29}\text{H}_{25}^{79}\text{Br}_1\text{N}_1\text{O}_5$ $[\text{M}+\text{H}]^+$: 546.1; found: 546.1, calcd for $\text{C}_{29}\text{H}_{25}^{81}\text{Br}_1\text{N}_1\text{O}_5$ $[\text{M}+\text{H}]^+$: 548.1; found: 548.1.

FluTPA1-ME.



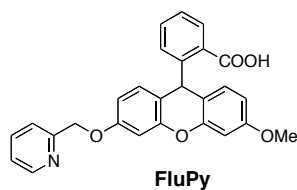
7.9, 0.9 Hz), 6.87 (m, 2H), 7.03 (t, 1H, $J = 7.6$ Hz), 7.08 (t, 1H, $J = 7.6$ Hz), 7.14 (ddd, 2H, $J = 7.6, 4.9, 1.0$ Hz), 7.27 (d, 1H, $J = 7.6$ Hz), 7.36 (d, 1H, $J = 7.6$ Hz), 7.50 (d, 2H, $J = 7.9$ Hz), 7.63 (m, 4H), 8.33 (ddd, 2H, $J = 4.9, 1.8, 0.9$ Hz); ^{13}C -NMR (126 MHz, CD_3OD) $\delta = 38.7, 55.9, 61.0, 61.1, 71.5, 102.1, 103.4, 111.1, 111.9, 118.4, 119.2, 121.6, 123.8, 123.9, 125.0, 126.9, 130.0, 131.8, 131.9, 132.1, 132.4, 133.3, 138.8, 139.0, 149.0, 149.3, 152.8, 152.8, 157.8, 159.4, 159.6, 160.8, 173.1$; HR-FAB-MS m/z calcd for $\text{C}_{40}\text{H}_{35}\text{N}_4\text{O}_5$ $[\text{M}+\text{H}]^+$: 650.2607; found: 650.2613.

2-[3-(2-pyridylmethoxy)-6-methoxy-9H-xanthen-9-yl]benzoic acid methyl ester, **5**.



This compound was prepared according to the same procedure for the synthesis of alcohol **3** by using 2-(chloromethyl)pyridine hydrochloride (98 mg, 0.6 mmol) in place of 6-bromomethyl-2-pyridinemethanol. Yield: 72 %; ^1H -NMR (500 MHz, CDCl_3) $\delta = 3.77$ (s, 3H), 3.94 (s, 3H), 5.18 (s, 2H), 6.20 (s, 1H), 6.52 (dd, 1H, $J = 8.5, 2.4$ Hz), 6.61 (dd, 1H, $J = 8.5, 2.4$ Hz), 6.64 (d, 1H, $J = 2.4$ Hz), 6.73 (d, 1H, $J = 2.4$ Hz), 6.92 (d, 1H, $J = 8.5$ Hz), 6.95 (d, 1H, $J = 8.5$ Hz), 7.10 (dd, 1H, $J = 7.6, 1.2$ Hz), 7.16-7.21 (m, 2H), 7.31 (td, 1H, $J = 7.6, 1.2$ Hz), 7.50 (d, 1H, $J = 7.6$ Hz), 7.69 (td, 1H, $J = 7.6, 1.8$ Hz), 7.78 (dd, 1H, $J = 7.6, 1.2$ Hz), 8.58 (ddd, 1H, $J = 4.8, 1.8, 0.9$ Hz); ESI-MS m/z calcd for $\text{C}_{28}\text{H}_{24}\text{N}_1\text{O}_5$ $[\text{M}+\text{H}]^+$: 454.2; found: 454.2.

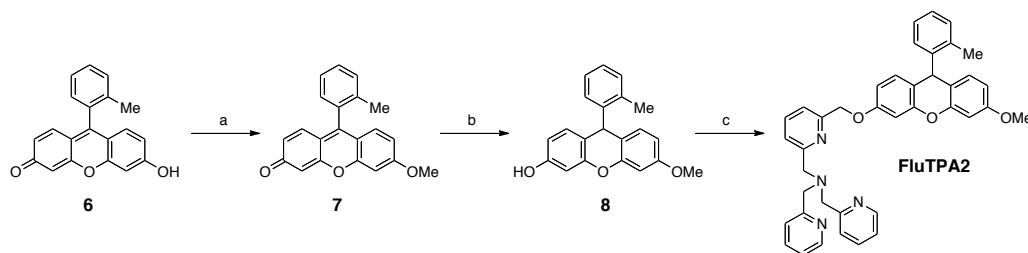
FluPy.



This compound was prepared according to the same procedure for the synthesis of **FluTPA1** by using compound **5** (45.3 mg, 0.1 mmol). Yield: 91 %; ^1H -NMR (500 MHz, CD_3OD) $\delta = 3.67$ (s, 3H), 5.06 (s, 2H), 5.88 (s, 1H), 6.44 (dd, 1H, $J = 8.5, 2.4$ Hz), 6.52 (d, 1H, $J = 2.4$ Hz), 6.54 (dd, 1H, $J = 8.5, 2.4$ Hz), 6.61 (d, 1H, $J = 2.4$ Hz), 6.68-6.70 (m, 1H), 6.93-6.97 (m, 2H), 7.10 (d, 1H, $J = 8.5$ Hz), 7.14 (d, 1H, $J = 8.5$ Hz), 7.26 (dd, 1H, $J = 7.9, 5.0$ Hz), 7.32-7.34 (m, 1H), 7.49 (d, 1H, $J = 7.9$ Hz), 7.76 (td, 1H, $J = 7.9, 1.5$ Hz), 8.44 (ddd, 1H, $J = 7.8, 1.5, 0.9$ Hz); ^{13}C -NMR (126 MHz, CD_3OD) $\delta = 39.5, 55.8, 71.4,$

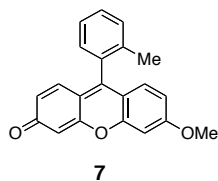
101.9, 103.1, 110.9, 111.6, 119.0, 119.9, 123.3, 124.4, 126.5, 127.7, 129.3, 130.7, 132.3, 132.5, 139.0, 141.7, 145.8, 149.8, 152.9, 152.9, 158.3, 159.2, 160.7, 178.7; HR-FAB-MS m/z calcd for $C_{27}H_{22}N_1O_5$ $[M+H]^+$: 440.1498; found: 440.1492.

Scheme S2. Synthesis of FluTPA2



(a) MeI, K₂CO₃/MeCN, 78 % (b) NaBH₄/MeOH (c) **6CITMPA**, K₂CO₃/MeCN, 52 % (for 2 steps).

6-methoxy-9-(2-tolyl)-3H-xanthen-3-one, **7**.



A mixture of **6** (83 mg, 0.27 mmol), iodomethane (72 mg, 0.5 mmol), and K₂CO₃ (276 mg, 2 mmol) in acetonitrile (10 mL) was refluxed overnight. After evaporation, the residue was dissolved in AcOEt (50 mL), and the organic layer was washed water (30 mL × 2) and brine (30 mL), dried over MgSO₄, and concentrated. The residue was purified by SiO₂ chromatography (CHCl₃:MeOH = 19:1) to give methylated compound **7** as a red-brown solid (67 mg, 78 %). ¹H-NMR (270 MHz, CDCl₃) δ = 2.07 (s, 3H), 3.93 (s, 3H), 6.46 (d, 1H, J = 2.0 Hz), 6.57 (dd, 1H, J = 9.9, 2.0 Hz), 6.77 (dd, 1H, J = 9.1, 2.4 Hz), 6.94 (d, 1H, J = 9.9 Hz), 6.96 (d, 1H, J = 2.4 Hz), 6.98 (d, 1H, J = 9.1 Hz), 7.15-7.18 (m, 1H), 7.34-7.49 (m, 3H).

mixtures were vortexed and kept in dark at room temperature for 3 h. All fluorescence spectra were measured with an excitation wavelength at 470 nm.

3. Product Analysis

To a 1 μM solution of FluTPA1 in 50 mM HEPES buffer (pH 7.20) containing 2 mM GSH was added a 20 μM solution of $[\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4]\text{PF}_6$ in CH_3CN under aerobic conditions. The mixture was stirred for 3 h at room temperature and analyzed with a reverse phase HPLC (Waters Delta 600 system, 4.6×150 mm, XBridgeTMC₁₈) eluted with $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1 % NH_3). The retention time was compared with that of an authentic sample 3'-O-methylfluorescein (OMF). The yield of the production of OMF was calculated from the integrated areas of the emission for OMF to be 70 ± 2 %. For ESI-MS analysis, a 10 μM solution of $[\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4]\text{PF}_6$ in CH_3CN or CuSO_4 in water was added to a 2 μM solution of FluTPA1 in 10 mM ammonium acetate containing 100 μM GSH under aerobic conditions. The mixture was stirred for 2 h at room temperature. The samples were directly measured using micrOTOF II focus (Bruker).

4. Cell Culture Experiments

Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS, Gibco) and 1% Antibiotic-Antimycotic (Gibco) at 37 °C in a 5% $\text{CO}_2/95\%$ air incubator. For fluorescence imaging, cells (5×10^4) were passed on a glass-bottomed dish coated with poly-L-lysine (MW: 7,000-15,000) and incubated for 4 days. Copper uptake experiments were performed in the same medium except the overnight supplementation of 200 μM CuCl_2 at the end of the incubation period noted above. Immediately before the staining experiments, cells were washed twice with PBS buffer containing 200 μM EDTA, incubated with 5 μM 2Me-FITPA (in DMEM) for 3 h at 37 °C, washed twice with PBS, and then the dish was filled with 1.5 mL of HBS for fluorescence imaging. The cells were imaged with a FLUOVIEW confocal laser-scanning microscope (OLYMPUS) with Alexa Fluor 488 channel (488 nm excitation, 500-600 nm emission filter).

5. References

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- (2) Mugerli, L.; Burchak, O. N.; Chatelain, F.; Balakirev, M. Y. *Bioorg. Med. Chem. Lett.* **2006**, *16*,

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- (3) Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 4888-4894.
- (4) Lucchese, B.; Humphreys, K. J.; Lee, D. H.; Incarvito, C. D.; Sommer, R. D.; Rheingold, A. L.; Karlin, K. D. *Inorg. Chem.* **2004**, *43*, 5987-5998.

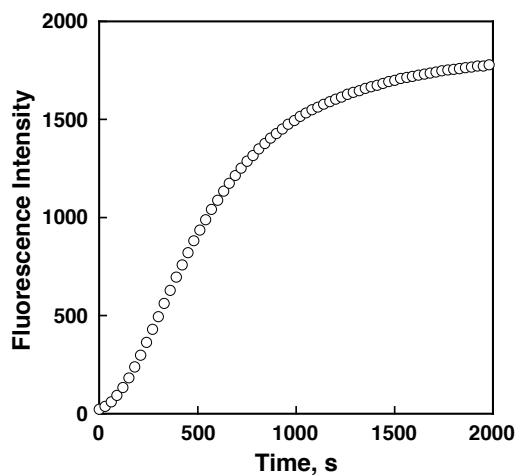


Figure S1. Time course of the fluorescence intensity change at 510 nm ($\lambda_{\text{ex}} = 470$ nm) in the reaction of FluTPA1 (1 μM) and Cu^{I} (20 μM) in 50 mM HEPES (pH 7.20) containing 2 mM glutathione (GSH).

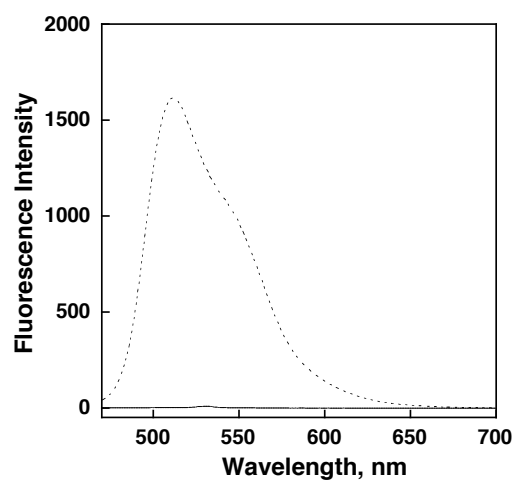


Figure S2. (a) Fluorescence response of 1 μM FluPy (solid line) and FluTPA1 (dotted line) to 20 μM $[\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4]\text{PF}_6$. These spectra were recorded after 2 h reaction with Cu^{I} in 50 mM HEPES (pH 7.20) containing 2 mM GSH. The excitation wavelength was 470 nm.

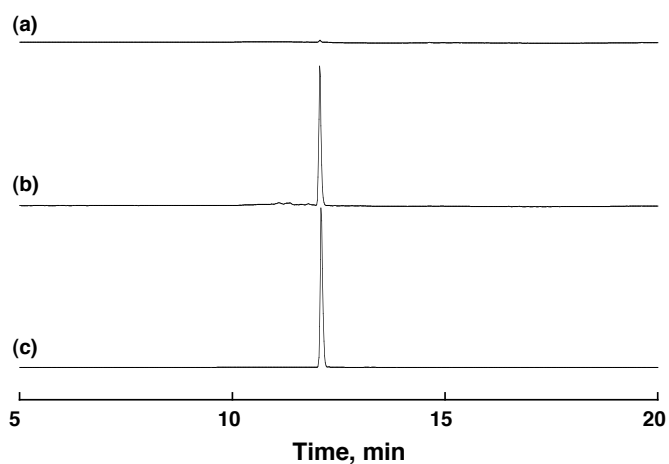


Figure S3. HPLC chromatograms of the reaction mixture of FluTPA1 and $[\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4]\text{PF}_6$. (a) Before addition of $[\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4]\text{PF}_6$. (b) Reaction mixture after incubation for 2 h at room temperature. (c) 3'-O-Methylfluorescein (OMFP) prepared as the authentic sample. The reaction was carried out in 50 mM HEPES (pH 7.20) containing 2 mM GSH. The HPLC gradient was as follows: solvent A (0.1 % NH_3 in water), solvent B (0.1 % NH_3 and 0.2 % water in CH_3CN); 5-20 min, 0-100% B. The peaks were monitored by UV absorbance at 450 nm.

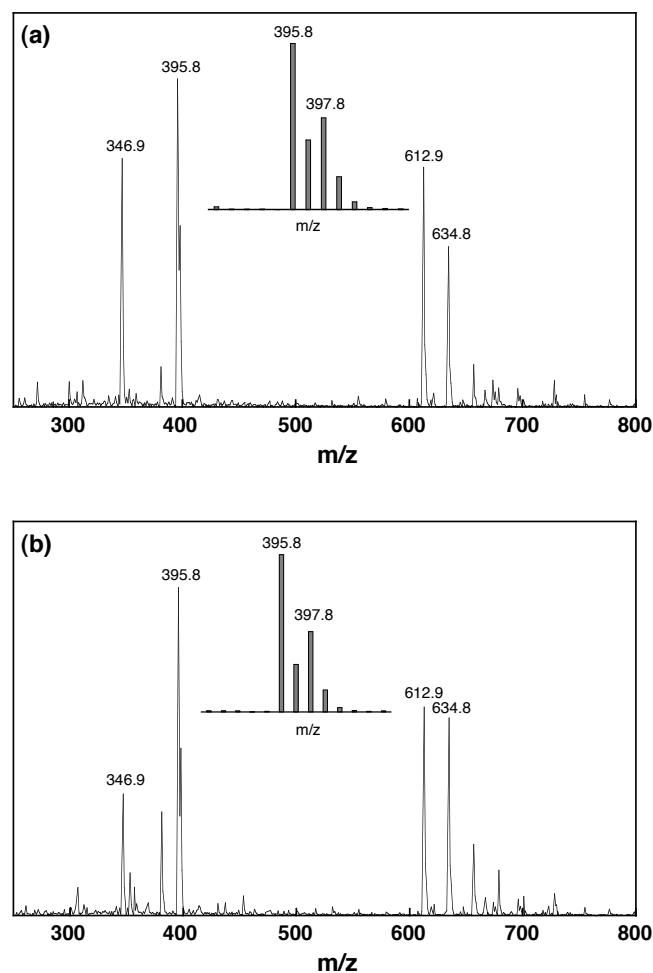


Figure S4. ESI mass spectra (positive ion mode) for the reaction of 2 μM FluTPA1 (a) with $[\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4]\text{PF}_6$ (10 μM) and (b) with CuSO_4 (10 μM) in 10 mM NH_4OAc in the presence of 100 μM GSH. The incubation time was 2 h. Inset: expansion of ESI-MS chromatogram in the range between 391 and 403. Mass peak observed at 346.9 ($[\text{M} + \text{H}]^+$) was attributed to 3'-*O*-methylfluorescein, and peaks at 612.9 ($[\text{M} + \text{Na}]^+$) and 634.8 ($[\text{M} + \text{K}]^+$) were attributed to the oxidized form of GSH (GS-SG).

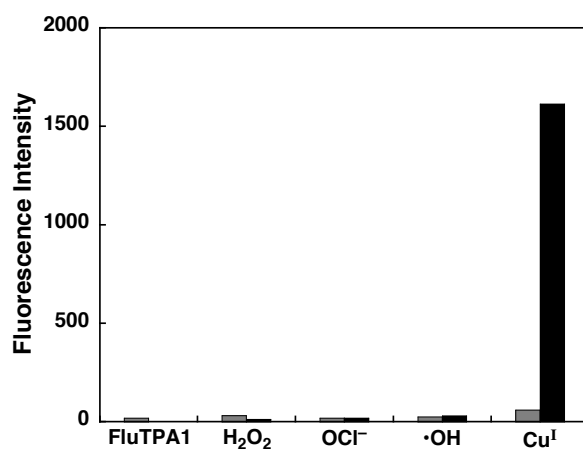


Figure S5. Fluorescence response of 1 μM FluTPA1 to H_2O_2 , hypochloride (OCl^-), hydroxyl radical ($\bullet\text{OH}$), and Cu^{I} in 50 mM HEPES (pH 7.20). Gray and black bars represent the fluorescence intensities at 513 nm observed in the absence and in the presence of 2 mM GSH, respectively. All spectra were obtained after incubation at room temperature for 2 h. Reaction conditions are as follows. H_2O_2 : 0.1 M H_2O_2 was added to give a final concentration of 300 μM . OCl^- : 0.3 mM NaOCl was added to give a final concentration of 300 μM . $\bullet\text{OH}$: 0.1 M H_2O_2 (200 μM of the final concentration) was added to the probe solution containing 200 μM EDTA and 20 μM $\text{Fe}(\text{OAc})_2$.

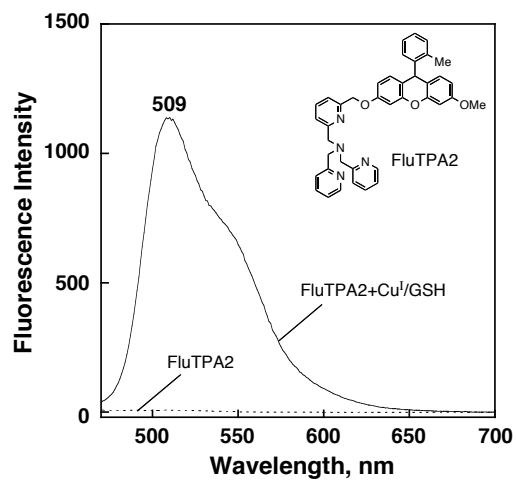


Figure S6. Fluorescence response of 1 μM FluTPA2 (dotted line) to 20 μM $[\text{Cu}^{\text{I}}(\text{CH}_3\text{CN})_4]\text{PF}_6$. A solid spectrum was recorded after a 2 h reaction of FluTPA2 with Cu^{I} in 50 mM HEPES (pH 7.20) containing 2 mM glutathione (GSH). The excitation wavelength was 470 nm.

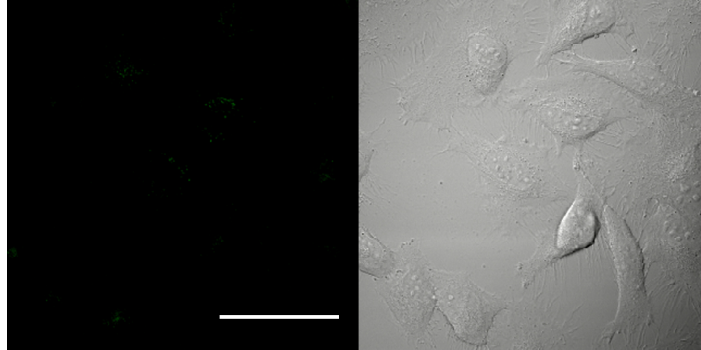


Figure S7. Confocal fluorescence images of HeLa cells incubated with 5 μM FluTPA2 for 30 min at 37 $^{\circ}\text{C}$ followed by addition of 50 μM H_2O_2 then further incubated for 3 h at 37 $^{\circ}\text{C}$. Bright-field transmission image of the same cells is shown in right. Scale bar indicates 50 μm .

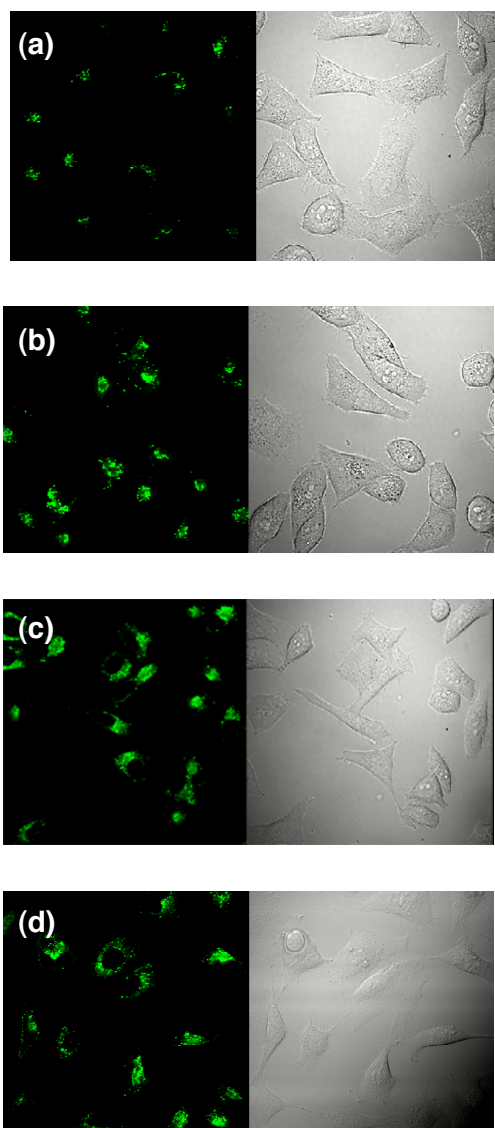


Figure S8. Confocal fluorescence images of HeLa cells supplemented with (a) 20 μM , (b) 50 μM , (c) 100 μM , and (c) 200 μM of CuCl_2 for 8 h. Before imaging, cells were incubated with 5 μM FluTPA2 for 3 h at 37 $^\circ\text{C}$. Bright-field transmission image of the same cells were shown in right.