Supporting Information

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SI Materials and Methods

Commercial Reagents for Synthesis of Dyes. All reactions using air- or moisture-sensitive reagents were performed in oven-dried glassware under an atmosphere of dry N_2. THF used for anhydrous reactions were dried and stored over 4-Å molecular sieves. Other reagents were used without further purification. Silica gel P60 (SiliCycle) was used for column chromatography and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical TLC and visualized with UV light and/or staining with *p*-anisaldehyde. Bis(2-((2-(ethylthio)ethyl)thio)ethyl)amine (1) and 3,6-bis(trifluoromethanesulfonyl)-xanthone (2) were synthesized according to literature procedures. All other reagents were purchased from Sigma-Aldrich. ^{1}H NMR and ^{13}C NMR spectra were collected in $CDCl_3$ or MeOD (Cambridge Isotope Laboratories) at 25 °C on Bruker AVQ-400 or Bruker AVB-400 spectrometers at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard δ notation of parts per million using the peak of residual proton signals of $CDCl_3$ or MeOD as an internal reference. Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets. Low-resolution electrospray ionization mass spectrometry (LRESI-MS) were carried out using a liquid chromatography mass spectrometer (Agilent Technology 6130, Quadrupole LC/MS). High-resolution electrospray ionization mass spectrometry (HRESI-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

3-(Pyrrolidin-1-yl)-6-trifluoromethanesulfonyl-xanthone (2). A solution of 3,6-bis(trifluoromethanesulfonyl)-xanthone, **1** (1.0 g, 2.03 mmol), in DMSO (5 mL) was preheated to 85 °C and treated with pyrrolidine (133.4 μL, 1.62 mmol). After 2 h, the reaction was cooled to room temperature, poured into brine (30 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography (1:2 vol/vol EtOAc/hexanes) to afford the titled compound, **2**, as an off-white solid (455 mg, 70% yield). ^{1}H NMR (400 MHz, 1:1 vol/vol MeOD:$CDCl_3$): δ 8.34 (d, J = 8.0 Hz, 1H), 8.04 (d, J = 8.0 Hz, 1H), 7.86 (s, 1H), 7.56 (d, J = 1.3 Hz, 1H), 7.37 (dd, J = 8.0, 2.2 Hz, 1H), 6.76 (dd, J = 8.0, 1.2 Hz, 1H), 6.51 (d, J = 2.2 Hz, 1H), 3.47 (t, J = 5.0 Hz, 4H), 2.10 (t, J = 5.0 Hz, 4H). ^{13}C NMR (100 MHz, 1:1 vol/vol MeOD:$CDCl_3$): δ 173.97, 158.46, 156.32, 155.66, 152.13, 128.88, 127.85, 121.99, 116.44, 111.88, 111.76, 110.65, 98.58, 48.23, 25.18, 24.22. LRESI-MS calculated for $[M+H]^+$ 398.07, found 398.2.

3-((*tert*-Butyldimethylsilyl)oxy)-6-(pyrrolidin-1-yl)-9H-xanthen-9-one (3). A suspension of **2** (455 mg, 1.10 mmol) in 1,4-dioxane (20 mL) was treated with a solution of Et_4NOH in MeOH (1.5 M, 1.47 mL, 2.20 mmol). The resultant solution was stirred at room temperature for 2 h. After the reaction was complete, as judged by TLC, the volatiles were removed under reduced pressure to afford a yellow solid, which was treated with imidazole (748.9 mg, 11 mmol) and CH_2Cl_2 (30 mL). The solution was cooled to 0 °C, then treated with *tert*-butyldimethylsilyl chloride (497 mg, 3.3 mmol) and stirred at room temperature for 3 h. The volatiles were removed under reduced pressure, and the crude residue was purified via flash chromatography using an EtOAc/hexanes gradient (1:5–1:4 vol/vol EtOAc/hexanes) to afford compound **3** as a yellow solid (323.4 mg, 74.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (d, J = 8.8 Hz, 1H), 8.14 (d, J = 9.2 Hz, 1H), 6.90 (dd, J = 8.8, 2.4 Hz, 1H), 6.85 (dd, J = 8.8, 2.4 Hz, 1H), 6.83 (d, J = 2.0 Hz, 1H), 6.68 (d, J = 2.0 Hz, 1H), 3.42 (m, 4H), 1.69 (m, 4H), 1.04 (s, 9H), 0.30 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.27, 160.87, 158.47, 157.65, 155.48, 127.99, 127.75, 117.08, 116.79, 112.56, 111.63, 107.29, 99.32, 25.73, 25.61, 25.30, 24.37, 18.29, −4.32. LRESI-MS calculated for $[M+H]^+$ 396.20, found 396.2.

4-Bromo-3-(methylphenyl)methanol (5). A solution of methyl 4-bromo-3-methylbenzoate **4** (10.0 g, 43.6 mmol) in anhydrous THF (200 mL) was cooled to 0 °C in an ice bath. After addition of $LiAlH_4$ (2.1 g, 54.5 mmol) in portions, the reaction was warmed to room temperature and stirred for 2 h. Then 1 M NaOH (50 mL) was added and the solution was extracted with EtOAc (3 × 100 mL). The combined organic layers were dried (Na_2SO_4) and concentrated to afford compound **5** as a colorless liquid (9.35 g, 100% yield). ^{1}H NMR, (400 MHz, $CDCl_3$); δ 2.36 (s, 3H), 3.36 (s, 1H), 4.48 (s, 2H), 6.94 (d, 1H, J = 7.6 Hz), 7.13 (s, 1H), 7.45 (d, 1H, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$); δ 22.8, 64.1, 123.7, 125.8, 129.3, 132.3, 137.8, 140.0. LRESI-MS calculated for $[M^+]$ 199.98, found 200.2.

1-Bromo-4-(chloromethyl)-2-methylbenzene (6). Thionyl chloride (1.5 mL, 19.9 mmol) was added to a solution of **5** (2.0 g, 9.9 mmol) in CH_2Cl_2 (50 mL) at room temperature. After overnight stirring, the reaction was concentrated and purified by flash column chromatography (1:9 vol/vol EtOAc/hexanes) to afford compound **6** as a yellow liquid (1.99 g, 91% yield), which was used without further purification.

***N*-(4-Bromo-3-methylbenzyl)-3,6,12,15-tetrathia-9-monoazaheptadecane (8).** A solution of **6** (1.75 g, 8.0 mmol), bis(2-((2-(ethylthio)ethyl) thio)ethyl)amine **7** (5.0 g, 16.0 mmol), and K_2CO_3 (2.2 g, 16.0 mmol) in MeCN (50 mL) was stirred overnight at reflux. The volatiles were removed under reduced pressure, and the residue was dissolved in CH_2Cl_2. The solution was washed with water (2 × 50 mL), dried (Na_2SO_4), and concentrated. Purification by flash column chromatography (1:7 vol/vol EtOAc/hexanes) afforded compound **8** as a clear oil (1.25 g, 45%). ^{1}H NMR, (400 MHz, $CDCl_3$): δ 1.21 (t, 6H, J = 7.4 Hz), 2.35 (s, 3H), 2.50 (q, 4H, J = 7.4 Hz), 2.64 (m, 16H), 3.52 (s, 2H, 7.00 (d, 1H, J = 8.0 Hz), 7.17 (s, 1H), 7.41 (d, 1H, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$); δ 14.8, 22.9, 26.0, 30.0, 31.7, 32.4, 57.8, 57.9, 123.3, 127.6, 131.0, 132.1, 137.6, 138.4. LRESI-MS calculated for $[M+H]^+$ 496.08, found 496.1.

***N*-(4-Bromo-3-methylbenzyl)-*N*-octyloctan-1-amine (14).** A solution of **6** (875 mg, 4.0 mmol), dioctylamine **13** (1.9 g, 8.0 mmol), and K_2CO_3 (1.1 g, 8.0 mmol) in MeCN (25 mL) was stirred overnight at reflux. The volatiles were removed under reduced pressure, and the residue was dissolved in CH_2Cl_2. The solution was washed with water (2 × 50 mL), dried (Na_2SO_4), and concentrated. Purification by flash column chromatography (1:7 vol/vol EtOAc/hexanes) afforded compound **7** as a clear oil (1.315 g, 77% yield). ^{1}H NMR (400 MHz, $CDCl_3$): 7.47 (d, J = 8 Hz, 1H), 7.24 (s, 1H), 7.05 (d, J = 8 Hz, 1H), 3.49 (s, 2H), 2.43–2.40 (m, 7H), 1.48–1.46 (m, 4H), 1.36–1.29 (m, 20H), 0.93 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 137.35, 131.92, 131.25, 127.81, 58.01, 53.84, 31.90, 29.55, 29.36, 27.47, 26.95, 22.86, 22.70, 14.13. LRESI-MS calculated for $[M+H]^+$ 424.26, found 424.4.

CR3. An oven-dried, two-neck round-bottom flask was charged with **7** (62.8, 0.13 mmol) and anhydrous THF (1 mL). The solution

was cooled to −78 °C and treated with a solution of *tert*-BuLi in pentane (1.7 M, 74 μL, 0.13 mmol) under a N_2 atmosphere. After stirring at the same temperature for 30 min, a solution of compound **3** (25 mg, 0.06 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was warmed to room temperature, stirred for a 1 h, and then quenched with aqueous (aq.) HCl (2 M, 2 mL). After 2 h, the reaction was poured into saturated (sat.) $NaHCO_3$ (30 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography using a MeOH/CH_2Cl_2 gradient (1:49–1:19 vol/vol MeOH/CH_2Cl_2) to afford Copper Rhodol-3 (CR3) as a red solid (27 mg, 62.9% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (m, 2H), 7.12 (d, J = 7.6 Hz, 1H), 6.93 (s, 1H), 6.91 (s, 1H), 6.61–6.49 (m, 4H), 3.73 (s, 2H), 3.45 (m, 4H), 2.84–2.82 (m, 4H), 2.74 (m, 12H), 2.57 (q, J = 7.2 Hz, 4H), 2.10 (m, 4H), 2.06 (s, 3H), 1.26 (t, J = 7.6 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 136.22, 130.54, 129.14, 126.11, 105.07, 99.74, 98.31, 97.08, 96.22, 93.62, 58.17, 53.91, 48.09, 32.44, 31.76, 30.05, 26.06, 25.35, 19.63, 14.80. HRESI-MS calculated for $C_{37}H_{49}N_2O_2S_4$ $[M+H]^+$ *m/z* 681.2671, found 681.2663.

Ctrl-CR3. An oven-dried two-neck round-bottom flask was charged with **8** (30 mg, 0.06 mmol) and anhydrous THF (1 mL). The solution was cooled to −78 °C and treated with a solution of *tert*-BuLi in pentane (1.7 M, 35 μL, 0.06 mmol) under a N_2 atmosphere. After stirring at the same temperature for 30 min, a solution of compound **3** (10 mg, 0.03 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was warmed to room temperature, stirred for 1 h, and then quenched with aq. HCl (2 M, 2 mL). After 2 h, the reaction was poured into sat. $NaHCO_3$ (30 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography using a MeOH/CH_2Cl_2 gradient (1:49–1:19 vol/vol MeOH/CH_2Cl_2) to afford **Ctrl-CR3** as a red solid (5.5 mg, 35% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (m, 2H), 7.10 (d, J = 7.2 Hz, 1H), 6.91 (s, 1H), 6.09 (s, 1H), 6.55–6.51 (m, 2H), 6.46–6.43 (m, 2H), 3.61 (s, 2H), 3.44 (m, 4H), 2.48 (m, 4H), 2.10 (m, 4H), 2.06 (s, 3H), 1.26 (m, 24H), 0.88 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 136.22, 130.53, 129.12, 126.08, 105.07, 99.75, 98.31, 97.08, 96.20, 93.62, 58.20, 53.80, 31.85, 29.50, 29.30, 27.42, 26.90, 22.88, 22.68, 14.08. HRESI-MS calculated for $C_{41}H_{57}N_2O_2$ $[M+H]^+$ *m/z* 609.4415, found 609.4409.

(4-Bromo-3-(trifluoromethyl)phenyl)methanol (10). A solution of methyl 4-bromo-3-(trifluoromethyl)benzoate **9** (6.5 g, 22.9 mmol) in anhydrous THF (50 mL) was treated with $LiAlH_4$ (1.04 g, 27.4 mmol). The suspension was warmed to room temperature and stirred for 2.5 h. Water (10 mL), 1 M NaOH (10 mL), and water (10 mL) were added sequentially to the reaction. Lithium salts were removed via filtration, and the filtrate was diluted with EtOAc (50 mL) and washed with brine (3 × 25 mL). The organic layer was dried (Na_2SO_4) and concentrated to afford compound **10** as a clear syrup (5.86 g, 100% yield), which was used without further purification.

1-Bromo-4-(chloromethyl)-2-(trifluoromethyl)benzene (11). A solution of compound **10** (1.5 g, 5.88 mmol) in thionyl chloride (20 mL, 275 mmol) was refluxed for 16 h. After cooling to room temperature, the solution was slowly added to cold sat. $NaHCO_3$ (250 mL) and extracted with CH_2Cl_2 (100 × 50 mL). The combed organic fractions were dried (Na_2SO_4) and concentrated under reduced pressure. The crude residue was purified via flash chromatography (1:9 vol/vol EtOAc/hexanes) to afford compound **11** as a light-brown liquid (801 mg, 49.9% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71–7.66 (m, 2H), 7.39 (dd, J = 10.8, 2.4 Hz, 1H), 4.55 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 137.22, 135.41, 132.97, 127.98, 127.93, 127.88, 126.83, 124.11, 121.39, 119.86, 44.50. LRESI-MS calculated for $[M+H]^+$ 272.93, found 273.1.

***N*-(4-Bromo-3-(trifluoromethyl)benzyl)-2-((2-(ethylthio)ethyl)thio)-*N*-(2-((2-(ethylthio)ethyl)thio)ethyl)ethan-1-amine (12).** A round-bottom flask charged with compound **11** (307.2 mg, 1.12 mmol), bis(2-((2-(ethylthio)ethyl)thio)ethyl)amine **7** (702.5 mg, 2.24 mmol), KI (371.8 mg, 2.24 mmol), K_2CO_3 (464.4 mg, 3.36 mmol), and MeCN (10 mL) was stirred at 55 °C for 16 h, cooled to room temperature, and concentrated. The crude residue was treated with water (50 mL) and extracted with EtOAc (3 × 100 mL). The combined organic layers were dried (Na_2SO_4), concentrated, and purified via flash chromatography (1:8 vol/vol EtOAc/hexanes) to afford compound **12** as a light-brown syrup (411 mg, 66.6% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.70 (d, J = 2 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.42 (d, J = 8.4 Hz, 1H), 3.63 (s, 2H), 2.73–2.62 (m, 16H), 2.53 (q, J = 7.6 Hz, 4H), 1.24 (t, J = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 139.46, 134.83, 133.01, 130.03, 129.72, 127.85, 127.80, 124.33, 121.61, 118.20, 57.57, 53.75, 32.52, 32.46, 32.32, 32.29, 31.74, 30.24, 30.14, 26.03, 14.82. LRESI-MS calculated for $[M+H]^+$ 550.05, found 550.2.

***N*-(4-Bromo-3-(trifluoromethyl)benzyl)-*N*-octyloctan-1-amine (15).** A round-bottom flask charged with compound **11** (327.7 mg, 1.20 mmol), dioctylamine **13** (579.5 mg, 2.40 mmol), KI (398.4 mg, 2.40 mmol), K_2CO_3 (497.5 mg, 3.60 mmol), and MeCN (10 mL) was stirred at 55 °C for 16 h, cooled to room temperature, and concentrated. The crude residue was treated with water (50 mL) and extracted with EtOAc (3 × 100 mL). The combined organic layers were dried (Na_2SO_4), concentrated, and purified via flash chromatography (1:19 vol/vol EtOAc/hexanes) to afford compound **15** as a yellow syrup (544.7 mg, 82.4% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (m, 1H), 7.66 (d, J = 8.4 Hz, 1H), 7.40 (d, J = 7.2 Hz, 1H), 3.56 (s, 2H), 2.42 (t, J = 7.2 Hz, 4H), 1.48 (m, 4H), 1.34–1.29 (m, 20H), 0.92 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 134.57, 133.01, 127.95, 57.82, 53.94, 31.86, 29.52, 29.32, 27.40, 27.06, 22.68, 14.10. LRESI-MS calculated for $[M+H]^+$ 478.23, found 478.3.

Copper Fluor-3, CF3. An oven-dried two-neck round-bottom flask was charged with **12** (53 mg, 0.10 mmol) and anhydrous THF (1 mL). The solution was cooled to −78 °C and treated with a solution of *tert*-BuLi in pentane (1.7 M, 55 μL, 0.10 mmol) under a N_2 atmosphere. After stirring at the same temperature for 10 min, a solution of compound **3** (25 mg, 0.06 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was warmed to room temperature, stirred for a further 1 h, and then quenched with aq. HCl (2 M, 2 mL). After 2 h, the reaction was poured into sat. $NaHCO_3$ (30 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography using MeOH/CH_2Cl_2 (1:19 vol/vol MeOH/CH_2Cl_2) to afford **CF3** as a red solid (11.7 mg, 25.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.92 (1H), 7.74 (d, J = 8 Hz, 1H), 7.28 (m, 1H), 6.77 (d, J = 9.2 Hz, 2H), 6.60–6.56 (m, 2H), 6.50–6.48 (m, 2H), 3.83 (s, 2H), 3.44 (m, 4H), 2.85–2.81 (m, 4H), 2.75–2.70 (m, 12H), 2.58 (q, J = 7.6 Hz, 4H), 2.09 (m, 4H), 1.26 (t, J = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.12, 155.70, 152.52, 142.14, 132.11, 131.36, 130.98, 130.85, 130.22, 129.68, 126.88, 116.11, 112.00, 111.58, 105.44, 97.32, 58.17, 54.20, 48.49, 32.89, 32.71, 32.66, 32.12, 32.08, 30.66, 30.57, 30.02, 26.44, 25.69, 15.14. HRESI-MS calculated for $C_{37}H_{46}F_3N_2O_2S_4$ $[M+H]^+$ *m/z* 735.2389, found 735.2385.

Control Copper Fluor-3, Ctrl-CF3. An oven-dried two-neck round-bottom flask was charged with **13** (50 mg, 0.11 mmol) and anhydrous THF (1 mL). The solution was cooled to −78 °C

and treated with a solution of *tert*-BuLi in pentane (1.7 M, 57 μL, 0.11 mmol) under a nitrogen atmosphere. After stirring at the same temperature for 10 min, a solution of compound **3** (25 mg, 0.06 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was warmed to room temperature, stirred for a further 1 h, and then quenched with aq. HCl (2 M, 2 mL). After 2 h, the reaction was poured into sat. $NaHCO_3$ (30 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography using MeOH/CH_2Cl_2 (1:19 vol/vol MeOH/CH_2Cl_2) to afford **Ctrl-CF3** as a red solid (13.8 mg, 34.7% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (1H), 7.78 (m, 1H), 7.30 (m, 1H), 6.7 (m, 2H), 6.61–6.58 (m, 2H), 6.5–6.48 (m, 2H), 3.79 (s, 2H), 3.47 (m, 4H), 2.57 (m, 4H), 2.13–2.10 (m, 4H), 1.58–1.33 (m, 24H), 0.91 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.21, 155.75, 152.56, 142.34, 132.21, 131.56, 130.90, 130.83, 130.22, 129.88, 126.83, 116.31, 111.99, 111.68, 105.14, 97.62, 58.36, 48.09, 31.84, 29.29, 25.40, 22.67, 14.11. HRESI-MS calculated for $C_{41}H_{54}F_3N_2O_2$ $[M+H]^+$ *m/z* 663.4132, found 663.4126.

3-(Morpholino)-6-trifluoromethanesulfonyl-xanthone (16). A solution of 3,6-bis(trifluoromethanesulfonyl)-xanthone, **1** (1.0 g, 2.03 mmol), in DMSO (10 mL) was preheated to 85 °C and treated with morpholine (140.0 μL, 1.62 mmol). After 2 h, the reaction was cooled to room temperature, poured into brine (30 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography (1:1 vol/vol EtOAc/hexanes) to afford the titled compound, **16**, as an off-white solid (276 mg, 32% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.32 (d, J = 8.8 Hz, 1H), 8.07 (d, J = 8.8 Hz, 1H), 7.29 (d, J = 2.0 Hz, 1H), 7.19 (dd, J = 8.8, 1.6 Hz, 1H), 6.84 (dd, J = 8.8, 1.6 Hz, 1H), 6.61 (s, 1H), 3.84 (t, J = 4.4 Hz, 4H), 3.34 (t, J = 4.4 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.17, 158.12, 156.33, 155.84, 152.27, 129.00, 127.90, 121.88, 116.69, 113.11, 111.68, 110.79, 99.34, 66.29, 47.02. LRESI-MS calculated for $[M+H]^+$ 429.05, found 429.2.

3-((*tert*-Butyldimethylsilyl)oxy)-6-(morpholino)-9H-xanthen-9-one (17). A suspension of **16** (276 mg, 0.64 mmol) in 1,4-dioxane (6 mL) was treated with a solution of Et_4NOH in MeOH (1.5 M, 856 μL, 1.28 mmol). The resultant solution was stirred at room temperature for 2 h. After the reaction was complete, as judged by TLC, the volatiles were removed under reduced pressure to afford a yellow solid, which was treated with imidazole (408 mg, 6.0 mmol) and CH_2Cl_2 (20 mL). The solution was cooled to 0 °C, then treated with *tert*-butyldimethylsilyl chloride (290.3 mg, 1.93 mmol) and stirred at room temperature for 1 h. The volatiles were removed under reduced pressure, and the crude residue was purified via flash chromatography using an EtOAc/hexanes gradient (1:5–1:4 vol/vol EtOAc/hexanes) to afford compound **17** as a yellow solid (35.7 mg, 13.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (t, J = 8.4 Hz, 2H), 6.92 (dd, J = 9.2, 2.4 Hz, 1H), 6.86 (m, 2H), 6.74 (d, J = 2.4 Hz, 1H), 3.91 (t, J = 4.8 Hz, 4H), 3.40 (t, J = 4.8 Hz, 4H), 1.05 (s, 9H), 0.32 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.43, 161.13, 158.19, 157.69, 155.50, 128.09, 127.88, 117.32, 116.70, 113.89, 111.39, 107.34, 99.96, 66.52, 47.51, 25.60, 18.30, -4.31. LRESI-MS calculated for $[M+H]^+$ 411.19, found 411.2.

CR1. An oven-dried, two-neck round-bottom flask was charged with **7** (86.2, 0.17 mmol) and anhydrous THF (1 mL). The solution was cooled to −78 °C and treated with a solution of *tert*-BuLi in pentane (1.7 M, 102 μL, 0.17 mmol) under a N_2 atmosphere. After stirring at the same temperature for 30 min, a solution of compound **17** (35.7 mg, 0.09 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was warmed to room temperature, stirred for a 1 h, and then quenched with aq. HCl (2 M, 2 mL). After 2 h, the reaction was poured into sat. $NaHCO_3$ (30 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography using MeOH/CH_2Cl_2 (1:19 vol/vol MeOH/CH_2Cl_2) to afford **CR1** as a red solid (32.7 mg, 54.1% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (m, 1H), 7.34 (m, 2H), 7.11 (d, J = 7.6 Hz, 1H), 6.93–6.73 (m, 4H), 6.59 (d, J = 9.6 Hz, 1H), 6.52 (s, 1H), 3.74 (s, 2H), 3.49 (s, 4H), 2.82–2.74 (m, 20H), 2.58 (m, 4H), 2.07 (s, 3H), 1.26 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.13, 155.74, 154.70, 140.48, 136.22, 131.65, 130.57, 129.68, 129.13, 128.12, 126.12, 115.90, 111.86, 111.53, 105.17, 98.80, 60.34 25.33, 24.25, 19.65, 14.78, 14.15. HRESI-MS calculated for $C_{37}H_{49}N_2O_2S_4$ $[M+H]^+$ *m/z* 696.2548, found 696.2812.

3-(Bis(2-methoxyethyl)amino)-6-trifluoromethanesulfonyl-xanthone (18). A solution of 3,6-bis(trifluoromethanesulfonyl)-xanthone, **1** (1.0 g, 2.03 mmol) in DMSO (3 mL) was preheated to 90 °C and treated with bis(2-methoxyethyl)amine (300 μL, 2.03 mmol). After overnight stirring, the reaction was cooled to room temperature, poured into brine (30 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography (1:1 vol/vol EtOAc/hexanes) to afford the titled compound, **18**, as a yellow solid (253.9 mg, 26.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.35 (d, J = 8.8 Hz, 1H), 8.07 (d, J = 9.2 Hz, 1H), 7.33 (d, J = 2 Hz, 1H), 7.21 (dd, J = 8.8, 2.0 Hz, 1H), 6.77 (dd, J = 8.8, 2.0 Hz, 1H), 6.55 (d, J = 2.0 Hz, 1H), 3.71–3.61 (m, 8H), 3.38 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.01, 158.47, 156.38, 153.78, 152.18, 128.95, 128.11, 122.06, 116.51, 111.40, 110.68, 110.32, 96.93, 69.88, 59.10, 51.22. LRESI-MS calculated for $[M+H]^+$ 475.09, found 475.2.

3-((*tert*-Butyldimethylsilyl)oxy)-6-(bis(2-methoxyethyl)amino)-9H-xanthen-9-one (19). A suspension of **18** (253.9 mg, 0.53 mmol) in 1,4-dioxane (10 mL) was treated with a solution of Et_4NOH in MeOH (1.5 M, 712 μL, 1.07 mmol). The resultant solution was stirred at room temperature for 1.5 h. After the reaction was complete, as judged by TLC, the volatiles were removed under reduced pressure to afford a yellow solid, which was treated with imidazole (182 mg, 2.67 mmol) and CH_2Cl_2 (15 mL). The solution was cooled to 0 °C, then treated with *tert*-butyldimethylsilyl chloride (241.5 mg, 1.6 mmol) and stirred at room temperature for 1 h. The volatiles were removed under reduced pressure, and the crude residue was purified via flash chromatography using EtOAc/hexanes (1:1 vol/vol EtOAc/hexanes) to afford compound **19** as a yellow syrup (219 mg, 89.6% yield). ^{1}H NMR (400 MHz, 1:1 vol/vol MeOD:$CDCl_3$): δ 8.22–8.14 (m, 2H), 6.97–6.92 (m, 3H), 6.70 (d, J = 8.4 Hz, 1H), 3.81 (s, 4H), 3.75 (s, 4H), 3.51 (s, 6H), 1.14–1.03 (m, 9H), 0.19 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): 162.71, 158.10, 157.68, 152.79, 127.16, 127.03, 126.99, 116.71, 112.64, 110.60, 109.37, 109.17, 106.71, 101.67, 96.40, 69.32, 58.16, 50.45, 24.01, 24.78, 24.71, −4.82, −5.33. LRESI-MS calculated for $[M+H]^+$ 457.23, found 457.3.

CR2. An oven-dried, two-neck round-bottom flask was charged with **7** (81.4, 0.16 mmol) and anhydrous THF (1 mL). The solution was cooled to −78 °C and treated with a solution of *tert*-BuLi in pentane (1.7 M, 96.4 μL, 0.16 mmol) under a N_2 atmosphere. After stirring at the same temperature for 30 min, a solution of compound **19** (25 mg, 0.05 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was warmed to room temperature, stirred for a 1 h, and then quenched with aq. HCl (2 M, 2 mL). After 2 h, the reaction was poured into sat. $NaHCO_3$ (30 mL) and extracted with EtOAc (3 × 10 mL). The

combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography using MeOH/CH_2Cl_2 (1:19 vol/vol MeOH/CH_2Cl_2) to afford **CR2** as a red solid (23.2 mg, 57.8% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.31 (m, 2H), 7.08 (d, J = 10.4 Hz, 1H), 6.90 (dd, J = 12.4, 5.2 Hz, 2H), 6.64–6.57 (m, 3H), 6.50 (s, 1H), 3.70–3.59 (m, 10H), 3.34 (s, 6H), 2.80–2.72 (m, 14H), 2.55 (q, 9.6 Hz, 4H), 2.05 (s, 3H), 1.24 (t, J = 9.6H, 8H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.10, 163.63, 159.93, 158.39, 156.07, 155.79, 154.33, 141.17, 135.94, 131.37, 131.08, 130.85, 130.14, 128.87, 127.68, 127.19, 126.36, 115.56, 113.46, 112.46, 112.40, 111.44, 104.28, 102.08, 99.59, 98.66, 66.33, 66.16, 58.10, 53.91, 32.14, 31.60, 29.75, 25.68, 19.08, 14.26. HRESI-MS calculated for $C_{37}H_{49}N_2O_2S_4$ $[M+H]^+$ m/z 742.2966, found 742.2817.

3-(Piperidyl)-6-trifluoromethanesulfonyl-xanthone (20). A solution of 3,6-bis(trifluoromethanesulfonyl)-xanthone, **1** (1.0 g, 2.03 mmol) in DMSO (5 mL) was preheated to 85 °C and treated with piperidine (160.4 μL, 1.62 mmol). After 16 h, the reaction was cooled to room temperature, poured into brine (30 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography (1:3 vol/vol EtOAc/hexanes) to afford the titled compound, **20**, as a yellow solid (336.4 mg, 78% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.31 (d, J = 8.8 Hz, 1H), 8.02 (d, J = 8.8 Hz, 1H), 7.28 (d, J = 2.0 Hz, 1H), 7.16 (dd, J = 8.8, 1.6 Hz, 1H), 6.81 (dd, J = 8.8, 1.6 Hz, 1H), 6.56 (d, J = 2.0 Hz, 1H), 3.39 (1, 4H), 1.66 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.97, 158.46, 156.32, 155.66, 152.13, 128.88, 127.85, 121.99, 116.44, 111.88, 111.76, 110.65, 98.58, 48.23, 25.18, 24.22. LRESI-MS calculated for $[M+H]^+$ 427.07, found 427.1.

3-((*tert*-Butyldimethylsilyl)oxy)-6-(piperidyl)-9H-xanthen-9-one (21). A suspension of **20** (336.4 mg, 0.78 mmol) in 1,4-dioxane (10 mL) was treated with a solution of Et_4NOH in MeOH (1.5 M, 1.04 mL, 1.57 mmol). The resultant solution was stirred at room temperature for 2 h. After the reaction was complete, as judged by TLC, the volatiles were removed under reduced pressure to afford a yellow solid, which was treated with imidazole (370 mg, 5.44 mmol) and CH_2Cl_2 (25 mL). The solution was cooled to 0 °C, then treated with *tert*-butyldimethylsilyl chloride (250.0 mg, 1.66 mmol) and stirred at room temperature for 1 h. The volatiles were removed under reduced pressure, and the crude residue was purified via flash chromatography using EtOAc/hexanes (1:3 vol/vol EtOAc/hexanes) to afford compound **21** as a yellow syrup (237.9 mg, 74.4% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (d, J = 8.8 Hz, 1H), 8.13 (d, J = 9.2 Hz, 1H), 6.89 (dd, J = 8.8, 2.4 Hz, 1H), 6.84 (m, 2H), 6.68 (d, J = 2.0 Hz, 1H), 5.31 (s, 1H), 3.42 (s, 4H), 1.69 (s, 6H), 1.04 (s, 9H), 0.30 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.27, 160.87, 158.47, 157.65, 155.48, 127.99, 127.75, 117.08, 116.79, 112.56, 111.63, 107.29, 99.32, 48.59, 25.73, 25.61, 25.30, 24.37, 18.29, −4.32. LRESI-MS calculated for $[M+H]^+$ 409.21, found 409.3.

CR4. An oven-dried, two-neck round-bottom flask was charged with **7** (60.6, 0.12 mmol) and anhydrous THF (1 mL). The solution was cooled to −78 °C and treated with a solution of *tert*-BuLi in pentane (1.7 M, 72 μL, 0.12 mmol) under a N_2 atmosphere. After stirring at the same temperature for 30 min, a solution of compound **19** (25 mg, 0.06 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was warmed to room temperature, stirred for a 1 h, and then quenched with aq. HCl (2 M, 2 mL). After 2 h, the reaction was poured into sat. $NaHCO_3$ (30 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography using MeOH/CH_2Cl_2 (1:19 vol/vol MeOH/CH_2Cl_2) to afford **CR4** as a red solid (23.0 mg, 54.2% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39–7.37 (m, 2H), 7.14 (d, J = 7.6 Hz, 1H), 6.96–6.92 (m, 2H), 6.83 (s, 1H), 6.80 (d, J = 9.6 Hz, 1H), 6.60 (d, J = 9.6 Hz, 1H), 6.51 (s, 1H), 3.76 (s, 2H), 3.52 (s, 4H), 2.85 (m, 4H), 2.77–2.74 (m, 12H), 2.60 (q, J = 7.2 Hz, 4H), 2.11 (s, 3H), 1.74 (s, 6H), 1.30 (t, J = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.12, 155.70, 152.52, 142.14, 132.11, 131.36, 130.98, 130.85, 130.22, 129.68, 126.88, 116.11, 112.00, 111.58, 105.44, 97.32, 58.17, 54.20, 48.49, 32.89, 32.71, 32.66, 32.12, 32.08, 30.66, 30.57, 30.02, 26.44, 25.69, 15.14. HRESI-MS calculated for $C_{37}H_{49}N_2O_2S_4$ $[M+H]^+$ m/z 694.2755, found 694.2563.

3-(4-Cbz-piperazinyl)-6-trifluoromethanesulfonyl-xanthone (22). A solution of 3,6-bis(trifluoromethanesulfonyl)-xanthone, **1** (1.0 g, 2.03 mmol), in DMSO (3 mL) was preheated to 90 °C and treated with 1-z-piperazine (391.5 μL, 2.03 mmol). After 45 min, the reaction was cooled to room temperature, poured into brine (30 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified via flash chromatography (2:3 vol/vol EtOAc/hexanes) to afford the titled compound, **22**, as a yellow syrup (336.4 mg, 40.4% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (d, J = 8.0 Hz, 1H), 7.87 (d, J = 8.0 Hz, 1H), 7.19–7.10 (m, 5H), 6.99 (d, J = 8.0 Hz, 1H), 6.63 (d, J = 8 Hz, 1H), 6.40 (s, 1H), 5.07 (s, 1H), 4.99 (s, 2H), 3.50 (s, 4H), 3.22 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.10, 158.16, 156.35, 155.37, 155.14, 152.33, 136.45, 129.00, 128.58, 128.22, 128.02, 121.92, 120.31, 117.11, 116.76, 113.03, 112.06, 110.84, 99.62, 67.45, 53.54, 46.76, 43.12. LRESI-MS calculated for $[M+H]^+$ 562.10, found 562.30.

3-((*tert*-Butyldimethylsilyl)oxy)-6-(4-Cbz-piperazinyl)-9H-xanthen-9-one (23). A suspension of **22** (461.0 mg, 0.82 mmol) in 1,4-dioxane (10 mL) was treated with a solution of Et_4NOH in MeOH (1.5 M, 1.1 mL, 1.64 mmol). The resultant solution was stirred at room temperature for 2 h. After the reaction was complete, as judged by TLC, the volatiles were removed under reduced pressure to afford a yellow solid, which was treated with imidazole (280 mg, 4.1 mmol) and CH_2Cl_2 (15 mL). The solution was cooled to 0 °C, then treated with *tert*-butyldimethylsilyl chloride (309 mg, 2.05 mmol) and stirred at room temperature for 1 h. The volatiles were removed under reduced pressure, and the crude residue was purified via flash chromatography using EtOAc/hexanes (2:3 vol/vol EtOAc/hexanes) to afford compound **23** as an off-white solid (393.9 mg, 88.2% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (d, J = 8.4 Hz, 1H), 8.14 (d, J = 8.8 Hz, 1H), 7.41–7.39 (m, 5H), 6.86–6.82 (m, 3H), 6.66 (d, J = 2.4 Hz, 1H), 5.20 (s, 2H), 3.70 (t, J = 4.8 Hz, 4H), 3.40 (m, 4H), 1.04 (s, 9H), 0.31 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.27, 161.10, 158.12, 157.64, 155.17, 154.95, 136.48, 128.60, 128.22, 128.05, 127.88, 117.31, 116.68, 113.74, 111.76, 107.35, 100.24, 67.43, 47.15, 25.62, 18.30, −4.29. LRESI-MS calculated for $[M+H]^+$ 544.24, found 544.4.

CR5. An oven-dried, two-neck round-bottom flask was charged with **7** (60.6, 0.12 mmol) and anhydrous THF (1 mL). The solution was cooled to −78 °C and treated with a solution of *tert*-BuLi in pentane (1.7 M, 72 μL, 0.12 mmol) under a N_2 atmosphere. After stirring at the same temperature for 30 min, a solution of compound **19** (25 mg, 0.06 mmol) in anhydrous THF (5 mL) was added dropwise. The reaction was warmed to room temperature, stirred for a 1 h, and then quenched with aq. HCl (2 M, 2 mL). After 2 h, the reaction was poured into sat. $NaHCO_3$ (30 mL) and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The crude

residue was purified via flash chromatography using MeOH/CH_2Cl_2 (1:19 vol/vol MeOH/CH_2Cl_2) to afford **CR5** as a red solid (23.0 mg, 54.2% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.07 (d, J = 8.4 Hz, 2H), 7.40 (m, 2H), 7.10 (d, J = 7.6 Hz, 2H), 7.01 (d, J = 9.2 Hz), 6.91–6.79 (m, 5H), 6.71 (s, 1H), 6.58 (d, J = 8.8 Hz, 1H), 6.49 (s, 1H), 4.29 (s, 2H), 3.73 (s, 2H), 3.54 (s, 4H), 3.43 (s, 4H), 2.80–2.69 (m, 16H), 2.56–2.52 (m, 4H), 2.04 (s, 3H), 1.23 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.27, 161.10, 158.12, 157.64, 155.17, 154.95, 136.48, 128.60, 128.22, 128.05, 127.88, 117.31, 116.68, 113.74, 111.76, 107.35, 100.24, 67.43, 47.15, 25.62, 18.30, −4.29. HRESI-MS calculated for $C_{37}H_{49}N_2O_2S_4$ $[M+H]^+$ m/z 829.3075, found 829.2725.

Storage and Handling of CF3 and Ctrl-CF3 Reagents. A protocol for the storage and handling of CF3 and Ctrl-CF3 reagents is given here. For all spectroscopic and cell imaging experiments, CF3 and Ctrl-CF3 were freshly prepared and purified according to the synthetic procedures described above. Aliquots of each dye were prepared by dissolving in anhydrous methanol or in ethyl acetate to a concentration of 20 mM, and, following the removal of solvent in vacuo at room temperature, the dried aliquots were stored over desiccant at −80 °C. Storage at higher temperatures and/or without desiccant will potentially lead to a shorter shelf life for the dyes. These dried aliquots were used within 2 mo, and the quality of the samples was checked by NMR and fluorescence spectroscopy before performing each experiment. For each spectroscopic or imaging experiment, a fresh dried aliquot of dye was reconstituted in anhydrous reagent-grade DMSO on the day of use to provide a stock solution with concentrations ranging from 0.5 mM to 5 mM. The DMSO stocks were then immediately diluted in aqueous buffers to working concentrations of 0.5–5 μM. Aliquots are mixed by either pipetting up and down several times or subjecting to gentle vortex mixing for less than 5 s. Suitable buffers include PBS and Hepes (20–50 mM). Solutions freshly prepared in this manner are optically transparent and are suitable for use that same day; most measurements were completed within 3 h. Notably, CF3 and Ctrl-CF3 are not stored as DMSO stock solutions nor as aqueous buffers. Prolonged exposure to heat above and/or centrifugation at speeds greater than the maximum RPM on a microcentrifuge for 30 s can potentially result in precipitation of aqueous solutions or attenuation of fluorescence responses. Excitation and emission wavelengths for spectroscopic and imaging are provided in the respective experimental sections.

Spectroscopic Materials and Methods. Milli-Q water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM Hepes buffer, pH 7, or in PBS, pH 7.4. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer, and fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scan spectrofluorometer equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photocounting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for absorption and emission measurements were contained in 1-cm × 1-cm quartz cuvettes with or without septum and sealing caps (1.4-mL or 3.5-mL volume; Starna). Fluorescence quantum yields were determined by reference to Rhodamine B in ethanol (Φ = 0.49) (3). The binding affinities of Cu^+ to CR3 and CF3 were measured using thiourea as a competitive ligand to provide buffered Cu^+ solutions. Stability constants for thiourea binding were taken from the literature: $\beta_{12} = 2.0 \times 10^{12}$, $\beta_{13} = 2.0 \times 10^{14}$, $\beta_{14} = 3.4 \times 10^{15}$ (4). Cu^+ was delivered in the form of $[Cu(MeCN)_4][PF_6]$ from an acetonitrile stock solution (2 mM). Metals used in the selectivity assay were derived from their chloride salts. The apparent dissociation constants (K_d) were determined using the following equation: $(F - F_{min})/(F_{max} - F_{min}) = [Cu^+]/(K_d + [Cu^+])$, where F is the observed fluorescence, F_{max} is the fluorescence for the Cu^+:dye complex, and F_{min} is the fluorescence for the free CR3 or CF3 dye. Detection limit experiments were performed as described in the caption of Fig. S3*E*.

Measurement of Partition Coefficients. A modified version of the shake method was used to determine partition coefficients or log D values (5). Briefly, PBS (500 μL), 1-octanol (500 μL), and 2 mM solution of dye in DMSO (1 μL) were added to a 1.5-mL microcentrifuge tube. The dye was partitioned between the layers via vortexing for a maximum of 15–30 s. The microcentrifuge tube was centrifuged on a bench-top minifuge for a maximum of 15–30 s to separate the layers. Prolonged vortexing or centrifuging above these time limits or speeds can lead to inconsistent measurements and transfer of dye. A portion of the 1-octanol layer (250 μL) was transferred to a 96-well plate, and the absorbance from 450–700 nm was measured. The concentration of dye in the 1-octanol layer was determined by comparison with a standard curve constructed from at least four data points.

In Vitro Glutathione Experiments. This series of experiments shows that CF3, but not Ctrl-CF3, is able to reversibly respond to copper and chelation in the presence of glutathione (GSH), a major redox buffer and potential competing copper ligand in biological contexts. All manipulations were carried out in an anaerobic chamber (VAC glovebox, Pd catalyst, atmosphere 10% H_2 with N_2 balance.) Buffer (20 mM Hepes, pH 7) was deoxygenated on a Schlenk line by freeze–pump–thawing before being brought into the anaerobic chamber. Solutions of 5 mM GSH and 2.5 mM GSSG (glutathione disulfide) in anaerobic buffer were prepared using 1 and 2 equivalents of sodium hydroxide (from a 1 M deoxygenated stock solution), respectively, to maintain pH 7. The stock solutions were mixed to yield the indicated GSH/GSSG concentrations in 1-cm × 1-cm path length Semimicro Septum Cap and Screw Cap Starna Fluorometer cells (Fig. S3). A 2-mM stock solution of CF3 (or Ctrl-CF3) was prepared anaerobically in deoxygenated DMSO, and this stock was added to GSH/GSSG-containing buffer, with stock and buffer solutions made according to procedures documented in the storage and handling protocol to yield a final concentration of 5 μM CF3 (or Ctrl-CF3). After measuring background fluorescence, a 2-mM Cu^+ stock was prepared by anaerobically dissolving $Cu(MeCN)_4PF_6$ in deoxygenated MeCN. Cu^+ was added from this stock solution to the buffered solution for a final Cu^+ concentration of 5 μM. Five minutes after Cu^+ addition, fluorescence intensity was measured. Neocuproine was prepared as a DMSO stock at 40 mM and added to final concentration of 400 μM.

The fluorescence response of 5 μM CF3-Cu^+ and 5 μM Ctrl-CF3-Cu^+ complexes to GSH was determined by preforming the CF3-Cu^+ or Ctrl-CS3-Cu^+ complex from relevant stock solutions. An initial fluorescence spectrum was obtained, and subsequent scans were taken 5 min after addition of GSH at 0 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, and 1 mM. Solutions of 10 mM and 100 mM GSH in anaerobic buffer were prepared using 1 and 2 equivalents of sodium hydroxide (from 1 M deoxygenated stock solution), respectively, to maintain pH 7.

In Vitro Model Liposome Experiments. This series of experiments show that CF3, but not Ctrl-CF3, is able to reversibly respond to copper and chelation in the presence of model liposome agents, including ones of well-defined size and composition. All manipulations and spectra were acquired at 23 °C. Samples for emission measurements were prepared to a final volume of 1 mL contained in 1-cm × 1-cm path length quartz cuvettes. Buffer (20 mM Hepes, pH 7) was rigorously deoxygenated in cycles of evacuation and refilling with nitrogen on a Schlenk line. A 2-mM stock solution of CF3 or Ctrl-CF3 was prepared in DMSO, and this stock was added to buffer to yield a final concentration of 2 μM CF3 or

Ctrl-CF3 according to procedures documented in the storage and handling protocol.

For experiments with commercial, preformed neutral liposomes of well-defined size (FormuMax, DPPC/CHOL 55:45 mol/mol, 100 nm), liposomes were added to the buffered solution of CF3 or Ctrl-CF3 to afford final liposome concentrations of 50 μM, 100 μM, or 200 μM. The CF3/liposome or Ctrl-CF3/liposome formulation was allowed to equilibrate for 6.5 h before a fluorescence spectrum was obtained (λ_{ex} = 534 nm, λ_{em} collected from 540 nm to 700 nm). Cu^+ was added to the buffered solution from a 2-mM Cu^+ stock solution prepared by dissolving $Cu(MeCN)_4PF_6$ in MeCN to yield a final concentration of 2 μM Cu^+. A fluorescence scan was obtained after a 15-min equilibration time period, at which point neocuproine was added from a 40-mM stock solution in DMSO to yield a final concentration of 400 μM. For experiments using DMPC (1,2-dimyrisotyl-sn-glycero-3-phosphocholine) as a model liposome forming agent, a DMPC stock solution (10 mM) was prepared in buffer, and this reagent was added to the buffered solution of CF3 or Ctrl-CF3 to afford final DMPC concentrations of 25 μM, 50 μM, 100 μM, and 200 μM. The CF3/DMPC or Ctrl-CF3/DMPC formulation was allowed to equilibrate for 30 min before a fluorescence spectrum was obtained (λ_{ex} = 534 nm, λ_{em} collected from 540 to 700 nm). Cu^+ was added to the buffered solution from a 2-mM Cu^+ stock prepared by dissolving $Cu(MeCN)_4PF_6$ in MeCN to yield a final concentration of 2 μM Cu^+. A fluorescence scan was obtained after a 15-min equilibration time period, at which point neocuproine was added from a 40-mM stock solution in DMSO to yield a final concentration of 400 μM.

In Vitro BSA Model Protein Experiments. This series of experiments show that CF3, but not Ctrl-CF3, is able to reversibly respond to copper and chelation in the presence of BSA as a model protein. Buffer (20 mM Hepes, pH 7) was rigorously degassed in cycles of evacuation and refilling on a Schlenk line. A 2-mM stock solution of CF3 or Ctrl-CF3 was prepared in DMSO, and this stock was added to buffer to yield a final concentration of 2 μM CF3 or Ctrl-CF3. A 5% BSA (Fisher Scientific; BP1605-100) stock solution was prepared in buffer and was then added to the buffered solution of CF3 or Ctrl-CF3 to afford a 0.1% BSA solution. The CF3/BSA or Ctrl-CF3/BSA solution was allowed to equilibrate for 90 min before a fluorescence scan was obtained (λ_{ex} = 534 nm, λ_{em} collected from 540 to 700 nm). Cu^+ was added to the buffered solution from a 2-mM Cu^+ stock prepared by dissolving $Cu(MeCN)_4PF_6$ in MeCN to yield a final concentration of 2 μM Cu^+. A fluorescence scan was obtained, at which point tris [(ethylthio)ethyl]amine (TEMEA) (1) was added from a 50-mM stock solution in DMSO to yield a final concentration of 100 μM.

Cell Lysate Experiments. This series of experiments show that CF3, but not Ctrl-CF3, is able to reversibly respond to copper and chelation within cell lysates, which contain all of the chemical components such as lipids, proteins, and GSH redox buffer/ligands in combination. All manipulations were carried out in an anaerobic chamber (VAC glovebox, Pd catalyst, 10% H_2 in N_2 atmosphere). PBS was deoxygenated on a Schlenk line by freeze–pump–thaw cycles before being brought into the anaerobic chamber. HEK293T cells were cultured as previously described (1) and grown until confluent. Cells were washed three times in cold PBS, harvested by scraping, and subsequently resuspended in an appropriate amount of PBS. The cells were lysed anaerobically by freeze–pump–thaw cycles and then brought into the anaerobic chamber. These lysates were separated by centrifugation at 7,200 × *g* for 10 min, after which the supernatant was collected and the pellet was discarded. Protein content was quantified by the Bradford assay, and lysates were diluted to a final protein content of 1 mg/mL.

Lysates were added to Semimicro Septum Cap or Screw Cap Fluorimeter cells (Starna). A 2-mM stock solution of CF3 or Ctrl-CF3 was prepared anaerobically in deoxygenated DMSO as described in *Storage and Handling of CF3 and Ctrl-CF3 Reagents*, and this stock solution was added to cell lysates to give a final probe concentration of 2 μM. A 10-mM Cu^+ stock solution was prepared by dissolving $[Cu(MeCN)_4][PF_6]$ in deoxygenated MeCN, and Cu^+ was added from this stock solution to give final Cu^+ concentrations of 10 μM, 50 μM, and 100 μM. Treated lysates were allowed to equilibrate for 45 min, at which point fluorescence intensity was measured (λ_{ex} = 534 nm, λ_{em} collected from 540 to 700 nm). Neocuproine was added (from a 1-mM stock to yield a final concentration of 2 μM) to lysates treated with 100 μM Cu^+, and fluorescence intensity was measured immediately.

Dissociated Hippocampal Neuronal Cell Cultures. Primary hippocampal neuron cultures were prepared using the method described by Goslin et al. (6). Timed pregnant Sprague–Dawley rats were ordered from Charles River. Neuronal cultures were prepared from the brains of rats at embryonic days 18–21, plated on 12 mm poly-L-lysine coated coverslips in serum-based medium with B27 supplement (Life Technologies), and then replaced and maintained in Neurobasal media with B27 and GlutaMAX supplements. All animal care and experimental protocols were approved by the Animal Care and Use Committees at the University of California, Berkeley.

Imaging of Dissociated Hippocampal Neuron Cultures. For copper imaging, DIV 10–13 cultured hippocampal neurons were washed with HBSS, incubated with 2 μM CF3 or Ctrl-CF3 in HBSS for 20 min at 37 °C, 5% CO_2, and placed in fresh HBSS perfusion, or HBSS with 200 μM bathocuproine disulfonate disodium salt (BCS; Sigma). Images were taken on a custom-modified two-photon microscope (Fluoview 300; Olympus America) using a 60× objective (Olympus LUMPlanFl/IR ×60/0.90W) with excitation laser tuned to 900 nm. For confocal imaging, an Argon laser was used for excitation. In both cases, emission was collected at wavelengths longer than 515 nm. Images were thresholded at a common pixel value for comparison between control and chelated conditions (ImageJ; National Institute of Neurological Disorders and Stroke). To quantitatively compare fluorescence levels across conditions, the image was subdivided into nonoverlapping regions, and the average fluorescence was computed by averaging over these regions. Note that imaging experiments were done over the course of 30 s to 1 min after initial staining, treatment, and washing.

For calcium imaging (Fig. 4), DIV 12–15 cultured hippocampal neurons were incubated with 5 μM Ca^{2+}-sensitive dye, OGB (Life Technologies) in Hepes buffered saline (in mM: 5 glucose, 120 NaCl, 10 Hepes, 2 $CaCl_2$, 2.5 KCl, 2 $MgCl_2$) for 30 min, then washed with HBSS for 10 min. Images were acquired on a Leica confocal imaging system (CS SP) equipped with an argon gas ion laser and a Leica inverted microscope (DM IRBE) fitted with a Leica 63x objective (HCX PL Apo; NA, 1.32). OGB was excited at 488 nm, and the fluorescence signal was collected at 500–540 nm. Images were acquired every 1.45 s at 512 × 512 pixels. BCS was bath applied. Images were corrected for motion artifacts using the Turboreg ImageJ plugin. The 5 × 5 pixel regions of interest were manually selected within all cells in the field of view using custom MATLAB scripts. Fluorescence signals were averaged within these regions over time. We quantified the correlation structure of spontaneous calcium transients using the following approach. First, we identified cell events as the time points when the change in fluorescence exceeded 10% of the baseline fluorescence ($\Delta F/F > 10\%$). Second, we identified network events as the time points where more than 10% of cells exhibited events. Last, we computed the fraction of cells that also

exhibited above-threshold transients in 1-s bins for a total duration of ± 5 s (Fig. 4*B*).

For pharmacological manipulations, cultured hippocampal neurons (DIV 12–15) were incubated in either HBSS or GSH monoethyl ester (GSH-MEE, 500 μM) for 2 h. Neurons were then loaded with either CF3 or Ctrl-CF3 (2 μM) and imaged, using identical imaging parameters. Pixels were pooled across images and histogrammed for distribution changes in pixel intensity. As positive control experiments to establish that BCS perfusion alone does not trigger oxidative stress, neurons were loaded with 500 nM Rhodamine 123 (Life Technologies) and placed in perfusion either with low O_2 (not aerated) HBSS or 200 μM BCS. Images were acquired at 30 min, 90 min, and 120 min. Note that imaging experiments were done over the course of 30 s to 1 min after initial staining, treatment, and washing.

Retinal Preparation. P10–P12 C57BL/6 mice of either sex were deeply anesthetized with isoflurane and decapitated. Eyes were removed, and retinas were isolated in oxygenated artificial cerebrospinal fluid [aCSF; containing (in mM) 119.0 NaCl, 26.2 $NaHCO_3$, 11 glucose, 2.5 KCl, 1.0 K_2HPO_4, 2.5 $CaCl_2$, and 1.3 $MgCl_2$]. For Ca^{2+} and copper imaging, retinas were mounted GCL-up on filter paper (Millipore) and perfused continuously with oxygenated aCSF. CTR1 heterozygous mice were maintained and genotyped according to published procedures (7). All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Berkeley, CA and conformed with guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals,* the Public Health Service Policy, and the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research.

Retinal Imaging. For two-photon imaging of copper levels, retinas were bolus loaded either CF3 or a Ctrl-CF3 at 0.4 μM, with excitation wavelength of 910 nm, and emission was collected below 515 nm (8). For nucleic acid colabeling, Syto 17 Red (Life Technologies) was used at 0.4 μM. Pixel intensities for CF3, Ctrl-CF3, and Syto 17 Red were histogrammed and plotted on the same axis to demonstrate CF3-specific intensity changes. For two-photon imaging of intracellular calcium concentration, retinas were loaded with OGB using the multicell bolus loading technique (8). Two-photon calcium imaging of neurons in the GCL was preformed using a custom-modified two-photon microscope (Fluoview 300, Olympus America, Inc.). XYZ scans were used to localize neurons in the GCL. Time series images were acquired at 1 Hz using a 60× objective (Olympus LUMPlanFI/IR 60×/0.90W) with the excitation laser tuned to 790 nm. For pharmacological experiments, aCSF containing 200 μM BCS or 15 μM ATN-224 and/or 50 μM D-(-)-2-amino-5-phosphonopentanoic acid (AP5) were bath applied on stage.

Images were corrected for motion artifacts using the Turboreg ImageJ plugin. The 10 × 10 pixel regions of interest were manually selected within all cells in the field of view using custom MATLAB scripts. Fluorescence signals were averaged within these regions over time. Cell events were identified when the change in fluorescence exceeded 15% of the cell's baseline fluorescence within 1 s. Cells were categorized as participating in a retinal wave if cell events correlated with neighboring cells.

Preparation of Retinal Extracts and Immunoblotting. A representative protocol is as follows: retinas were isolated, and one retina for each sample was homogenized for 30 s and vortexed for 30 s three times in 200 μL of radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors (Roche Complete MiniTab) and phosphatase inhibitors (Thermo Scientific Halt Phosphatase Inhibitor Mixture), lysed for 30 min on ice, and cleared by centrifugation at 16,100 × *g* for 10 min at 4 °C. Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce), 5 μL of 4× Laemmli buffer (0.25 mM Tris, 2% SDS, 40% glycerol, 20% beta-mercaptoethanol, 0.04% bromophenol blue) was added, the samples were denatured at 37 °C for 10 min, and 12 μg of total protein were loaded and separated on a 4–12% Bis-Tris gel (NuPAGE Novex; Life Technologies). Proteins were transferred to PVDF membranes (Immobilon; Millipore) with a semidry transfer system (Biorad) at constant voltage (15 V) for 35 min for CTR1. The blots were blocked in 5% BSA in wash buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20) overnight at 4 °C, and incubated for 2 d at 4 °C in anti-CTR1 1:1,000, which was kindly provided by Prof. Dennis J. Thiele. Membranes were washed 5 times for 5 min in wash buffer, incubated at room temperature with 1:2,000 secondary antibody (Santa Cruz) for 1 h, washed 5 times for 5 min in wash buffer, and then visualized using enhanced chemiluminescence (Luminata Classico Western HRP Substrate; Millipore) recorded on a BioRad GelDoc imaging station. The blot was stripped using Restore Plus stripping buffer (Thermo Scientific) according to manufacturer's instructions. The blot was then washed 5 times for 5 min in wash buffer, and then probed for anti-GAPDH 1:2,000 (Abcam) and developed as described above.

ICP-MS. P10–P12 C57BL/6 mice of either sex were deeply anesthetized with isoflurane, and decapitated. Eyes were then removed and lyophilized. The average of at least six samples is reported with SD. Samples (~1 mg) were digested with 100 μL concentrated HNO_3 (trace metal grade, Fisher Scientific) in borosilicate tubes at 90 °C in water baths for 2 h. After cooling, 100 μL H_2O_2 (Optima grade, Fisher Scientific) was added. The digested samples were brought to a total volume of 1 mL with 1% HNO_3 (trace metal grade, Fisher Scientific).

ICP-MS analysis was carried out in the Elemental Analysis Core at Oregon Health & Science University using an Agilent 7700x equipped with an ASX 250 autosampler. The system was operated at a radio frequency power of 1550 W, an argon plasma gas flow rate of 15 L/min, and Ar carrier gas flow rate of 1.08 L/min. Elements were measured in kinetic energy discrimination mode using He gas (4.2 mL/min).

For measurement, 5× dilutions of samples were prepared in 1% HNO_3 in acid-treated 15-mL conical tubes. Data were quantified using a 12-point calibration curve (0 ppb, 0.5 ppb, 1 ppb, 2 ppb, 5 ppb, 10 ppb, 50 ppb, 100 ppb, 400 ppb, 600 ppb, 800 ppb, 1,000 ppb (ng/g) for Ca, Cu, and Fe and a 9-point (0 ppb, 100 ppb, 400 ppb, 600 ppb, 800 ppb, 1,000 ppb, 2,000 ppb, 4,000 ppb, 8,000 ppb) calibration curve for S using external standards (Common Elements Mix 2 Multi-Element Aqueous Standard and Sulfur Single Elemental Standard; VHG Labs) in 1% HNO_3. For each sample, data were acquired in triplicate and averaged. A Ge-72 internal standard (Internal Standard Multi-Element Mix 3; VHG Labs) introduced with the sample was used to correct for plasma instabilities, and frequent measurements of a 10-ppb all-analyte solution as well as a blank (containing 1% HNO_3 only) were used as quality control and to determine the coefficient of variance. To access recovery rates of elements and probe background contamination from containers, a certified National Institutes of Standards and Technology standard reference material (bovine liver; 1577c) was digested and analyzed by the same method as the samples.

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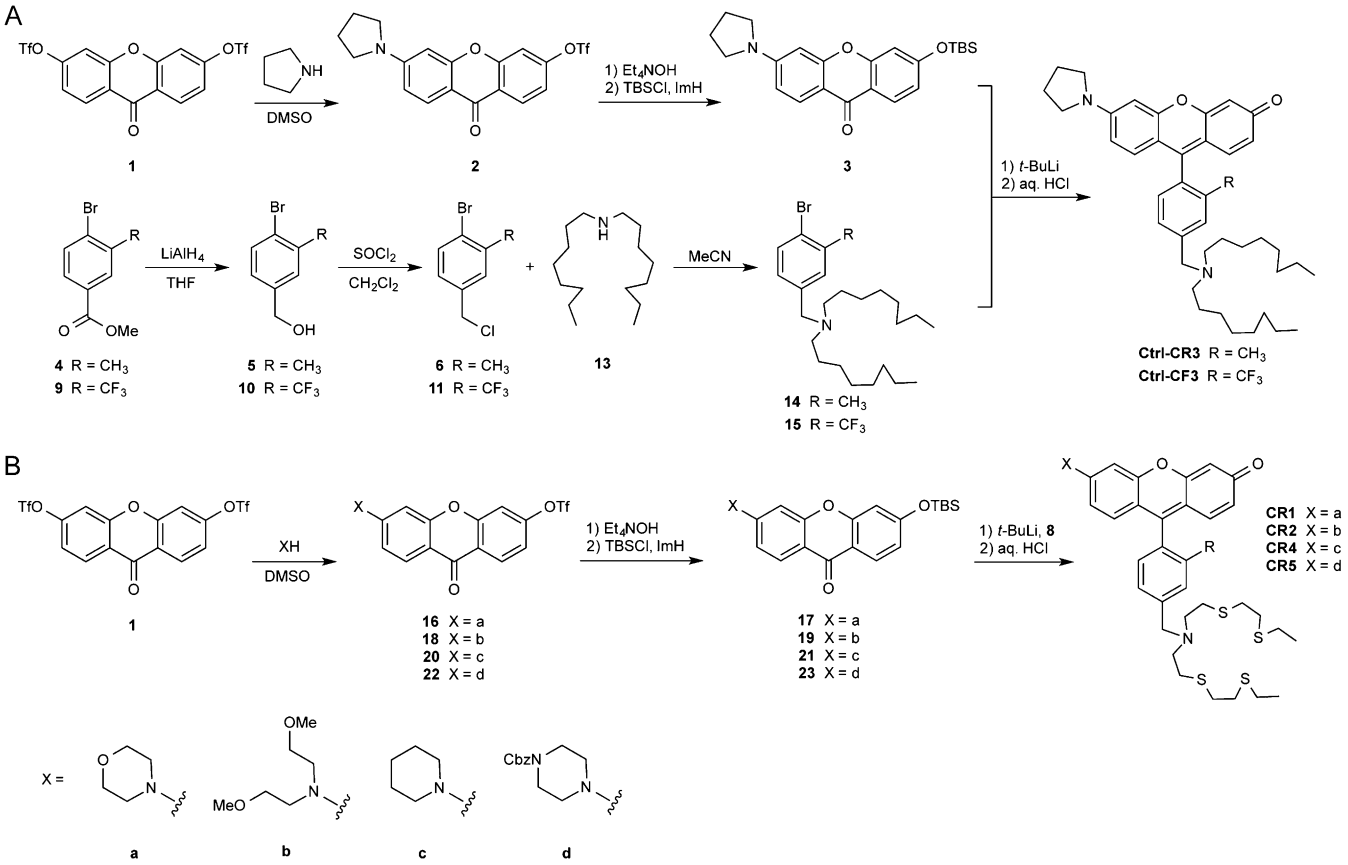

Fig. S1. (*A*) Synthesis of Ctrl-CR3 and Ctrl-CF3. (*B*) Synthesis of CR1, CR2, CR4, and CR5.

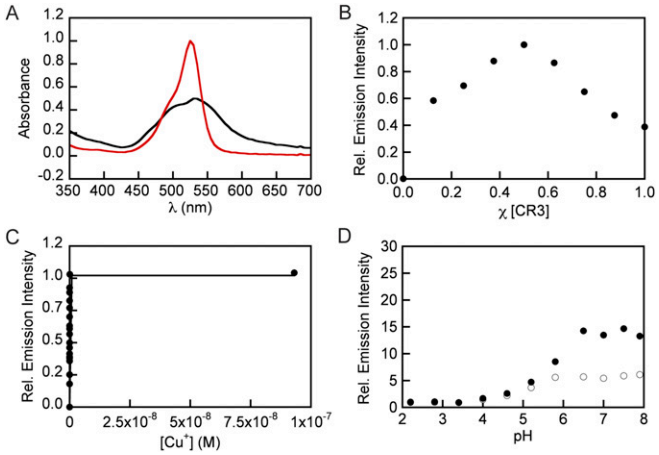

Fig. S2. Spectroscopic characterization of CR3. (*A*) UV-visible spectral change of 2 μM CR3 upon addition of 1 equivalent of Cu^{+}. (*B*) Job's plot of CR3 and Cu^{+}. The total concentrations of CR3 and Cu^{+} were kept at 4 μM. Excitation was provided at 525 nm and recorded at 545 nm. (*C*) Normalized fluorescence response of 2 μM CR3 to thiourea buffered Cu^{+} solutions for K_d value determination. The points are shown for free Cu^{+} buffered at 9.31×10^{-8} M, 4.88×10^{-9} M, 1.02×10^{-9} M, 6.73×10^{-10} M, 2.32×10^{-10} M, 1.67×10^{-12} M, 9.13×10^{-13} M, 5.70×10^{-13} M, 3.86×10^{-13} M, 2.77×10^{-13} M, 2.08×10^{-13} M, 1.60×10^{-13} M, 1.27×10^{-13} M, 1.03×10^{-13} M, 8.46×10^{-14} M, 7.06×10^{-14} M, 5.96×10^{-14} M, 4.38×10^{-14} M, and 2.59×10^{-14} M. The observed K_d is 1.3×10^{-13} M. Excitation was provided at 525 nm and collected from 530 to 650 nm. (*D*) Fluorescence intensities of 2 μM CR3 across a range of pH values. Open circles indicate emission intensities of CR3 itself, and filled circles indicate emission intensities of CR3 with equimolar Cu^{+}. Excitation was provided at 525 nm and emission was collected from 530 to 650 nm.

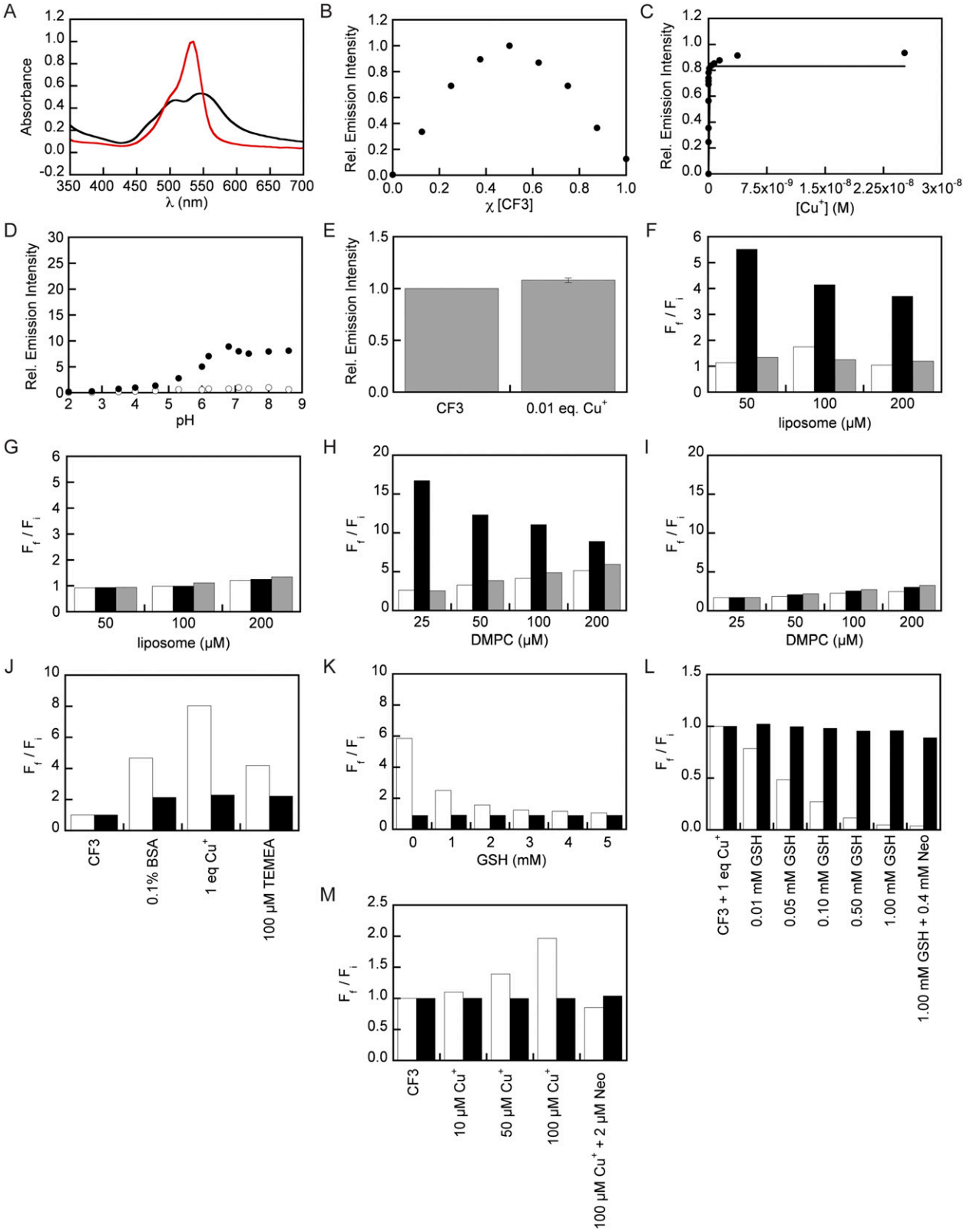

Fig. S3. Spectroscopic characterization of CF3. (*A*) UV-visible spectral change of 2 μM CF3 upon addition of 1 equivalent of Cu^{+}. (*B*) Job's plot of CF3 and Cu^{+}. The total concentrations of CF3 and Cu^{+} were kept at 4 μM. Excitation was provided at 534 nm and recorded at 557 nm. (*C*) Normalized fluorescence response of 2 μM CF3 to thiourea buffered Cu^{+} solutions for K_d value determination. The points are shown for free Cu^{+} buffered at 2.52×10^{-8} M, 3.73×10^{-9} M, 1.42×10^{-9} M, 7.44×10^{-10} M, 4.57×10^{-10} M, 1.06×10^{-10} M, 2.53×10^{-11} M, 1.10×10^{-11} M, 6.10×10^{-12} M, 3.85×10^{-12} M, 9.12×10^{-13} M, 2.07×10^{-13} M, and 8.45×10^{-14} M. The observed K_d is 3.3×10^{-13}. Excitation was provided at 534 nm and collected from 545 to 700 nm. (*D*) Fluorescence intensities of 2 μM CF3 across a range of pH values. Open circles indicate emission intensities of CF3 itself, and filled circles indicate emission intensities of CF3 with equimolar Cu^{+}. Excitation

Legend continued on following page

was provided at 534 nm and emission was collected from 545 to 700 nm. (*E*) Fluorescence spectral change of 2 μM CF3 upon addition of 0.01 equivalents (20 nM) of Cu^+ for the determination of the detection limit. Excitation was provided at 534 nm and collected from 545 to 700 nm. (*F*) Fluorescence response of 2 μM CF3 in the presence of neutral liposomes (FormuMax, DPPC/CHOL 55:45 mol/mol, 100 nm, 50–200 μM) (white), with subsequent addition of 2 μM Cu^+ to the solution (black), and a final addition of 400 μM neocuproine (gray). Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Excitation was provided at 534 nm and emission was collected over 540–700 nm. (*G*) Fluorescence response of 2 μM Ctrl-CF3 in the presence of neutral liposomes (FormuMax, DPPC/CHOL 55:45 mol/mol, 100 nm) (white), with subsequent addition of 2 μM Cu^+ to the solution (black), and a final addition of 400 μM neocuproine (gray). Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Excitation was provided at 534 nm, and emission was collected over 540–700 nm. (*H*) Fluorescence response of 2 μM CF3 in the presence of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, 25–100 μM), a compound used for liposome formation (white), with subsequent addition of 2 μM Cu^+ to the solution (black), and a final addition of 400 μM neocuproine (gray). Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Excitation was provided at 534 nm and emission was collected over 540–700 nm. (*I*) Fluorescence response of 2 μM Ctrl-CF3 in the presence of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, 25–100 μM), a compound used for liposome formation (white), with subsequent addition of 2 μM Cu^+ to the solution (black), and a final addition of 400 μM neocuproine (gray). Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Excitation was provided at 534 nm and emission was collected over 540–700 nm. The collective data in *F*–*I* show that CF3, but not Ctrl-CF3, can reversibly respond to Cu^+ in the presence of artificial lipids, albeit with diminished signal-to-noise compared with aqueous buffer alone. (*J*) Fluorescence response of 2 μM CF3 (white) and 2 μM Ctrl-CF3 (black) in 0.1% BSA and to subsequent addition of 2 μM Cu^+ and 100 μM TEMEA. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Excitation was provided at 534 nm, and emission was collected over 540–700 nm. The data in *J* show, unsurprisingly, that both dyes exhibit a fluorescence enhancement in the presence of 0.1% BSA as a model protein compared with pure aqueous buffer. However, only CF3 responds reversibly to addition of Cu^+ in the presence of BSA to give a turn-on response can be subsequently attenuated by the addition of the chelator TEMEA. Neither the addition of copper nor chelator alters the fluorescence responses of Ctrl-CF3. (*K*) Fluorescence response of 5 μM CF3 (white) and Ctrl-CF3 (black) to 5 μM Cu^+ at differing mole ratios of GSH with [GSH] + 2 [GSSG] = 5 mM. Cu^+ was added to the buffer containing GSH/GSSG and CF3 or Ctrl-CF3. Excitation was provided at 534 nm and collected from 540 to 700 nm. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). The data show that higher concentrations of GSH attenuate the fluorescence response of CF3 to added Cu^+, suggesting that CF3 can compete with GSH to sense Cu^+ within physiological concentration regimes. In contrast, Ctrl-CF3 shows no fluorescence dependence on Cu^+ or GSH. (*L*) Fluorescence response of a 5-μM preformed complex of CF3-Cu^+ (white) and 1:1 mixture of Ctrl-CF3 and Cu^+ (black) to GSH (0.01–1 mM) and neocuproine (Neo, 400 μM). Excitation was provided at 534 nm and collected from 540 to 700 nm. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Titration with the competing GSH ligand demonstrates the reversibility of the CF3-Cu^+ complex. (*M*) Fluorescence responses of 2 μM CF3 (white) and Ctrl-CF3 (black) to 10, 50, and 100 μM Cu^+, followed by addition of 2 μM neocuproine in HEK293T cell lysates (protein content of lysates: 1 mg/mL). Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Excitation was provided at 534 nm and emission was collected over 540–700 nm.

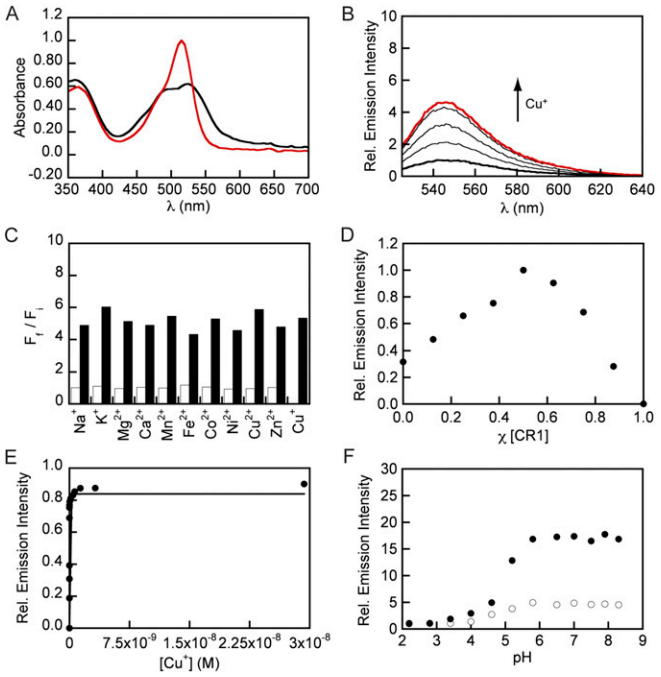

Fig. S4. Spectroscopic characterization of CR1. (*A*) UV-visible and (*B*) fluorescence spectral change of 2 μM CR1 upon addition of 1 equivalent of Cu^+. (*C*) Fluorescence responses of CR1 to various metal ions. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). White bars represent the addition of an excess of the indicated metal ion (2 mM for Na^+, K^+, Mg^{2+}, Ca^{2+}, and Zn^{2+}; 50 μM for all other cations) to a 2-μM solution of CR1. Black bars represent subsequent addition of 2 μM Cu^+ to the solution. (*D*) Job's plot of CR1 and Cu^+. The total concentrations of CR1 and Cu^+ were kept at 4 μM. (*E*) Normalized fluorescence response of 2 μM CR1 to thiourea buffered Cu^+ solutions for K_d value determination. The points are shown for free Cu^+ buffered at 2.93×10^{-8} M, 3.23×10^{-9} M, 1.37×10^{-9} M, 6.58×10^{-10} M, 4.57×10^{-10} M, 1.55×10^{-10} M, 5.07×10^{-11} M, 2.70×10^{-11} M, 9.33×10^{-12} M, 4.76×10^{-12} M, 9.23×10^{-13} M, 6.10×10^{-14} M, and 1.01×10^{-14} M. The observed K_d is 8.4×10^{-13} M. (*F*) Fluorescence intensities of 2 μM CR1 across a range of pH values. Open circles indicate emission intensities of CR1 itself, and filled circles indicate emission intensities of CR1 with equimolar Cu^+. For all assays, excitation was provided at 516 nm and emission was collected from 525 to 700 nm.

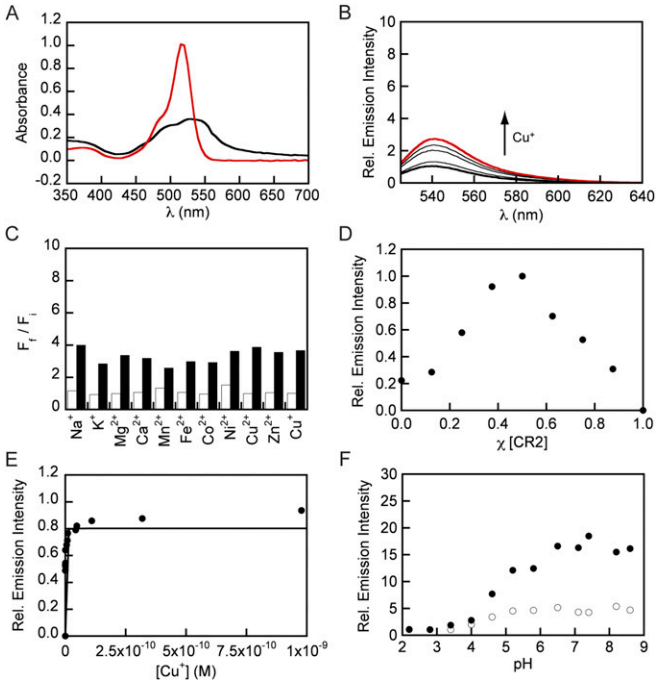

Fig. S5. Spectroscopic characterization of CR2. (*A*) UV-visible and (*B*) fluorescence spectral change of 2 μM CR2 upon addition of 1 equivalent of Cu^{+}. (*C*) Fluorescence responses of CR2 to various metal ions. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). White bars represent the addition of an excess of the indicated metal ion (2 mM for Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, and Zn^{2+}; 50 μM for all other cations) to a 2-μM solution of CR2. Black bars represent subsequent addition of 2 μM Cu^{+} to the solution. (*D*) Job's plot of CR2 and Cu^{+}. The total concentrations of CR2 and Cu^{+} were kept at 4 μM. (*E*) Normalized fluorescence response of 2 μM CR2 to thiourea buffered Cu^{+} solutions for K_d value determination. The points are shown for free Cu^{+} buffered at 1.53×10^{-9} M, 9.78×10^{-10} M, 3.19×10^{-10} M, 1.10×10^{-10} M, 4.80×10^{-11} M, 4.30×10^{-11} M, 1.04×10^{-11} M, 8.67×10^{-12} M, 5.60×10^{-12} M, 9.90×10^{-13} M, 5.84×10^{-13} M, 3.94×10^{-13} M, 1.77×10^{-13} M, and 5.50×10^{-14} M. The observed K_d is 9.5×10^{-14} M. (*F*) Fluorescence intensities of 2 μM CR2 across a range of pH values. Open circles indicate emission intensities of CR2 itself, and filled circles indicate emission intensities of CR2 with equimolar Cu^{+}. For all assays, excitation was provided at 518 nm and emission was collected from 525 to 700 nm.

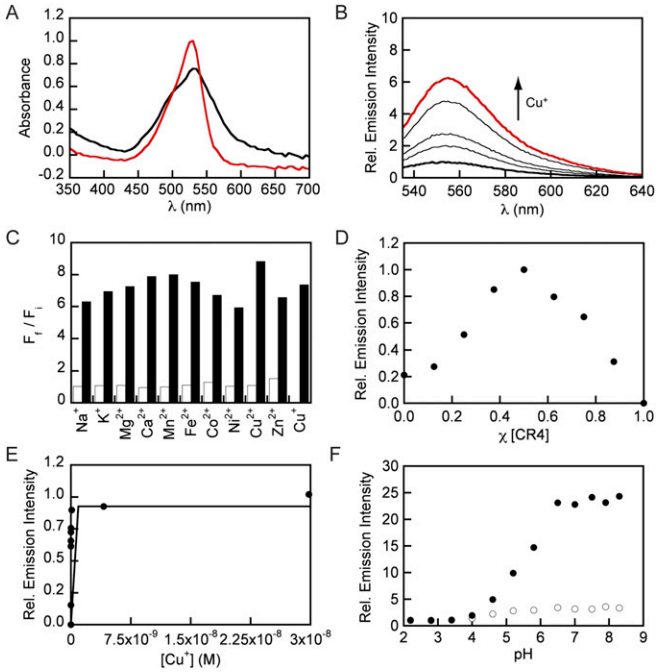

Fig. S6. Spectroscopic characterization of CR4. (*A*) UV-visible and (*B*) fluorescence spectral change of 2 μM CR4 upon addition of 1 equivalent of Cu^+. (*C*) Fluorescence responses of CR4 to various metal ions. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). White bars represent the addition of an excess of the indicated metal ion (2 mM for Na^+, K^+, Mg^{2+}, Ca^{2+}, and Zn^{2+}; 50 μM for all other cations) to a 2-μM solution of CR4. Black bars represent subsequent addition of 2 μM Cu^+ to the solution. (*D*) Job's plot of CR4 and Cu^+. The total concentrations of CR4 and Cu^+ were kept at 4 μM. (*E*) Normalized fluorescence response of 2 μM CR4 to thiourea buffered Cu^+ solutions for K_d value determination. The points are shown for free Cu^+ buffered at 5.32×10^{-8} M, 9.27×10^{-8} M, 2.98×10^{-8} M, 4.11×10^{-9} M, 1.01×10^{-10} M, 2.90×10^{-11} M, 1.40×10^{-11} M, 3.07×10^{-12} M, 9.11×10^{-13} M, and 1.97×10^{-13} M. The observed K_d is 7.9×10^{-13} M. (*F*) Fluorescence intensities of 2 μM CR4 across a range of pH values. Open circles indicate emission intensities of CR4 itself, and filled circles indicate emission intensities of CR4 with equimolar Cu^+. For all assays, excitation was provided at 530 nm and emission was collected from 535 to 700 nm.

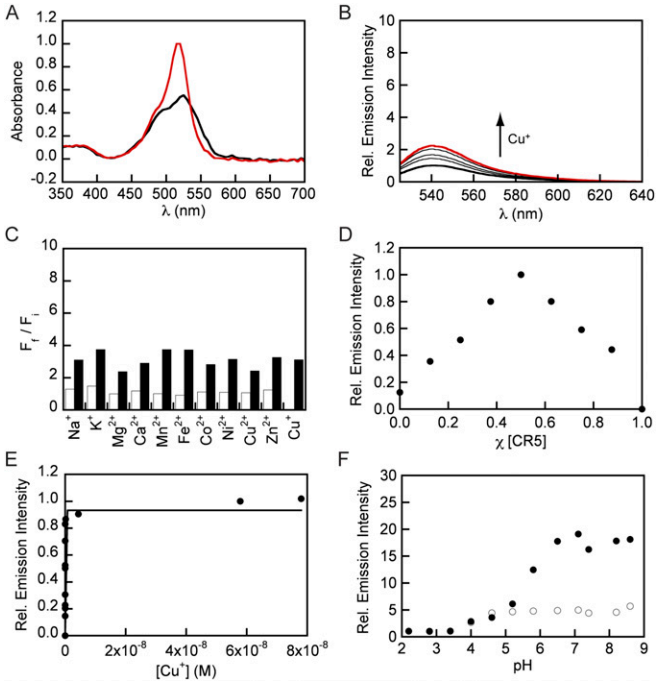

Fig. S7. Spectroscopic characterization of CR5. (*A*) UV-visible and (*B*) fluorescence spectral change of 2 μM CR5 upon addition of 1 equivalent of Cu^{+}. (*C*) Fluorescence responses of CR5 to various metal ions. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). White bars represent the addition of an excess of the indicated metal ion (2 mM for Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, and Zn^{2+}; 50 μM for all other cations) to a 2-μM solution of CR5. Black bars represent subsequent addition of 2 μM Cu^{+} to the solution. (*D*) Job's plot of CR5 and Cu^{+}. The total concentrations of CR5 and Cu^{+} were kept at 4 μM. (*E*) Normalized fluorescence response of 2 μM CR5 to thiourea buffered Cu^{+} solutions for K_d value determination. The points are shown for free Cu^{+} buffered at 5.79×10^{-8} M, 7.80×10^{-8} M, 4.40×10^{-9} M, 1.98×10^{-10} M, 6.77×10^{-12} M, 8.34×10^{-13} M, 7.22×10^{-13} M, 3.89×10^{-13} M, 1.78×10^{-13} M, 1.10×10^{-13} M, 7.71×10^{-14} M, and 5.08×10^{-14} M. The observed K_d is 3.6×10^{-13} M. (*F*) Fluorescence intensities of 2 μM CR5 across a range of pH values. Open circles indicate emission intensities of CR5 itself, and filled circles indicate emission intensities of CR5 with equimolar Cu^{+}. For all assays, excitation was provided at 520 nm and emission was collected from 525 to 700 nm.

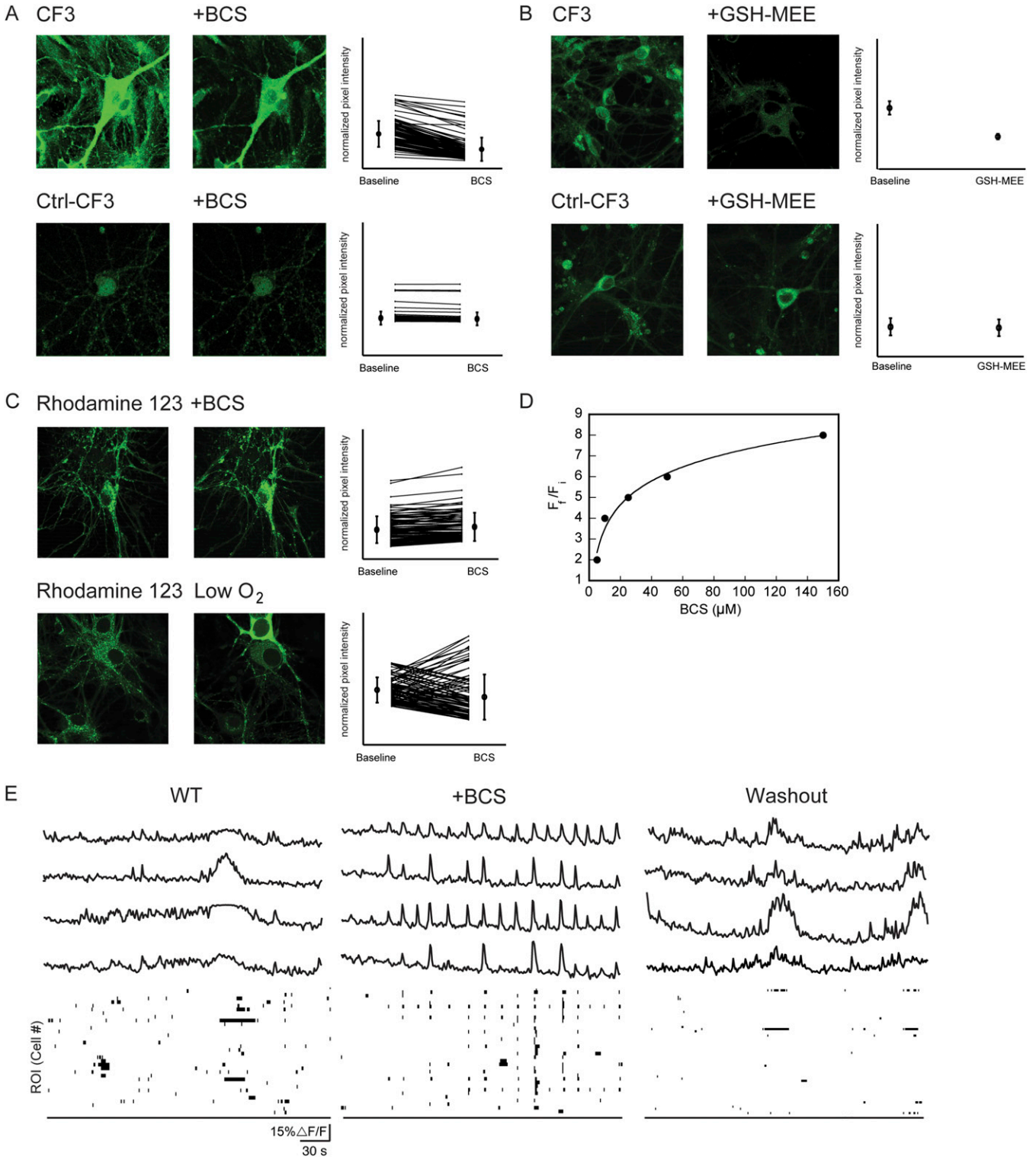

Fig. S8. Copper, calcium, and redox imaging in dissociated hippocampal cultures with BCS and GSH-MEE treatments. (*A*) Live-cell confocal imaging with CF3 and Ctrl-CF3 in dissociated hippocampal neurons suggests that acute BCS treatment alters intracellular labile Cu^{+}. Representative images are shown of hippocampal neurons that were incubated with 2 μM of the copper-responsive CF3 dye or Ctrl-CF3, which does not respond to copper, for 20 min. Fluorescence before and followed by 30 min of BCS perfusion were compared. To quantify the comparisons, the image was divided into a grid of 518 points. Each pair in the figure shows the effect of BCS on fluorescence at each point in the grid ($n = 3$ cultures). (*B*) CF3 staining of dissociated hippocampal neurons treated with GSH-MEE suggests that CF3 can respond to changes in the GSH-dependent copper pool. (*Top*) Representative images of hippocampal neurons stained with CF3 with and without GSH-MEE treatment (control $n = 6$ cultures, GSH-MEE $n = 6$ cultures). (*Bottom*) Representative images of hippocampal neurons stained with Ctrl-CF3 with and without GSH-MEE treatment show that this dye does not respond to changes in the GSH-dependent copper pool (control $n = 7$ cultures, GSH-MEE $n = 7$ cultures). Quantification was completed as described above in *A*. (*C*) Dissociated hippocampal neurons do not undergo oxidative stress with BCS treatment as assayed by the mitochondrial membrane potential marked Rhodamine 123. As a positive control, low-oxygen solutions were used to show an

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increase in mitochondrial fluorescence of Rhodamine 123. (*Top*) Representative image of a Rhodamine 123 stained hippocampal neuron before and after 2 h 200 μM BCS perfusion. (*Bottom*) Representative image of a Rhodamine 123 stained hippocampal neuron before and after perfusion with low-oxygen HBSS (Rhodamine 123 and BCS perfusion: $n = 3$ cultures; Rhodamine 123 and low-oxygen perfusion: $n = 4$ cultures). Quantification was completed as described above in *A*. (*D*) Dose dependence of BCS in dissociated hippocampal culture. Points represent the fluorescence response (F_f) with BCS treatment normalized to the baseline fluorescence (F_i). Experiments were done in duplicate. (*E*) The effects of BCS on spontaneous activity in dissociated hippocampal culture are reversible. Sample $\Delta F/F$ traces from four different cells loaded with OGB and raster plots of neuronal calcium transients greater than 10% $\Delta F/F$ for all cells in the field of view for WT, 200 μM BCS, and washout.

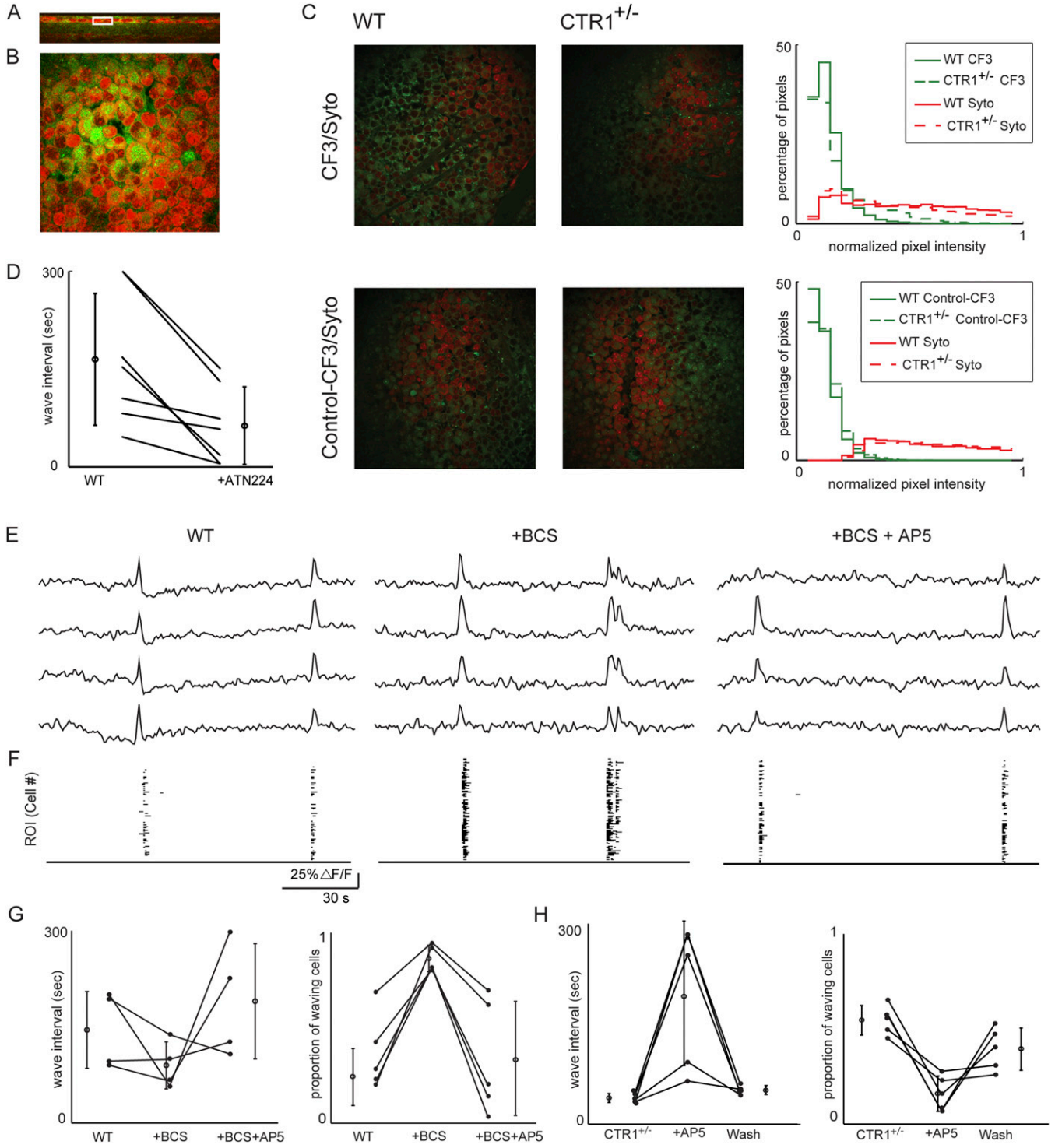

Fig. S9. Copper and calcium imaging in the developing retina. (*A*) CF3 (0.4 μM) coloaded with nucleic acid dye Syto 17 (0.4 μM), orthogonal projection of two-photon Z-stacks, shows dye penetration through to the inner nuclear layer. White box indicates depth at which *B* was imaged. (*B*) CF3 coloaded with nucleic acid dye Syto 17, ganglion cell layer, showing that CF3 is present in the cytosol as well as in the extracellular space. (*C*) $CTR1^{+/-}$ retinas show lower levels of labile Cu^{+} compared with WT retinas as visualized by the copper-responsive dye CF3, whereas the Ctrl-CF3 dye shows the same responses in both WT and $CTR1^{+/-}$ retina. (*Top*) Representative image of WT and $CTR1^{+/-}$ retina stained with CF3 (green) and Syto 17 (red) and normalized pixel intensity histogram for CF3 and Syto 17 fluorescence intensity. (*Bottom*) Representative image of WT and $CTR1^{+/-}$ retina stained with Ctrl-CF3 (green) and Syto 17 (red) normalized pixel intensity histogram for Ctrl-CF3 and Syto 17 fluorescence intensity (WT Ctrl-CF3: $n = 3$ retinas, CF3: $n = 4$ retinas; $CTR1^{+/-}$ Ctrl-CF3: $n = 3$ retinas, CF3: $n = 4$ retinas). (*D*) Treatment with another class of copper chelator, ATN-224, alters spontaneous activity in P11 mouse retina by increases in wave frequency. Lines connect values of median wave interval from retina in control versus chelation with 15 μM ATN-224 (control: $n = 7$ intervals; ATN-224: $n = 13$ intervals). (*E*) Blockade of *N*-methyl-D-aspartate receptors (NMDARs) occludes the effect of BCS on spontaneous activity in the developing retina. Sample ΔF/F traces from averaged regions within four different cells. Each trace row corresponds to the same cell, first in control solution (*Left*), followed by chelation with 200 μM BCS (*Middle*), and finally treatment with 200 μM BCS and 50 μM AP5 (*Right*). (*F*) Binary plots of neuronal calcium transients greater 15% ΔF/F for all cells in the field

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of view for WT (*Left*), 200 μM BCS (*Middle*), and 200 μM BCS + 50 μM AP5 (*Right*). (*G*) Application of AP5+BCS to WT retinas returns the frequency of calcium events and percentage of waving cells to pre-BCS levels. Summary of effects of BCS and AP5 on interevent interval for WT. Control: $n = 8$ intervals, median = 92 s, upper/lower quartile = 59/95; BCS: $n = 21$ intervals, median = 96 s, upper/lower quartile = 9/108; BCS+AP5: $n = 8$ intervals, median = 141 s, upper/lower quartile = 50/199. Lines connect values of interevent interval per retina in control versus BCS and BCS+AP5. Open circles are group means and SD. Summary of the effects of BCS and BCS+AP5 on the average proportion of cells that participated per wave. Control: $n = 5$ retinas, median = 36%, upper/lower quartile = 23/51; BCS: $n = 5$ retinas, median = 82%, upper/lower quartile = 81/93; BCS+AP5: $n = 5$ retinas, median = 20%, upper/lower quartile = 3.6/62. Lines connect values of average cell participation per retina in WT versus BCS and BCS+AP5. Open circles are group means and SD. (*H*) Application of AP5 to $CTR1^{+/-}$ retinas decreases wave frequency and cell participation. Summary of effects of AP5 on interevent interval for $CTR1^{+/-}$. $CTR1^{+/-}$ control: $n = 53$ intervals, median = 27 s, upper/lower quartile = 18/43; AP5: $n = 11$ intervals, median = 75 s, upper/lower quartile = 61/87; wash: $n = 21$ intervals, median = 46 s, upper/lower quartile = 34/69. Summary of the effects of AP5 on the average of proportion of cells that participated per wave. $CTR1^{+/-}$ control: $n = 5$ retinas, median = 54%, upper/lower quartile = 41/63; AP5: $n = 5$ retinas, median = 22%, upper/lower quartile = 41/63; wash: $n = 5$ retinas, median = 36%, upper/lower quartile = 20/43.

Table S1. Summary of spectroscopic properties and partition coefficients for CS3, CR1–CR5, CF3, Ctr1-CR3, and Ctr1-CF3

Sensor	Cu(I)	Φ	ε· M^{-1}·cm^{-1}	λ_{abs}, nm	λ_{em}, nm	Fold turn-on	K_d	Log D
CS3	–	0.007	3.1×10^4	550 511	560		8.9×10^{-14} M	3.46
	+	0.400	4.6×10^4	540		75		
CR1	–	0.022	2.5×10^4 and 2.6×10^4	490 and 525	543		8.4×10^{-13} M	0.97
	+	0.138	5.5×10^4	515	543	5.3		
CR2	–	0.024	1.7×10^4	534	541		9.5×10^{-14} M	0.96
	+	0.098	4.9×10^4	518	541	3.7		
CR3	–	0.011	4.2×10^4	535	545		1.3×10^{-13} M	0.96
	+	0.15	8.5×10^4	529	545	12.6		
CR4	–	0.009	3.9×10^4	534	554		7.9×10^{-13} M	0.99
	+	0.144	4.7×10^4	530	554	7.4		
CR5	–	0.025	4.0×10^4 and 4.4×10^4	490 and 525	540		3.6×10^{-13} M	1.14
	+	0.068	8.1×10^4	515	540	3.1		
CF3	–	0.026	3.3×10^4 and 3.7×10^4	510 and 550	557		3.3×10^{-13} M	1.15
	+	0.219	6.9×10^4	534	557	40.4		
Ctrl-CR3	–	0.003	4.1×10^4	505	545		N/A	0.87
	+	0.003	4.1×10^4	505	545	N/A		
Ctrl-CF3	–	0.007	3.7×10^4 and 4.7×10^4	510 and 545	557		N/A	1.04
	+	0.007	3.7×10^4 and 4.7×10^4	510 and 545	557	N/A		

N/A, not applicable.