Dynamic fluxes of s-block metals like potassium, sodium, and calcium are of broad importance in cell signaling. In contrast, the concept of mobile transition metals triggered by cell activation remains insufficiently explored, in large part because metals like copper and iron are typically studied as static cellular nutrients and there are a lack of direct, selective methods for monitoring their distributions in living cells. To help meet this need, we now report Coppersensor-3 (CS3), a bright small-molecule fluorescent probe that offers the unique capability to image labile copper pools in living cells at endogenous, basal levels. We use this chemical tool in conjunction with synchotron-based microprobe X-ray fluorescence microscopy (XRFM) to discover that neuronal cells move significant pools of copper from their cell bodies to peripheral processes upon their activation. Moreover, further CS3 and XRFM imaging experiments show that these dynamic copper redistributions are dependent on calcium release, establishing a link between mobile copper and major cell signaling pathways. By providing a small-molecule fluorophore that is selective and sensitive enough to image labile copper pools in living cells under basal conditions, CS3 opens opportunities for discovering and elucidating functions of copper in living systems.

Along these lines, molecular imaging with copper-responsive fluorescent sensors offers a potentially powerful methodology for interrogating its cell biology by allowing the specific tracking of copper pools in living cells with spatial and temporal resolution (12, 26–32). In this regard, analogous tools have revolutionized the study of calcium in a variety of biological settings (1) and hold promise for interrogating other cellular metals (26). However, fluorescent-based sensing of Cu$^+$, the oxidation state stabilized in reducing cytosolic environments, presents several additional challenges that make it more difficult to detect compared to other abundant metal ions in cells (e.g., Na$^+$, K$^+$, Cu$^{2+}$, Mg$^{2+}$, Zn$^{2+}$). The most prominent of these challenges include (i) redox specificity over Cu$^{2+}$, the other major oxidation state for biological copper, (ii) the propensity for Cu$^+$ in water to disproportionate to Cu$^{2+}$ and Cu metal, and (iii) the ability of Cu$^+$ to quench fluorescence by electron and/or energy transfer. Indeed, of the growing number of reported strategies for fluorescence copper detection (12, 26), only three synthetic sensors, CTAP-I (29), CS1 (30, 31), and RCS1 (32), and two protein-based sensors (33, 34) have been validated for live-cell imaging with Cu$^{2+}$. Moreover, the relatively low quantum efficiencies of the first-generation synthetic reagents (Φ ≤ 0.14 in Cu$^{2+}$-bound forms) have limited their use to date for cellular imaging under conditions of prolonged copper overload or depletion.

Here, we present the synthesis, properties, and applications of Coppersensor-3 (CS3), a bright fluorescent sensor that now offers the unique ability to detect labile copper pools at basal, endogenous levels in living cells. This BODIPY-based probe features high selectivity over competing cellular metal ions, including redox differentiation between Cu$^+$ and Cu$^{2+}$, visible wavelength excitation and emission profiles, and a 75-fold fluorescence turn-on response with high quantum efficiency (Φ = 0.40) for Cu$^+$ detection. By using this chemical tool in conjunction with synchotron-based microprobe X-ray fluorescence microscopy (XRFM) in a combined imaging study, an approach that has been successfully employed for monitoring resting copper distributions in mammalian cells (29), we have discovered that neuronal cells trigger a marked translocation of copper pools from their cell bodies to extended outer processes when activated by depolarization. Moreover, additional CS3 and microprobe XRFM studies show that these dynamic copper movements are dependent on the


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Calcium-dependent copper redistributions in neuronal cells revealed by a fluorescent copper sensor and X-ray fluorescence microscopy

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Metals are essential components of all living cells, and in many cases cells trigger and utilize dynamic metal movements for signaling purposes. Such processes are well established for alkali and alkaline earth metals like potassium, sodium, and calcium (1–3) but not for transition metals like copper and iron, which are traditionally studied for their roles as static cofactors in enzymes (4–6). We have initiated a program aimed at exploring the concept of mobile transition metals and their contributions to cell physiology and pathology, and in this context, brain neurons offer an attractive model for this purpose owing to their widespread use of potassium and sodium ion channels and calcium release for signaling events (7), as well as a high requirement for dynamic fluxes of s-block metals like potassium, sodium, and calcium are of broad importance in cell signaling. In contrast, the concept of mobile transition metals triggered by cell activation remains insufficiently explored, in large part because metals like copper and iron are typically studied as static cellular nutrients and there are a lack of direct, selective methods for monitoring their distributions in living cells. To help meet this need, we now report Coppersensor-3 (CS3), a bright small-molecule fluorescent probe that offers the unique capability to image labile copper pools in living cells at endogenous, basal levels. We use this chemical tool in conjunction with synchotron-based microprobe X-ray fluorescence microscopy (XRFM) to discover that neuronal cells move significant pools of copper from their cell bodies to peripheral processes upon their activation. Moreover, further CS3 and XRFM imaging experiments show that these dynamic copper redistributions are dependent on calcium release, establishing a link between mobile copper and major cell signaling pathways. By providing a small-molecule fluorophore that is selective and sensitive enough to image labile copper pools in living cells under basal conditions, CS3 opens opportunities for discovering and elucidating functions of copper in living systems.

fluorescent sensor | molecular imaging | mobile metals | transition metal signaling

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release of calcium, establishing a link between mobile copper and major cell signaling pathways. The combined advances in optical brightness and turn-on response for CS3 afford a host of opportunities for studying the cell biology of copper by providing the ability to visualize labile copper pools in living cells under basal and stimulated conditions.

Results and Discussion
Design, Synthesis, and Spectroscopic Evaluation of Coppersensor-3 (CS3), a Bright Fluorophore for Selective Cu(II) Detection. We previously reported Coppersensor-1 (CS1), a first-generation, selective turn-on fluorescent sensor for aqueous Cu\(^{2+}\) with visible excitation and emission profiles, and demonstrated its utility for live-cell imaging (30). This reporter shows good selectivity for Cu\(^{2+}\) over other cellular metal ions at physiologically relevant concentrations, a robust 10-fold fluorescence enhancement upon Cu\(^{2+}\) complexation, and allows for the visualization of Cu\(^{2+}\) in live mammalian cells under conditions of acute copper overload. However, attempts to use CS1 to interrogate the dynamics of endogenous cellular copper pools at basal levels were limited by the relatively low quantum yield of the CS1:Cu\(^{2+}\) complex (Φ = 0.13). Seeking to maintain high Cu\(^{2+}\) specificity while improving optical brightness values and turn-on responses, we reasoned that increasing electron density on the fluorophore reporter would favor a greater turn-on enhancement through a brighter Cu\(^{2+}\)-dye complex. Nagano’s laboratory has previously shown that substitution of fluoro substituents with metthoxy groups on the boron center of BODIPY fluorophores offers a practical strategy for tuning electronic properties of these fluorescent dyes (35). Fig. 1 details the synthetic route to Coppersensor-3 (CS3) based on these design considerations.

Spectroscopic analysis of apo CS3 in 20 mM HEPES buffered to pH 7 reveals one major peak in the visible region at 550 nm (ε = 3.1 × 10^4 cm\(^{-1}\) M\(^{-1}\)) with a shoulder at 511 nm. Maximal emission occurs at 560 nm with weak fluorescence (Φ = 0.007). Addition of Cu\(^{2+}\) leads to a blue shift in the absorption band to 540 nm (ε = 4.6 × 10^4 cm\(^{-1}\) M\(^{-1}\)) and a large 75-fold increase in fluorescence intensity (Φ = 0.40), with a corresponding hypsochromic shift in emission maximum to 548 nm (Fig. 2A). These values represent a significant improvement over the first-generation CS1 dye (Cu\(^{2+}\)-bound Φ = 0.13, 10-fold turn-on response) that has implications for its practical utility in cell imaging experiments (vide infra). Binding analysis using the method of continuous variations (Job’s plot) indicates that a 1:1 Cu\(^{2+}\):dye complex is responsible for the turn-on fluorescence response observed for CS3 (SI Text). The apparent dissociation constant for the CS3:Cu\(^{2+}\) complex is 8.9(3) × 10^{-14} M in HEPES buffer at pH 7 (SI Text).

CS3 exhibits high selectivity for Cu\(^{2+}\), even in the presence of physiologically relevant concentrations of competing metal ions (Fig. 2B). The fluorescence response of CS3 to Cu\(^{2+}\) is not affected by the presence of 2 mM Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\), nor do these metal ions cause increases in the fluorescence signal. Moreover, other biologically abundant transition metal ions (50 μM Co\(^{2+}\), Fe\(^{3+}\), Mn\(^{2+}\), or Ni\(^{2+}\)) do not trigger false positives, nor do they interfere with Cu\(^{2+}\)-induced fluorescence enhancements for CS3. More importantly, 50 μM Cu\(^{2+}\) neither causes a fluorescence increase nor hinders the response of CS3 to Cu\(^{2+}\), indicating that CS3 maintains oxidation state specificity for Cu\(^{2+}\) over Cu\(^{2+}\). Finally, owing to the thioether groups in the sensor we have included a panel of trace soft heavy metal ions for selectivity studies, including Hg\(^{2+}\), Ag\(^{+}\), Tl\(^{+}\), and Pb\(^{2+}\) (6) (SI Text). Of these heavy metal ions, CS3 does show some turn-on response to Ag\(^{+}\) at high, nonphysiological levels, but the addition of Cu\(^{2+}\) reveals that Cu\(^{2+}\) can displace Ag\(^{+}\) from the sensor. The large fluorescent turn-on response of CS3 to Cu\(^{2+}\), in conjunction with its high selectivity in the presence of interfering ions, suggests that this tool is a promising reagent for imaging basal levels of exchangeable Cu\(^{2+}\) pools in living cells.

CS3 Is Capable of Imaging Labile Pools of Copper in Living Cells at Basal and Copper-Depleted Levels. The two previously reported turn-on small-molecule fluorescent probes for live-cell Cu\(^{2+}\) detection, CTAP-1 and CS1, are capable of detecting changes in labile intracellular copper levels, but their relatively low quantum efficiencies limit their use to visualizing differences under situations of acute or prolonged copper overload (29, 30). We reasoned that CS3, with its improved brightness and turn-on response to Cu\(^{2+}\), would provide the ability to report pools of intracellular, exchangeable Cu\(^{2+}\) at basal levels. We therefore sought to test whether this chemical tool could image labile copper stores under both basal and copper-depleted conditions. To this end we depleted cells of their endogenous copper stores by culturing them in media containing the membrane-impermeable chelator bathocuproine disulfonate (BCS). This treatment has been shown to mildly decrease copper levels within mammalian cells without compromising their viability (36). Accordingly, human embryonic kidney (HEK 293T) cells were grown either in normal media or in media containing 200 μM BCS for 20 h to make them copper depleted, stained with 2 μM CS3 for 10 min, and subsequently imaged by confocal microscopy (Fig. 3). Cells grown in normal control media exhibit markedly higher fluorescence signals compared to cells grown in the presence of BCS (Fig. 3 A and B), indicating that CS3 can respond to changes in basal, endogenous levels of exchangeable Cu\(^{2+}\) as well as sense differences between copper-depleted and copper-normal conditions. To provide further support that BCS targets copper selectively, we treated HEK 293T cells with BCS and then stained them with the Zn\(^{2+}\)-responsive dye FluoZin-3 AM. We find that the zinc levels as measured by the zinc probe are not statistically different in the BCS-treated cells relative to the control (SI Text). In addition, we treated HEK 293T cells with BCS and imaged total metal pools by XRFM. We find that copper levels are significantly decreased in BCS-treated cells relative to control cells, whereas phosphorus and zinc show the opposite trend (SI Text).

We also examined whether CS3 could report more acute changes in exchangeable intracellular copper pools by treating cells with 100 μM of the competing cell-permeable Cu\(^{2+}\)-chelator tris(ethylthio)ethylamine (TEMEA) and CS3 for 10 min. (Fig. 3C). The observed fluorescence signal was muted upon introduction of this competing copper ligand, indicating that CS3 responds reversibly to Cu\(^{2+}\) and can sense dynamic variations in kinetically labile Cu\(^{2+}\) pools in living mammalian cells. In addition, nuclear staining with Hoechst 33342 affirms that the viability of HEK 293T cells is not affected by CS3 staining or the manipulation of cellular copper status.

![Fig. 1. Synthesis of CS3.](image-url)
intracellular Cu that CS3 is capable of reporting dynamic changes in endogenous 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 appropriate metal ion (2 mM for Ca^{2+}, Mg^{2+}, and Zn^{2+}; 50 μM for all other cations) to a 4 μM solution of CS3. Black bars represent the subsequent addition of 4 μM Cu^{2+} to the solution. Excitation was provided at 530 nm, and the collected emission was integrated over 540 to 700 nm.

Fig. 2. Spectroscopic responses and selectivity of CS3. All spectra were acquired in 20 mM HEPES, pH 7, at 25 °C. (A) Fluorescence response of 4 μM CS3 to Cu^{2+}. Spectra shown are for buffered [Cu^{2+}] of 0, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 μM. (B) Fluorescence responses of CS3 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 appropriate metal ion (2 mM for Ca^{2+}, Mg^{2+}, and Zn^{2+}; 50 μM for all other cations) to a 4 μM solution of CS3. Black bars represent the subsequent addition of 4 μM Cu^{2+} to the solution. Excitation was provided at 530 nm, and the collected emission was integrated over 540 to 700 nm.

Discovery of Depolarization-Induced Movements of Copper Pools in Neurons with Live-Cell CS3 Molecular Imaging. With data showing that CS3 is capable of reporting dynamic changes in endogenous intracellular Cu^{2+} stores by molecular imaging and that this probe is sensitive enough to visualize labile pools under basal conditions, we then sought to use this chemical tool to probe copper homeostasis in brain neurons. To this end, live hippocampal dissociated cultured neurons stained with 2 μM CS3 show a diffuse fluorescent signal pattern, localized mainly in the soma (Fig. 4A). To evaluate the spatial distributions of labile Cu^{2+} pools in these neuronal cells under basal conditions, we measured the emission intensity ratio of dendritic copper to somatic copper regions (D_{Cu}:S_{Cu}). Quantification of the dendritic and somatic fluorescence intensity signals provides a D_{Cu}:S_{Cu} ratio signal of 0.24 ± 0.04 in these resting neurons.

We then moved on to characterize the distribution of labile copper pools in activated neuronal cells. Interestingly, we observed that neurons treated with 50 mM KCl for 2 min to induce their depolarization and stained with 2 μM CS3 show a marked redistribution of labile copper pools from their somatic cell bodies to peripheral processes, quantified by a patent increase in D_{Cu}:S_{Cu} ratio to 0.35 ± 0.04 (Fig. 4E). These imaging data, which provide direct evidence that the spatial distributions of copper change upon neuronal activation, suggest that the redistribution of labile copper pools might be a result of a rise in dendritic levels and/or a decrease in somatic levels.

XRFM Provides an Independent and Complementary Method for Visualizing Mobile Copper Triggered in Depolarized Neuronal Cells. To study mobile copper in neuronal cells using an independent technique as well as verify our CS3-based molecular imaging results, we performed XRFM experiments at the Advanced Photon Source of the Argonne National Laboratory. XRFM affords, without any added reagents, a direct method for measuring total copper and other element distributions by their synchrotron-induced X-ray fluorescence signatures (29, 37–40). In particular, the instrument at the 2-ID-E beamline at the Advanced Photon Source of the Argonne National Laboratory boasts a spatial resolution of 200 nm, which makes it appropriate for examining the subcellular elemental distributions of single cells (41). We emphasize that the XRFM method measures total element content on fixed samples, thus providing a complementary approach to live-cell imaging of labile metal pools using fluorescent sensors. Moreover, the combination of small-molecule fluorescence imaging and XRFM has been exploited previously to provide a coherent picture of copper homeostasis in resting mammalian cells, setting the stage for studies of copper homeostasis in dynamic and stimulated situations in the present study (26, 27).

For the XRFM experiments, we utilized the same types of hippocampal neurons employed for the live-cell CS3 imaging studies but cultured on silicon nitride windows. These neuronal cell cultures were incubated either in buffer for 2 min as a baseline control or in buffer supplemented with 50 mM KCl to trigger their depolarization. The cells were then promptly fixed with paraformaldehyde (PFA) and examined by XRFM (Fig. 4). The elemental maps of total copper, zinc, and phosphorus pools are shown for baseline control neurons treated with buffer prior to fixation (Fig. 4 B–D). Phosphorus and zinc signals are concen-

Fig. 3. Molecular imaging of endogenous basal Cu in HEK 293T cells with CS3. (A) Control HEK 293T cells. (B) HEK 293T cells supplemented with 200 μM BCS in the growth medium for 20 h at 37 °C, and (C) HEK 293T cells treated with 100 μM TEMEA for 10 min. A, B, and C were stained with 2 μM CS3, 5 μM Hoechst 33342, and DMSO vehicle for TEMEA for 10 min at 37 °C in DMEM. (D) Graph showing the quantification of mean fluorescence intensity of each condition normalized to the control condition (n = 5 fields of cells per condition). Error bars represent the SEM. Asterisk (*) indicates P < 0.01 compared to control cells.
Brain neurons trigger movements of intracellular copper pools. Various methods that have allowed us to discover and establish that the CS3 and XRFM imaging experiments provide two independent, basal, untreated neurons (untreated for 2 min) show a statistically significant increase in KCl-stimulated neurons (Fig. 4 F–H), with copper pools displaying a marked redistribution from the cell body to peripheral processes. In contrast to the copper channel, similar elemental distributions of phosphorus and zinc are observed in the KCl-depolarized neurons compared to their unstimulated counterparts, revealing the relative mobility of copper pools in this model under these conditions. Quantification of the dendritic:somatic copper ratio from XRFM data collected from multiple Advanced Photon Source beam runs over 4 y shows a statistically significant increase in KCl-stimulated neurons (0.84 ± 0.04) compared to basal, untreated neurons (0.54 ± 0.07) (Fig. 4 J). Taken together, the CS3 and XRFM imaging experiments provide two independent methods that have allowed us to discover and establish that brain neurons trigger movements of intracellular copper pools upon their activation, causing a significant redistribution of neuronal copper stores from their somatic cell bodies to peripheral processes.

**CS3 Imaging and XRFM Show that Cellular Copper Movements are Dependent on Calcium release.** We next used CS3-based molecular imaging and XRFM to probe the effects of intracellular calcium release, a primary consequence of depolarization-induced neural activity, on the observed cellular copper movements. Multiple and distinct types of treatments to alter calcium release, including direct metal chelation or inhibition of cellular calcium entry channels or intracellular receptors, support a relationship between mobile copper and calcium signaling in this model.

First, intracellular calcium rises in neurons were blocked by treatment with the established intracellular Ca\(^{2+}\) chelator BAPTA, delivered in its membrane-permeable acetoxymethyl form (BAPTA-AM); this prochelator undergoes rapid hydrolysis by intracellular esterases to produce BAPTA (42). As shown in Fig. 5, Ca\(^{2+}\) chelation prevents KCl-induced redistribution of neuronal copper pools, as the observed $D_{Cu} \cdot S_{Cu}$ ratio in BAPTA-AM–treated neurons (n = 22) was significantly lower than in untreated neurons (n = 16) (Fig. 4 A–D).

**Fig. 4.** Molecular imaging of Cu distributions in resting and depolarized rat hippocampal neurons with CS3 and XRFM. (A) Live primary rat hippocampal neurons treated with extracellular solution (ECS) buffer for 2 min and then stained with 2 μM CS3 for 10 min. (B–D) Rat hippocampal neurons treated with ECS buffer for 2 min, fixed with 4% PFA and imaged by XRFM. Images shown are for (B) Cu, (C) Zn, and (D) P channels. (E) Live primary rat hippocampal neurons treated with 50 mM KCl in ECS buffer for 2 min and then stained with 2 μM CS3 for 10 min. (F–H) Rat hippocampal neurons treated with 50 mM KCl in ECS buffer for 2 min and then fixed with 4% PFA and imaged by XRFM. Images shown are for (F) Cu, (G) Zn, and (H) P channels. (I) Graph showing the blinded quantification of CS3-derived dendrite:soma fluorescence ratios for resting and depolarized neurons (n = 18). Error bars represent SEM ($P = 0.09$). (J) Graph showing the XRF dendrite:soma fluorescence ratios for resting and depolarized neurons. Error bars represent SEM. Asterisk (*) indicates $P < 0.05$.

**Fig. 5.** (A) Rat hippocampal neurons treated with ECS buffer for 2 min with 10 μM BAPTA-AM and then stained with 2 μM CS3 for 10 min. (B–D) Rat hippocampal neurons treated with ECS buffer with 10 μM BAPTA-AM for 2 min and then fixed with 4% PFA and imaged by XRFM. Images shown are for (B) Cu, (C) Zn, and (D) P channels. (E) Live primary rat hippocampal neurons treated with 50 mM KCl in ECS buffer with 10 μM BAPTA-AM for 2 min and then stained with 2 μM CS3 for 10 min. (F–H) Rat hippocampal neurons treated with 50 mM KCl in ECS buffer with 10 μM BAPTA-AM for 2 min and then fixed with 4% PFA and imaged by XRFM. Images shown are for (F) Cu, (G) Zn and (H) P channels. (I) Graph showing the blinded quantification of CS3-derived dendrite:soma fluorescence ratios for BAPTA-AM-treated (n = 22) and BAPTA-AM/KCl-treated (n = 16) neurons. Error bars represent SEM. (J) Graph showing the XRF dendrite:soma fluorescence ratios for resting and depolarized BAPTA-AM–treated neurons. Error bars represent SEM.
A

B

C

D

E

F

G

H

Control

Dantrolene

Nifedipine

Dantrolene

Nifedipine

90 mM KCl

90 mM KCl

90 mM KCl

90 mM KCl

Fig. 6. Molecular imaging of Cu distributions in resting, depolarized, and inhibitor-treated rat hippocampal neurons with CS3. (A) Live primary rat hippocampal neurons treated with ECS buffer for 10 min, (B) treated with ECS buffer with 30 μM dantrolene for 10 min, (C) treated with ECS buffer with 100 μM nifedipine for 10 min, (D) treated with ECS buffer with 30 μM dantrolene and 100 μM nifedipine for 10 min, (E) treated with 90 mM KCl in ECS buffer for 2 min, (F) treated with 30 μM dantrolene in ECS buffer for 10 min and then 90 mM KCl in ECS buffer for 2 min, (G) treated with 100 μM nifedipine in ECS buffer for 10 min and then 90 mM KCl in ECS buffer for 2 min, and (H) treated with 30 μM nifedipine and 100 μM dantrolene in ECS buffer for 10 min and then 90 mM KCl in ECS buffer for 2 min. (I) Graph showing the blinded quantification of CS3-derived dendrite:soma fluorescence ratios for resting, depolarized, and inhibitor-treated neurons (n ≥ 11). Error bars represent the SEM. Asterisk (*) indicates P < 0.05.
in a native cellular context. Our findings, taken together with previous reports showing accumulation of mitochondria to neuronal filopodia and dendritic spines upon repeated depolarization (52), reversible trafficking of the P-type ATPase ATP7A from the perinuclear trans-Golgi to neuronal processes by NMDA receptor activation (23), and axonal localization of ATP7A potentially involved in process guidance (53, 54), point to the intriguing possibility that subcellular compartmentalization and transient reorganization of copper stores is essential to tuning dynamic neuronal activity. Furthermore, the requirement for calcium release to trigger copper movements provides an entry for connecting copper to canonical signal transduction pathways. We are actively pursuing an understanding of mobile copper as a poten-
tial new metal signal in the context of neuronal activity and other fundamental physiological processes.

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Supporting Information

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SI Text

Synthetic Materials and Methods. All reactions were carried out under a dry N₂ atmosphere and stirred magnetically. 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). BODIPY 2, receptor 4, and tris(ethyleneethio)ethamine (TEMEA) were synthesized according to literature procedures (1, 2). Houstec 33342, FluoroZin-3-AM, and Pluronic F-127 was purchased from Invitrogen. All other chemicals were purchased from Sigma-Aldrich and used as received.

³¹P NMR spectra were collected in CDCl₃ (Cambridge Isotope Laboratories) at 25 °C on a Bruker AV-300 or AV-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 CDCl₃ as an internal reference. Mass spectral analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

8-Chloromethyl-2,6-diethyl-4,4-dimethyl-1,3,5,7-tetramethyl-4-bora-3a-diaza-s-indacene (3). AlCl₃ (400 mg, 3.0 mmol) was added to a solution of 2 (353 mg, 1.0 mmol) in dichloromethane (10 mL) and sonicated at room temperature for 5 min. Methanol (5 mL) was added, and the reaction was allowed to stir for an additional 5 min. The mixture was diluted with ethyl acetate (50 mL), washed with water (2 × 20 mL) and brine (20 mL), and the organic layer was separated and dried over Na₂SO₄. Purification by column chromatography (silica gel, ethyl acetate) afforded 3 as a dark red solid (246 mg, 65%). ³¹P NMR (CDCl₃, 300 MHz): δ 6.87 (2H, s), 2.81 (6H, s), 2.50 (6H, s), 2.47 (6H, s), 2.42 (4H, q, J = 7.5 Hz), 1.06 (6H, t, J = 7.5 Hz). High-resolution fast atom bombardment (HRFAB)-MS calculated for [M⁺] 376.2089, found 376.2088.

8-[N-N-Bis(3',6'-dithiaocetyl)-aminomethyl]-2,6-diethyl-4,4-dimethyl-1,3,5,7-tetramethyl-4-bora-3a-diaza-s-indacene (Coppersensor-3, CS3, 1). A 25-mL Schlenk tube was charged with 3 (52 mg, 0.14 mmol), 4 (57 mg, 0.18 mmol), KI (23 mg, 0.14 mmol), and K₂CO₃ (39 mg, 0.28 mmol). CH₂CN (1.0 mL) was added via syringe, and the resulting solution was stirred at 45 °C for 3 h under a nitrogen atmosphere. The mixture was concentrated, and the residue was dissolved in dichloromethane (50 mL) and washed with water (2 × 20 mL). The organic phase was dried over Na₂SO₄, concentrated to dryness, and purified by column chromatography (silica gel, ethyl acetate) to provide 1 as an orange-red oil (50 mg, 55%). ³¹P NMR (CDCl₃, 300 MHz): δ 4.05 (s, 2H), 2.89 (4H, m), 2.79 (6H, s), 2.66–2.52 (16H, m), 2.49 (6H, s), 2.46–2.37 (10H, m), 1.24 (6H, t, J = 4.5 Hz), 1.04 (6H, t, J = 7.2 Hz). HRFAB-MS calculated for [M⁺] 653.3348, found 653.3340.

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. 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 fluorometer equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photocounting/analogue photomultiplier detection unit, and MD5020 motor driver. Samples for absorption and emission measurements were contained in 1-cm × 1-cm path length quartz cuv-ettes (1.4-mL or 3.5-mL volume, Starna). Fluorescence quantum yields were determined by reference to rhodamine 101 inner salt in methanol (Φ = 1.0). The binding affinities of Cu⁺ to CS3 were measured using thiourea as a competitive ligand to provide buffered Cu⁺ solutions. Stability constants for thiourea binding were taken from the literature: β_12 = 2.0 × 10^{12}, β_13 = 2.0 × 10^{12}, and β_14 = 3.4 × 10^{15}. (3). Cu(I) was delivered in the form of [Cu(MeCN)]PF₆, from an acetonitrile stock solution (2 mM). Measurements were carried out in 20 mM HEPES, pH 7. Excitation was provided at 530 nm and collected emission was integrated from 540 to 700 nm. 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⁺:CS complex, and F_{min} is the fluorescence for the free CS dye.

Cell Culture. HEK 293T cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and glutamine (2 mM). Three days before imaging, cells were passaged and plated in 4-well chambered coverglass slides coated with poly-L-lysine (50 µg/mL, Sigma). Dissociated embryonic rat hippocampal neurons (DIV 12–20) were plated at 200,000 cells mL⁻¹ on 12-mm poly-L-lysine-coated coverslips in serum-containing medium. All animal care and experimental protocols were approved by the Animal Care and Use Committee at the University of California, Berkeley.

Live-Cell Imaging. Confocal fluorescence imaging was performed with a Zeiss LSM 710 laser-scanning microscope, a 40x water-immersion objective lens, and at 37 °C with 5% CO₂ for the HEK 293T cells. Confocal fluorescence imaging was performed with a Zeiss LSM 510 NLO Axiovert 200 laser-scanning microscope and a 40x or 63x water-immersion objective lens for the rat hippocampal neurons. HEK 293T cells were treated with a water vehicle or BCS and a DMSO vehicle or TEMEA for chelator experiments. HEK 293T cells were loaded with CS3 (2 µM, 10 min) and Hoechst 33342 (5 µM, 10 min) in DMEM at 37 °C, washed and imaged in fresh DMEM, and excited at 543 nm with a HeNe laser for CS3 and excited at 405 nm with a diode laser for Hoechst 33342. For experiments with FluoZin-3, HEK 293T cells were stained with FluoZin-3 (2 µM, 10 min) and Pluronic F-127 (vol/vol) and Hoechst 33342 (5 µM, 10 min) in DMEM at 37 °C, washed and imaged in fresh DMEM, and excited at 488 with an Ar laser. The median fluorescence intensity of each field of cells was measured in ImageJ, the mean was calculated, and a two-tailed Student’s t test was used to establish statistical significance. Rat hippocampal neurons (DIV 14–20) were incubated in either extracellular solution (ECS) buffer (4) or ECS buffer containing 50 mM KCl for 2 min. The solution was aspirated and washed with ECS containing MgCl₂, and incubated with CS3 (2 µM, 10 min). In studies of neurons with calcium chelation, neurons were incubated with 10 µM BAPTA-AM for 30 min in media at 37 °C. The media was aspirated, and ECS buffer containing 10 µM BAPTA-AM or ECS buffer with 10 µM BAPTA-AM with 50 mM KCl was added. The neurons were allowed to sit in this solution for 2 min, at which point the buffer was removed and ECS with MgCl₂ added and the cells were incubated with CS3 (2 µM, 10 min). Neurons were imaged identically to HEK cells, except 2 stacks were acquired with a 1-μm slice thickness. Images of neurons were subjected to a blinded quantification with ImageJ, where the fluorescence intensity in a user-defined den-
drite and soma region was measured to determine the dendrite: soma CS3-ratio. Neuron studies with dantrolene and nifedipine treatments were carried out using DIV 12 cells. Coverslips containing neurons were washed once with ECS and then incubated with 30 μM dantrolene (30 mM stock in DMSO) and/or 100 μM nifedipine (100 mM stock in DMSO) for 10 min in ECS buffer at 37 °C. For KCl-treated cells, the KCl concentration was adjusted to 90 mM, and the cells were incubated for 2 min at 37 °C. After their respective treatments, all coverslips were washed with ECS (three times) and stained with CS3 (2 μM, 10 min) at room temperature. Neurons were subsequently washed with ECS containing MgCl₂ (three times) and imaged.

X-Ray Fluorescence Microscopy (XRFM) Samples. HEK 293T cells and rat hippocampal neurons were plated on poly-L-lysine coated silicon nitride windows (2 × 2 mm; thickness 500 nm) manufactured by Silson. After the treatments indicated in the text or supporting information, cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10 min. Residual PBS was removed by several washes in 20 mM PIPES, pH 7.2/200 mM sucrose followed by air drying. X-ray imaging was carried out with the scanning X-ray microprobe at beamline 2-ID-E at the Advanced Photon Source (Argonne National Laboratory) as previously described (5).


Fig. S1. Job’s plot of CS3 and Cu⁺. The total concentration of CS3 and Cu⁺ was kept at a constant 4 μM. Excitation was provided at 530 nm, and emission intensity was measured at 550 nm. Spectra were acquired in 20 mM HEPES, pH 7. The maximum fluorescence response at 0.5 mol fraction of CS3 indicates formation of a 1:1 Cu⁺:CS3 complex.

Fig. S2. Normalized fluorescence response of 4 μM CS3 to thiourea buffered Cu⁺ solutions for Kd value determination. Excitation was provided at 530 nm, and the collected emission was integrated from 540 to 700 nm. Spectra were acquired in 20 mM HEPES, pH 7. The points shown are for free Cu⁺ buffered at 9.22, 14.5, 15.1, 21.9, 22.7, 30.3, 38.9, 77.9, 101, 255, 383, 447, and 3,000 fM, respectively. The observed Kd value is 8.8(3) × 10⁻¹⁴ M.
Fig. S3. Fluorescence responses of CS3 to various heavy metal ions. Bars represent the final integrated fluorescence response ($F_f$) over the initial integrated emission ($F_i$). White bars represent the addition of the competing metal ion to a 4 μM solution of CS3. Black bars represent the subsequent addition of 4 μM Cu$^+$ to the solution. Excitation was provided at 530 nm, and the collected emission was integrated over 540 to 700 nm.

Fig. S4. Molecular imaging of endogenous basal Cu in HEK 293T cells with CS3. (A) Control HEK 293T cells, (B) HEK 293T cells supplemented with 200 μM BCS in the growth medium for 15 h at 37 °C, and (C) HEK 293T cells supplemented with 200 μM BCS in the growth medium for 25 h were stained with 2 μM CS3 and 5 μM Hoechst 33342 for 10 min at 37 °C in DMEM. (D) Graph showing the quantification of mean fluorescence intensity of each condition normalized to the control condition ($n = 4$ fields of cells per condition). Error bars represent the SEM. Asterisk (*) indicates $P < 0.01$ compared to control cells.
Fig. S5. (A) Control HEK 293T cells, (B) HEK 293T cells supplemented with 2 μL of vehicle in the growth medium for 15 h at 37 °C, and (C) HEK 293T cells supplemented with 2 μL of vehicle in the growth medium for 25 h at 37 °C were stained with 2 μM CS3 and 5 μM Hoechst 33342 for 10 min at 37 °C in DMEM. (D) Graph showing the quantification of the mean fluorescence intensity of each condition (n = 4 fields of cells per condition). Error bars represent the SEM.

Fig. S6. Molecular imaging of endogenous basal Zn in HEK 293T cells with FluoZin-3 AM. (A) Control HEK 293T cells, (B) HEK 293T cells supplemented with 200 μM BCS in the growth medium for 18 h at 37 °C, and (C) HEK 293T cells supplemented with 200 μM BCS in the growth medium for 29 h at 37 °C were stained with 2 μM FluoZin-3 AM and Pluronic F-127 (vol/vol) and 5 μM Hoechst 33342 for 30 min at 37 °C in DMEM. (D) Graph showing the quantification of the mean fluorescence intensity of each condition (n = 3 fields of cells per condition). Error bars represent the SEM.
Fig. S7. Molecular imaging of P, Cu, and Zn distributions in control HEK 293T cells and HEK 293T cells treated with 200 μM BCS in the growth medium for 40 h at 37 °C with XRFM. (A–D) Untreated HEK 293T cells were fixed with 4% PFA and imaged by XRFM. Images shown are for (A) Cu, (B) Zn, (C) P, and (D) S channels. (E–H) BCS-treated HEK 293T cells were fixed with 4% PFA and imaged by XRFM. Images shown are for (E) Cu, (F) Zn, (G) P, and (H) S channels. Quantification of the mean of the median fluorescence intensity of each condition (n = 8 cells per condition) (I) Cu (μg/cm²) normalized to S (μg/cm²), (J) Zn (μg/cm²) normalized to S (μg/cm²), and (K) P (μg/cm²) normalized to S (μg/cm²). Error bars represent the SEM. Asterisk (*) indicates \( P < 0.05 \) compared to control cells, and double asterisk (**) indicates \( P < 0.01 \).