Visualizing Ascorbate-Triggered Release of Labile Copper within Living Cells using a Ratiometric Fluorescent Sensor

Dylan W. Domaille, Li Zeng, and Christopher J. Chang*

Department of Chemistry and the Howard Hughes Medical Institute, University of California, Berkeley, CA 94720

Synthetic Materials and Methods. All reactions were carried out under an atmosphere of dry nitrogen and stirred magnetically. 2,2'-(Mesitylmethylene)bis(1*H*-pyrrole) **(2)** was synthesized according to the method of Laha *et al*.[1] Receptor **7** was synthesized according to our previously published procedure.[2] Tris((ethylthio)ethyl)amine (TEMEA) was synthesized according to a previously published procedure.[3] Silica gel P60 (SiliCycle) was used for column chromatography. Analytical thin layer chromatography was performed using SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. ^{1}H NMR spectra were collected in $CDCl_3$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker AV-300 or AVQ-400 spectrometer 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 $CDCl_3$ as an internal reference. Mass spectral analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

1,1'-Dichloro-5-mesityl-dipyrromethane (3). A solution of **2** (1.06 g, 4.0 mmol) in THF (80 mL) was wrapped in foil to protect it from light, purged with N_2 for 10 min, and cooled to -78 °C. *N*-Chlorosuccinimide (1.06 g, 8.0 mmol) in THF (80 mL) (also protected from light) was added via cannula transfer over the course of 1 h. The reaction was stirred for an additional 2 h at -78 °C, capped, and placed in the freezer (-20 °C) for 18 h. The olive-colored solution was concentrated, and the residue was diluted with dichloromethane (100 mL) and washed with water (2 × 20 mL). The layers were separated and the organic phase was dried over Na_2SO_4. Column chromatography (silica gel, 1:1 hexanes/dichloromethane) provided **3** as a brown oil (740 mg, 56%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.78 (2H, s), 6.92 (2H, s), 6.00 (2H, t, J = 3.6 Hz), 5.94 (2H, t, J = 2.7 Hz), 5.77 (1H, s), 2.32 (3H, s), 2.13 (6H, s). FAB-MS: calculated for [M^+] 332, found 332.

1,9-Dichloro-5-mesityl-dipyrromethene (4). Compound **3** (720 mg, 2.16 mmol) was dissolved in dichloromethane (25 mL) and the solution was purged for 5 min with N_2. A suspension of DDQ (539 mg, 2.38 mmol) in dichloromethane (10 mL) was added dropwise over the course of 5 min. The reaction was stirred for 1 h at room temperature whereupon saturated $NaHCO_3$ (20 mL) was added. The layers were separated, the organic phase washed with water (20 mL), dried over Na_2SO_4, and concentrated to a red-brown solid. Column chromatography (silica gel, 4:1 hexanes/dichloromethane) afforded **4** as reddish-brown needles (500 mg, 70%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.91 (2H, s), 6.33 (2H, d, J = 4.2 Hz), 6.18 (2H, d, J = 4.2 Hz), 2.27 (3H, s), 2.06 (6H, s). FAB-MS: calculated for [M^+] 331, found 331.

3,5-Dichloro-8-mesityl-BODIPY (5). To a solution of **4** (500 mg, 1.50 mmol) in dichloromethane (60 mL) was added NEt_3 (2.1 mL, 15.1 mmol) in one portion. The resulting mixture was stirred for 1 h at room temperature. $BF_3{\bullet}OEt_2$ (3.75 mL, 30.2 mmol) was added via syringe and the reaction was stirred in the dark for 12 h. Water (20 mL) was added and the layers were separated. The organic phase was dried over Na_2SO_4 and concentrated to dryness. Purification by column chromatography (silica gel, 1:1 dichloromethane/hexanes) gave **5** (525 mg, 92%) as an orange, crystalline solid. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.50 (2H, s), 6.60 (2H, d, *J* = 4.3 Hz), 6.36 (2H, d, *J* = 4.3 Hz), 2.35 (3H, s), 2.08 (6H, s). HR-FABMS calculated, 378.0673, found 378.0683.

3-Chloro-5-methoxy-8 mesityl-BODIPY (6). A solution of **5** (100 mg, 0.27 mmol) in THF (10 mL) was cooled to 0 °C and 30 wt % sodium methoxide (54 μL, 0.29 mmol) in methanol (15 mL) was added dropwise over 30 min. The reaction was maintained at 0 °C for 2 h, or until complete by TLC analysis. The mixture was diluted with dichloromethane (50 mL) and washed with water (2 × 20 mL). The organic phase was separated, dried over Na_2SO_4 and concentrated to dryness. Purification by flash chromatography (silica gel, 98:2 dichloromethane/ethyl acetate) delivered **6** as a bright orange solid (100 mg, 99%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.92 (2H, s), 6.70 (1H, d, *J* = 4.5 Hz), 6.31 (1H, d, *J* = 3.7 Hz), 6.20 (1H, d, *J* = 3.9 Hz), 6.10 (1H, d, *J* = 4.7 Hz), 4.14 (3H, s), 2.34, (3H, s), 2.07 (6H, s). HR-FABMS calculated, 374.1169, found 378.1178.

Ratio-Coppersensor-1, RCS1, (1). A 25-mL Schlenk tube was charged with **6** (100 mg, 0.267 mmol) and **7** (345 mg, 1.08 mmol). CH_3CN (1.0 mL) was added via syringe and the reaction was stirred at 45 °C, in the dark, for 72 h. Concentration of the reaction mixture and purification by column chromatography (silica gel, 20:1 toluene/acetonitrile, collected in 1 mL fractions) furnished sensor **1** as a dark red film (48 mg, 27%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.90 (2H, s), 6.46 (1H, d, *J* = 4.8 Hz), 6.15 (1H, d, *J* = 4.2 Hz), 5.95 (1H, d, *J* = 4.8 Hz), 5.64 (1H, d, *J* = 3.9 Hz), 3.98 (3H, s), 3.93 (4H, t, *J* = 7.2 Hz), 2.90 (12H, m), 2.58 (4H, q, *J* = 7.5 Hz), 2.33 (3H, s), 2.09 (6H, s), 1.25 (6H, t, *J* = 7.5 Hz). HR-FABMS calculated, 651.2428, found 651.2413.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7.0. Absorption spectra were recorded on a Varian Cary 50 spectrophotometer (Walnut Creek, CA) and fluorescence spectra were recorded on a Photo Technology International Quanta Master 4 L-format scan spectrofluorometer (Lawrenceville, NJ) 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 (3.5-mL volume, Starna, Atascadero, CA). Metals used in the selectivity assay were derived from their chloride salts, with the exception of Fe(II), which was derived from $[NH_4]_2[Fe][SO_4]_2{\bullet}6H_2O$. Fluorescence quantum yields were determined by reference to rhodamine 101 inner salt in methanol (Φ = 1.0).[4] The binding affinity of Cu^+ to RCS1 dye was measured using thiourea as a competitive ligand to provide a buffered Cu^+ solution. Briefly, a 2 μM solution of RCS1

was made in 20 mM HEPES, pH 7.0 and buffered with a known concentration of thiourea from a 500 mM stock in MilliQ H_2O. Cu(I) was delivered in the form of $[Cu(MeCN)_4][PF_6]$ from an acetonitrile stock solution (2 mM). The maximum acetonitrile concentration was 0.1%; this concentration of acetonitrile is not high enough to effectively compete with RCS1 for Cu^+. Stability constants for thiourea binding were taken from the literature: $\beta_{12} = 2.0 \times 10^{12}$, $\beta_{13} = 2.0 \times 10^{14}$, $\beta_{14} = 3.4 \times 10^{15}$.[5] Excitation was provided at 480 nm and the emission was integrated over 490-525 and 525-650 nm. The apparent dissociation constant (K_d) was 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^+:RCS1 complex; F_{min} is the fluorescence for RCS1; and $[Cu^+]$ is the 'free' Cu^+ available for complexation, which was calculated using the stability constants for thiourea and standard competition equilibrium expressions.

Fluorescence Imaging Experiments. Confocal imaging studies were performed on a Zeiss LSM510 NLO Axiovert 200 laser scanning microscope and a 40x Achroplan IR water-immersion objective lens at the Molecular Imaging Center at UC Berkeley. Excitation of RCS1-loaded cells at 488 nm was carried out with an Ar laser and emission between 506-720 nm was collected using a META detector operating in lambda mode with 10 nm collection windows. Excitation of Hoechst 33342 was carried out using a MaiTai two-photon laser at 780-nm pulses (mode-locked Ti:sapphire laser, Tsunami Spectra Physics) and emission was collected between 452-538 nm. Image analysis was performed with ImageJ.

Preparation and Staining of Cell Cultures. HEK 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). C6 cells were cultured in DMEM with GlutaMAX (Gibco) with 10% fetal bovine serum. One day before imaging, cells were passaged and plated on 18-mm coverslips in DMEM without Phenol Red containing 1 mM sodium pyruvate. For Cu-supplementation experiments, 100 μM $CuCl_2$ was added directly to the media 6 h prior to imaging. Untreated and treated coverslips were stained simultaneously in a single 35-mm Petri dish containing 2 μM RCS1 (from a 2 mM stock in DMSO) in DMEM. Coverslips were incubated at 37 °C for 10 minutes after which the dye-loaded media was replaced with fresh DMEM and imaged as described above. BCS (200 μM) and/or 1 mM L-ascorbic acid were added directly to the media 4 h prior to imaging and processed in an identical fashion to the Cu-supplemented coverslips. The TEMEA chelator was added directly to the imaging media from a 200 mM stock solution in DMSO and gently agitated to ensure a homogenous application.

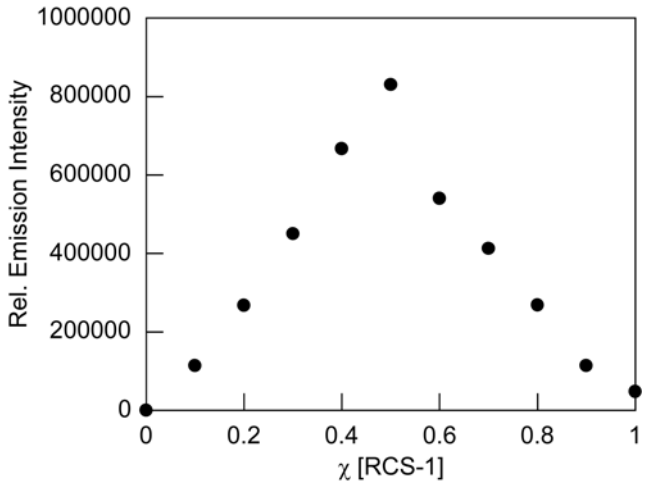

Figure S1: Job's plot of RCS1 and Cu^{+}. The total concentration of RCS1 and Cu^{+} were kept at a constant 2 μM. Excitation was provided at 490 nm and emission intensity was measured at 560 nm. Spectra were acquired in 20 mM HEPES, pH 7.0. The maximum fluorescence response at 0.5 mol fraction of RCS1 indicates formation of a 1:1 Cu^{+}:RCS1 complex.

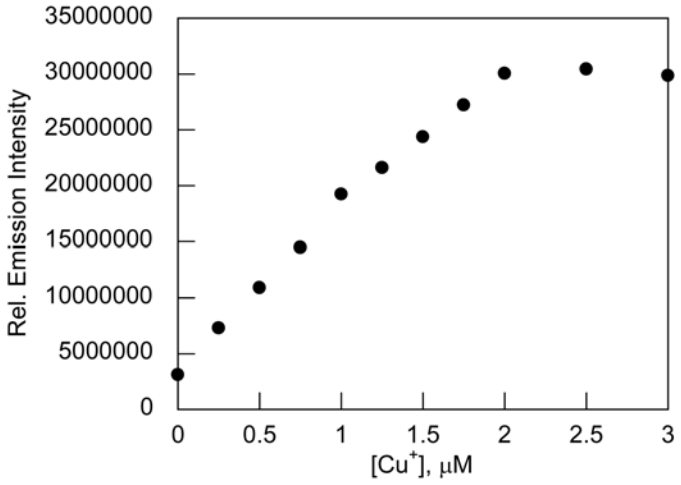

Figure S2: Normalized fluorescence response of 2 μM RCS1 to Cu^{+}. Excitation was provided at 490 nm and the emission intensity was measured at 560 nm. Spectra were acquired in 20 mM HEPES, pH 7.0. The break at 2 μM added Cu^{+} (1 equiv) is consistent with formation of a 1:1 Cu^{+}:RCS1 complex.

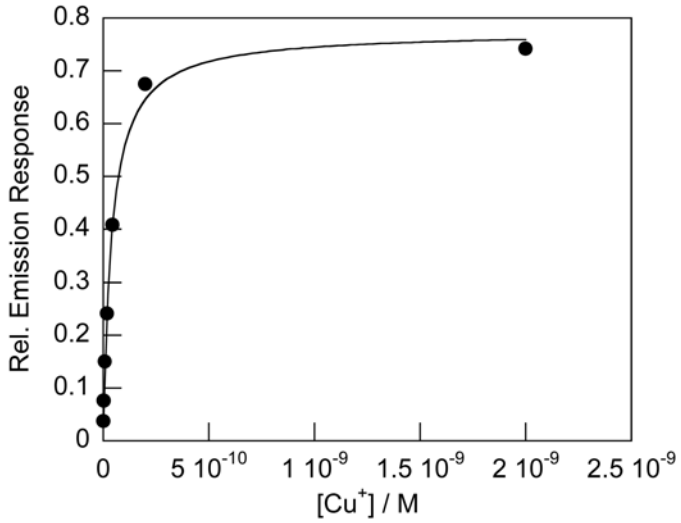

Figure S3: Normalized fluorescence response of 2 μM RCS1 to thiourea buffered Cu^+ solutions for K_d value determination. Excitation was provided at 490 nm and the collected emission was monitored at 560 nm. Spectra were acquired in 20 mM HEPES, pH 7.0. The points shown are for free Cu^+ buffered at 2.2, 4.4, 8.7, 20, 45, 200 and 2000 pM, respectively. The observed K_d value is 4.0(3) x 10^{-11} M.

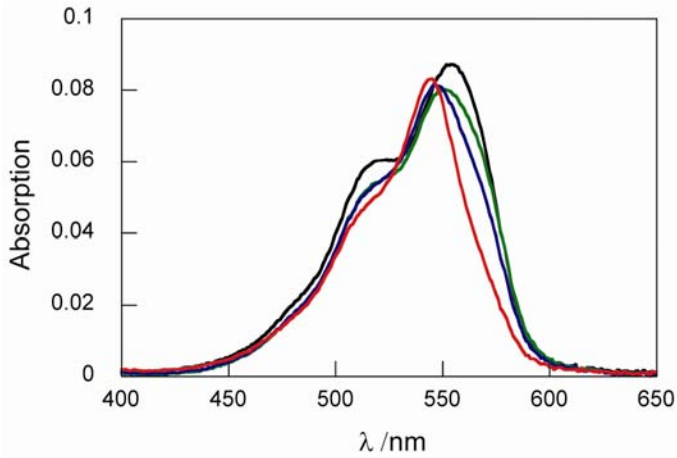

Figure S4: Absorption response of 2 μM RCS1 to Cu^{+}. Spectra shown are for 2 μM RCS1 only (black trace), and 2 μM RCS1 in the presence of 0.5 μM Cu^{+} (green trace), 1.0 μM Cu^{+}(blue trace), and 2.0 μM Cu^{+} (red trace). Spectra were acquired in 20 mM HEPES, pH 7.

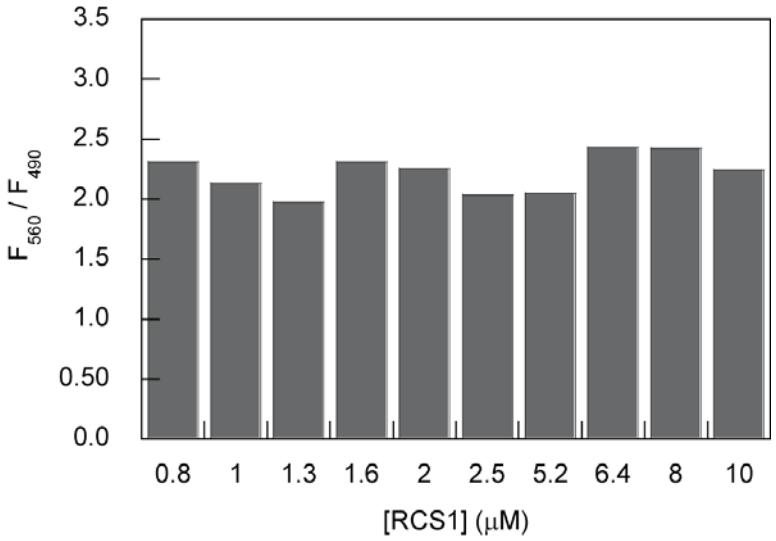

Figure S5: The fluorescence emission ratio from RCS1 over a range of concentations. Excitation was provided at 480 nm and the emission was collected over 490-650 nm. The integrated emission from 530-650 (F_{560}) was divided by the integrated emission from 490-530 nm (F_{490}) to provide the fluorescence ratio. Data were collected in 20 mM HEPES, pH 7.

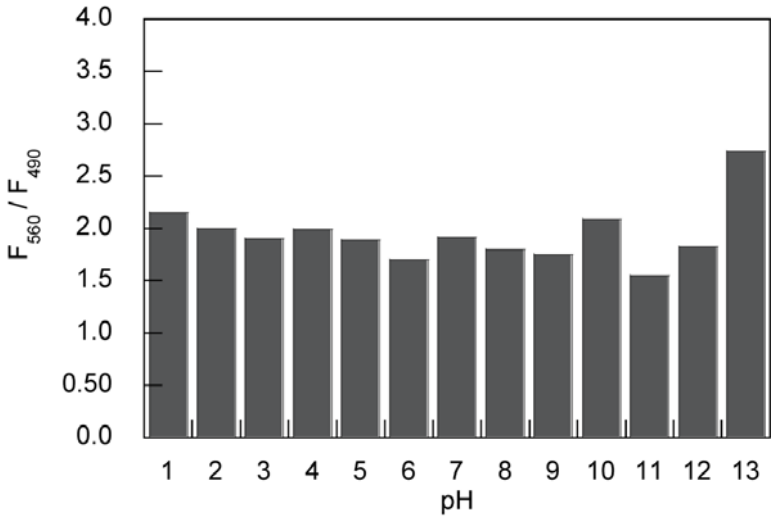

Figure S6: The fluorescence emission ratio from a 2 μM solution of RCS1 over a range of pH values. Bars represent the ratio generated from the integrated emission from 530 – 650 nm (F_{560}) over the integrated emission from 490 – 530 nm (F_{490}). Data were collected in 20 mM HEPES at the indicated pH.

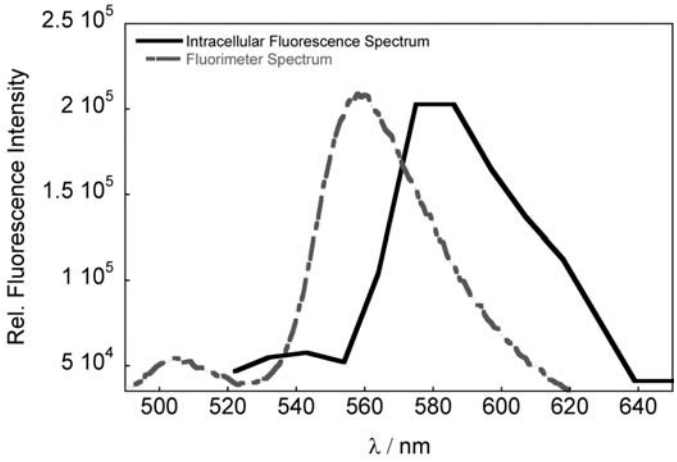

Figure S7: Normalized fluorescence emission scan (solid line) from RCS1 in Cu-supplemented HEK 293T cells. The median intensity was measured in 10 nm windows and plotted versus wavelength. A fluorimeter scan (dashed line) of RCS1 with 0.5 μM Cu^+ in 20 mM HEPES buffer, pH 7.0 is plotted on the same axes for comparison. The emission characteristics of the dye are red-shifted ca. 25 nm in a cellular environment relative to the emission characteristics of the dye in aqueous, buffered solution, but the ratio of the two bands remains constant between cuvette and cellular assays. The data obtained from the lambda scan were used to determine the META detector collection windows of 564 – 639 nm for the Cu^+-responsive peak, and 522 – 554 for the reference peak.

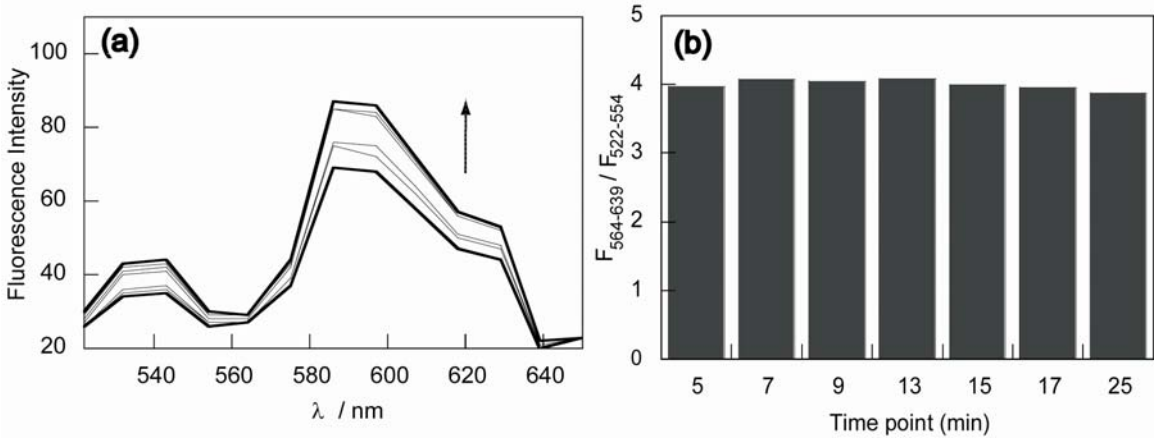

Figure S8: Fluorescence intensity and fluorescence ratio values from RCS1 over time upon loading into living cells. HEK 293T cells were incubated in DMEM with 2 μM RCS1 and λ scans were acquired at different time points during the dye uptake process. (a) λ scans showing a modest increase in intracellular fluorescence during the intracellular accumulation of RCS1 from a bath application of 2 μM RCS1 in DMEM. Intracellular spectra shown are for 5, 7, 9, 13, 15, 17, and 25 minutes after RCS1 addition. (b) Bars represent the ratio value obtained from the λ scans in (a). The ratio was generated by integrating the signal from 564 – 639 nm ($F_{564-639}$) and dividing it by the integrated signal from 522 - 554 nm ($F_{522-554}$). Notably, the ratio remains constant over time.

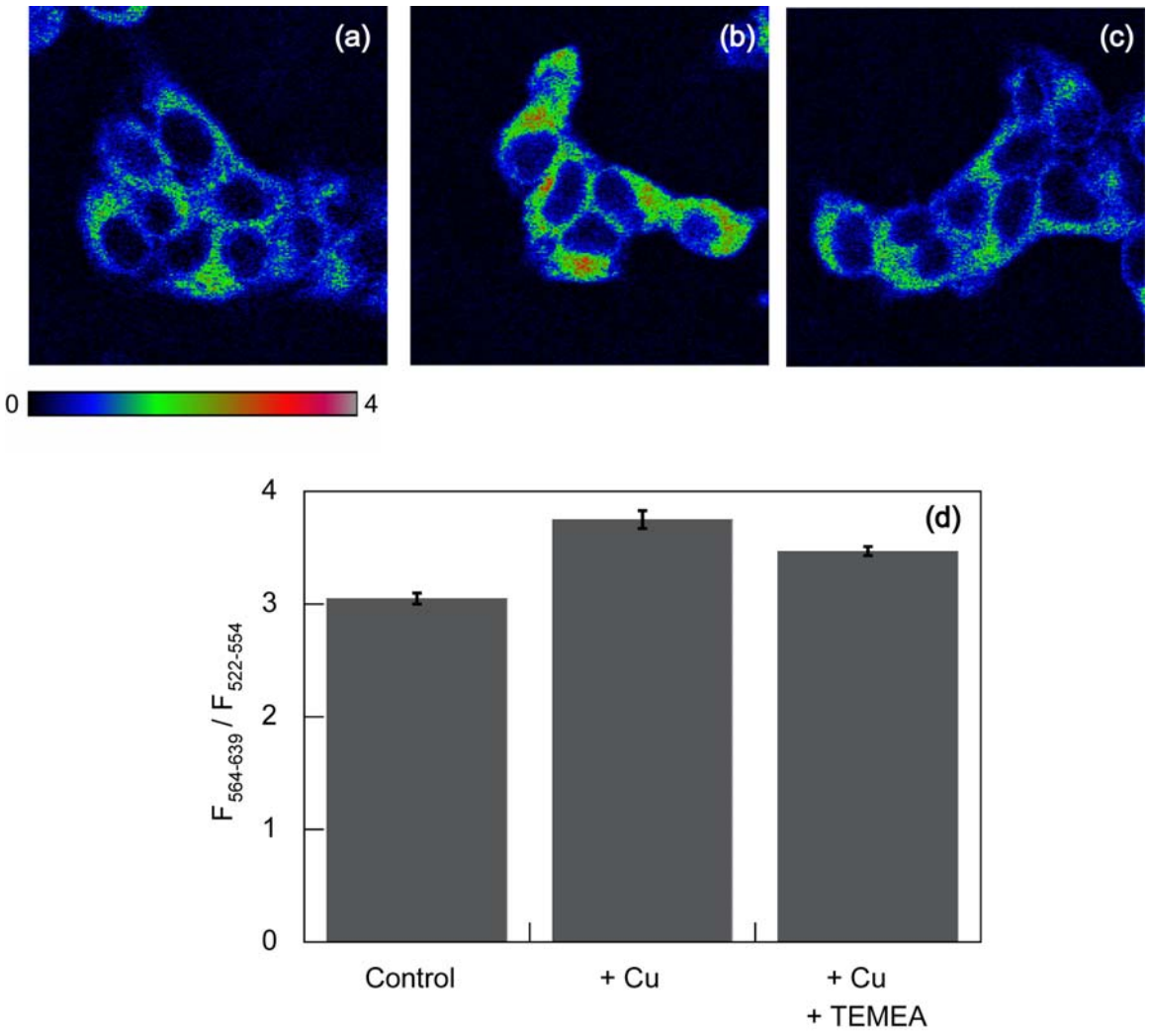

Figure S9: Ratio confocal microscopy images of Cu-supplemented HEK 293T cells with RCS1. Excitation was provided at 488 nm (79.2% laser power; 676 detector gain, 352 μm pinhole, 2.51 μs pixel time) and the emission was collected from 506-720 nm in 10 nm increments. Projection images were calculated in Image J by summing the total intensity between 522-554 nm (reference emission) and 564-639 nm (Cu-responsive peak). Ratio images were generated using the RatioPlus plugin ($I_{564\text{-}639} / I_{522\text{-}554}$) and the minimum and maximum display values were set to 2 and 10, respectively. All images were acquired with identical microscope settings. (a) HEK 293T cells grown in basal media and stained with 2 μM RCS1 for 10 min at 37 °C. (b) HEK 293T cells incubated with 100 μM $CuCl_2$ for 6 h at 37 °C and stained with 2 μM RCS1 for 10 min at 37 °C. (c) HEK 293T cells from condition (b) treated with 1 mM tris((ethylthio)ethyl)amine for 5 min by direct addition to the Petri dish on the microscope stage. (d) Bars represent the mean ratio generated from the total integrated density from 564-639 nm over the total integrated density from 522-554 nm. Values were generated from the analysis of five randomly selected fields of cells; error bars represent standard error measurement (s.e.m.).

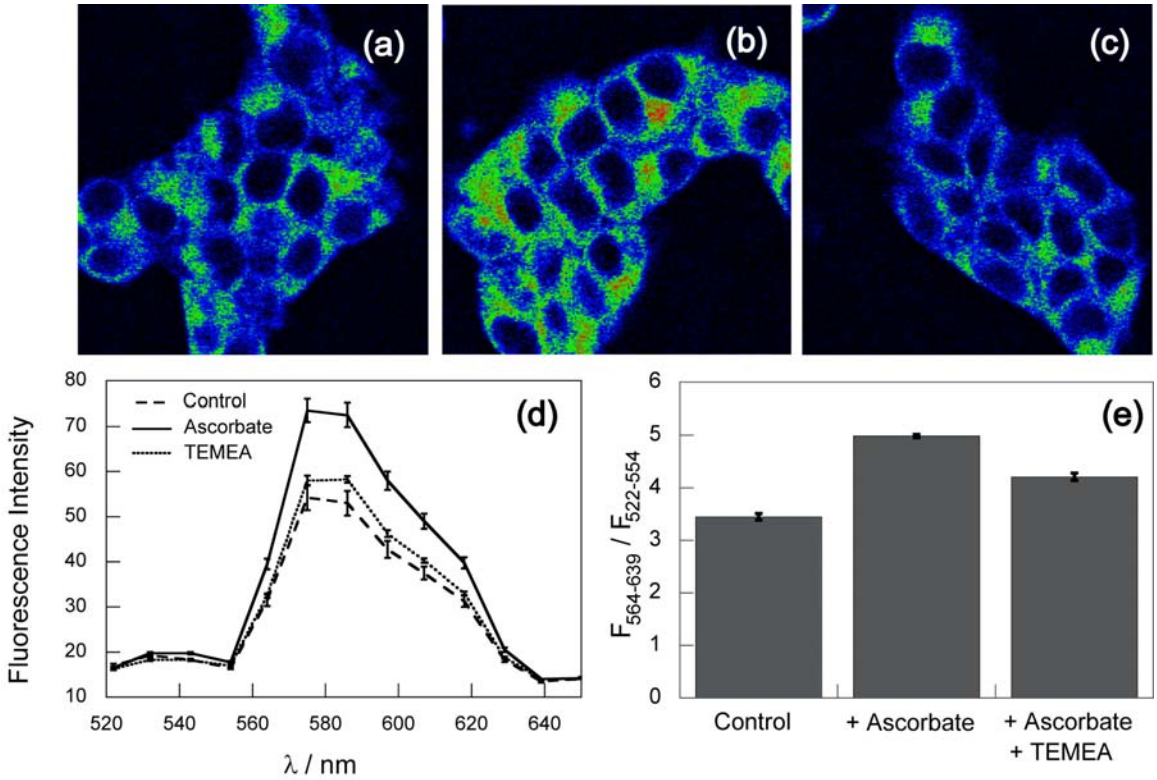

Figure S10: (a) Ratio confocal microscopy images of ascorbate-supplemented HEK 293T cells with RCS1. Excitation was provided at 488 nm (79.2% laser power; 676 detector gain, 352 μm pinhole, 2.51 μs pixel time) and the emission was collected from 506-720 nm in 10 nm increments. Projection images were calculated in Image J by summing the total intensity between 522-554 nm (reference emission) and 564-639 nm (Cu-responsive peak). Ratio images were generated using the RatioPlus plugin ($I_{564\text{-}639}$ / $I_{522\text{-}554}$) and the minimum and maximum display values were set to 2 and 10, respectively. All images were acquired with identical microscope settings. (a) HEK 293T cells stained with 2 μM RCS1 for 10 min at 37 °C. (b) HEK 293T cells incubated 1 mM ascorbic acid for 4 h at 37 °C and stained with 2 μM RCS1 for 10 min at 37 °C. (c) HEK 293T cells from condition (b) treated with 1 mM tris((ethylthio)ethyl)amine for 5 min by direct addition to the Petri dish on the microscope stage. (d) Lambda scans collected in 10 nm windows from 522 nm to 649 nm with fluorescence intensities measured in each frame and plotted versus wavelength. All values are the average fluorescence intensity from five randomly selected fields. Error bars represent standard error measurement (s.e.m). (e) Bar graph representing the integrated intensity from 564-649 nm over the integrated fluorescence intensity from 522-554 nm. Values are the mean ratio generated from the intensity from five randomly selected fields. Error bars represent standard error measurement (s.e.m).

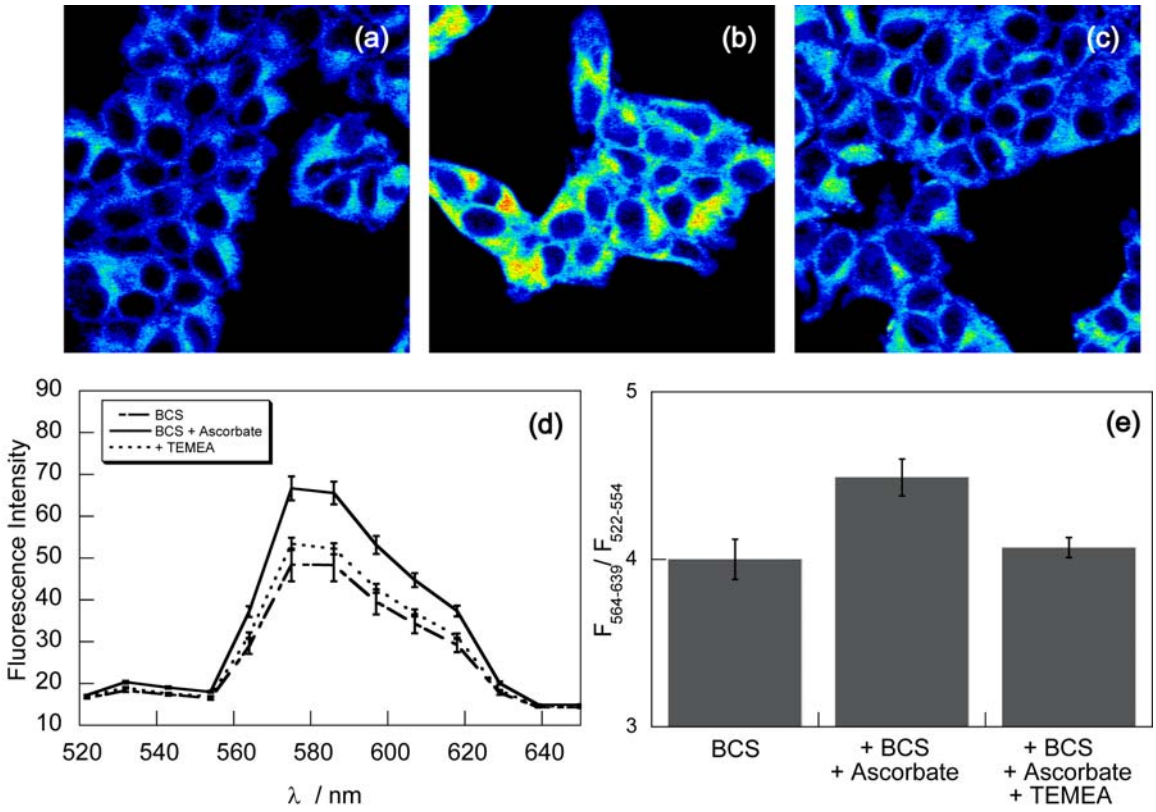

Figure S11: Ratio confocal microscopy images of ascorbate-supplemented HEK 293T cells with RCS1 with extracellular Cu^{+} chelation . Excitation was provided at 488 nm (79.2% laser power; 676 detector gain, 352 μm pinhole, 2.51 μs pixel time) and the emission was collected from 506-720 nm in 10 nm increments. Projection images were calculated in Image J by summing the total intensity between 522-554 nm (reference emission) and 564-639 nm (Cu-responsive peak). Ratio images were generated using the RatioPlus plugin ($I_{564\text{-}639}$ / $I_{522\text{-}554}$) and the minimum and maximum display values were set to 2 and 10, respectively. All images were acquired with identical microscope settings. (a) HEK 293T cells grown in media supplemented with 200 μM BCS for 4 h at 37 °C and stained with 2 μM RCS1 for 10 min at 37 °C. (b) HEK 293T cells incubated with 200 μM BCS and 1 mM ascorbic acid for 4 h at 37 °C and stained with 2 μM RCS1 for 10 min at 37 °C. (c) HEK 293T cells from condition (b) treated with 1 mM tris((ethylthio)ethyl)amine for 5 min by direct addition to the Petri dish on the microscope stage. (d) Lambda scans collected in 10 nm windows from 522 nm to 649 nm with fluorescence intensities measured in each frame and plotted versus wavelength. All values are the average fluorescence intensity from five randomly selected fields. Error bars represent standard error measurement (s.e.m). (e) Bars represent the mean ratio generated from the total integrated density from 564-639 nm over the total integrated density from 522-554 nm. Values were generated from the analysis of five randomly selected fields of cells; error bars represent standard error measurement (s.e.m.).

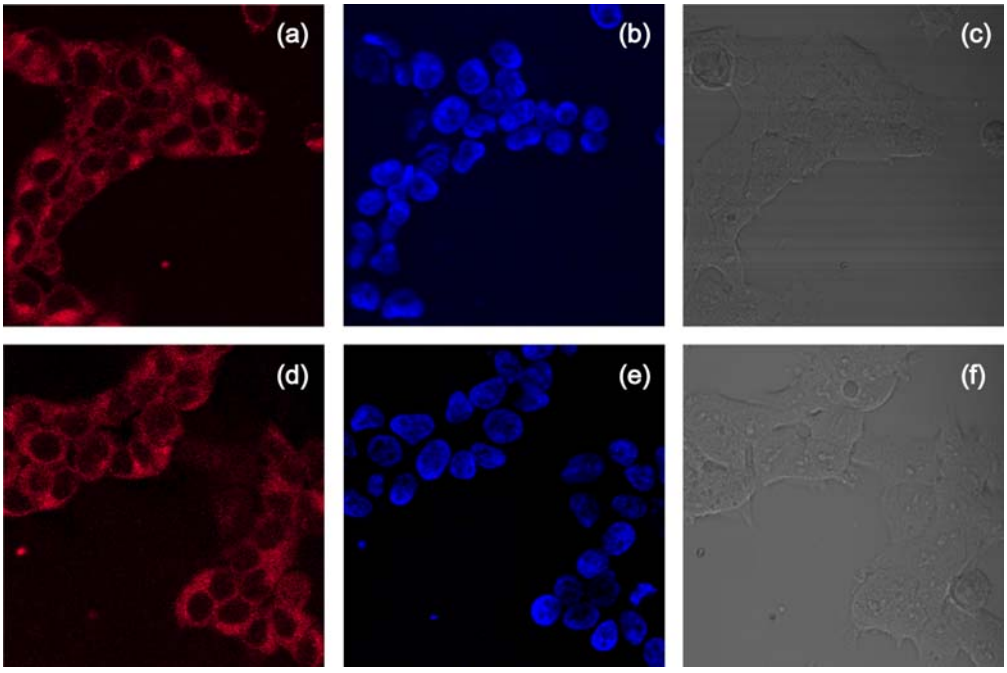

Figure S12: Viability studies of HEK 293T cells with ascorbate and Cu-supplementation. Shown above are signals from RCS1 (a), Hoescht 33342 (b) and a brightfield image (c) of cells treated with 1 mM ascorbic acid for 4 h and 1 mM tris((ethylthio)ethyl)amine for 5 min. Signals from RCS1 (d), Hoescht 33342 (e) and a brightfield image (f) of HEK 293T cells treated with 100 μM $CuCl_2$ for 6 h and 1 mM tris((ethylthio)ethyl)amine for 5 min.

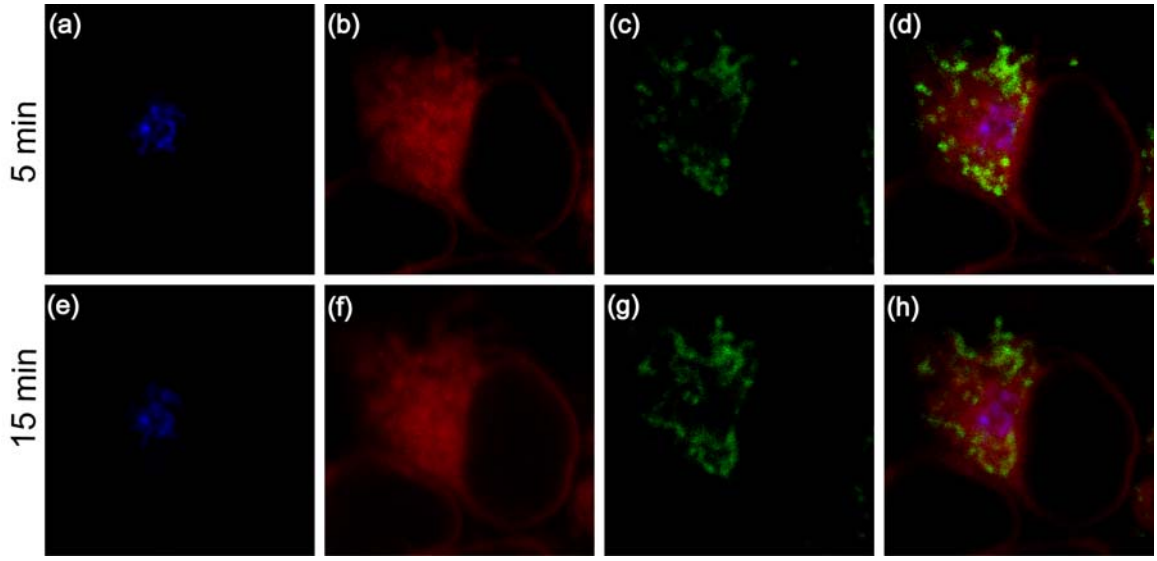

Figure S13: Time course examination of RCS1 distribution characteristics. HEK 293T cells were incubated with BODIPY FL C5-ceramide complexed to BSA (1.25 μM, 30 min) to label the Golgi, and MitoTracker Deep Red (50 nM, 30 min) to label the mitochondria. Organelle stains added directly to the culture media. After 30 min at 37 ºC, the coverslip was transferred to fresh DMEM containing 2 μM RCS1. Shown above are signals from (a) BODIPY ceramide (488 nm laser excitation, 68% laser power, 706 gain, 69 μm pinhole, 3.20 μs pixel time) with emission collected from 501 to 533 nm; (b) MitoTracker Deep Red (633 nm laser excitation, 58.4% laser power, 785 detector gain, 196 μm pinhole, 3.20 μs pixel time) with emission collected from 672 to 715 nm; (c) RCS1 (543 nm laser excitation, 75.3% laser power, 730 detector gain, 376 μm pinhole, 3.20 μs pixel time) with emission collected from 554 nm to 576 nm; (d) overlay of (a), (b), and (c). After RCS1 had been present for 15 min, the series of images were collected again. Images show signal from (e) BODIPY ceramide complexed to BSA (same settings as (a)), (f) MitoTracker Deep Red (same settings as (b)), (g) RCS1 (same settings as (c), except 680 detector gain), and (h) an overlay of (e), (f), and (g). The settings were adjusted at the 15 min time point to provide a similar intensity as the 5 min time point to facilitate the comparison of spatial distribution.

Table S1. Stability constants (logK) of metal chelators for Cu(I) and Cu(II) complexes

	$\log K_{Cu(I)L}$	$\log K_{Cu(II)L}$
Bathocuproine Disulfonate (BCS)[a]	~20	7.5
Tris((ethylthio)ethyl)amine (TEMEA)[b]	15.53	6.35
Ratio-Coppersensor-1 (RCS1)[c]	10.4	*n.d.*

[a] Cherny, R. A.; Barnham, K. J.; Lynch, T.; Volitakis, I.; Li, Q.-X.; McLean, C. A.; Multhaup, G.; Beyreuther, K.; Tanzi, R. E.; Masters, C. L.; Bush, A. I. *J. Struct. Biol.* **2000**, *130*, 209-216. [b] Ambundo, E. A.; Deydier, M.-V.; Grall, A. J.; Aguera-Vega, N.; Dressel, L. T.; Cooper, T. H.; Heeg, M. J.; Ochrymowycz, L. A.; Rorabacher, D. B. *Inorg. Chem.* **1999**, *38*, 4233-4242. [c] This work. *n.d.* = not determined.

References

(1) Laha, J. K.; Dhanalekshmi, S.; Taniguchi, M.; Ambroise, A.; Lindsey, J. S. *Org. Proc. Res. Dev.* **2003**, *7*, 799-812.

(2) Zeng, L.; Miller, E. W.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2006**, *128*, 10-11.

(3) Cooper, T. H.; Mayer, M. J.; Leung, K. H.; Ochrymowycz, L. A.; Rorabacher, D. B. *Inorg. Chem.* **1992**, *31*, 3796-3804.

(4) Karstens, T.; Kobs, K. *J. Phys. Chem.* **1980**, *84*, 1871-1872.

(5) Martell, A. E.; Smith, R. M. *Critical Stability Constants*; Plenum Press: New York, 1989.