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Molecular Imaging Approaches to Understanding the Roles of Copper in Biology

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Molecular Imaging Approaches to Understanding the Roles of Copper in Biology

By

Dylan Wythe Domaille

A dissertation submitted in partial satisfaction of the
requirements for the degree of
Doctor of Philosophy

in
Chemistry

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Graduate Division

of the
University of California, Berkeley

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Molecular Imaging Approaches to Understanding the Roles of Copper in Biology

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by Dylan Wythe Domaille
Abstract

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Cells exert precise control over their cellular copper pools through a sophisticated array of uptake, trafficking, and storage mechanisms that effectively maintain a low concentration of thermodynamically free copper ions while maintaining excellent kinetic lability of cellular copper stores. In higher eukaryotes and humans, and particularly in specialized cell types associated with brain, heart, intestine, and liver tissue, the molecular specifics of how kinetically labile copper pools are regulated at the subcellular level and the consequences of copper misregulation in aging and disease remain insufficiently understood. Biochemical and genetic studies have established a broad understanding of how cells acquire, maintain, redirect and release copper ions, while also identifying key proteins involved in these activities. The precise role of the copper ion, however, is more difficult to determine, owing mainly to a dearth of methods for directly following the fate of cellular copper stores. This dissertation describes the design, synthesis, and characterization of several new Cu(I)-responsive fluorophores. Through a targeted synthetic survey and comprehensive electrochemical study, the properties of our previously reported fluorophore, Coppersensor-1, were improved to yield a compound (Coppersensor-3) that exhibits the largest fluorescent response to Cu(I) to date. Along with X-ray fluorescence microscopy, CS3 was used to investigate disruptions in copper homeostasis in a cell model for Menkes disease. The following report describes the synthesis, characterization and applications of Ratio-Coppersensor-1 (RCS1), the first ratiometric fluorophore for live-cell imaging. This compound was used to investigate the effect of ascorbate on rat brain and human kidney cells, and proved able to be able to detect increases in endogenous labile Cu⁺ that occurs upon ascorbate treatment. A slight alteration to the ligand of RCS1 gave RCS2, which has similar spectroscopic properties to RCS1. Single-molecule X-ray crystallography and VT-NMR studies provide molecular dynamic details of RCS2 coordination to Cu⁺.
For Monica.
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Chapter 1:

Reagents and Modalities for Imaging Copper in Biological Systems
Abstract

Traditional methods for the detection of biological copper are often limited to whole-cell analysis (inductively-coupled plasmon mass spectrometry), fixed cells (histochemical analysis) or perturb cellular homeostasis (radioactive copper isotopes). This Chapter includes an overview of available small-molecule fluorophores, chemodosimeters, protein-based FRET devices, nanoparticles, enzyme-based assays, and magnetic resonance imaging contrast agents for detecting bioavailabile Cu^{+/2+} in living cells, tissues, and organisms.
**Introduction**

Synthetic chemists have discovered, and continue to discover, different ways that copper can catalyze different bond-making processes.\(^1\)\(^-\)\(^5\) However, they were scooped by Nature. To be fair, Nature had a bit of a head start, but it is clear from the attention paid to copper by synthetic methodology groups and from the ubiquity of copper in living organisms that rational humans and unthinking, lumbering evolution are both onto something. So why copper? Simply put, it’s soluble, abundant, and perhaps most important, it is capable of undergoing facile redox chemistry in a readily accessible redox window.\(^6\) For instance, a cupric ion can be easily reduced by, among other things, the abundant intracellular antioxidant, glutathione, or the physiological reductant, ascorbate; likewise, a cuprous ion can be oxidized to its cupric state with an oxidant as common as dioxygen.\(^7\)\(^,\)\(^8\) Accordingly, copper plays an important role as a catalytic co-factor to effect biologically relevant functionalizations (e.g. the hydroxylation of tyrosine in the biosynthesis of melanin) or perform maintenance roles (e.g. the catalytic decomposition of superoxide by superoxide dismutase).\(^9\)\(^-\)\(^15\)

Cells exert precise control over their cellular copper pools through a sophisticated array of uptake, trafficking, and storage mechanisms that effectively maintain a low concentration of thermodynamically free copper ions while maintaining excellent kinetic lability of cellular copper stores.\(^15\)\(^-\)\(^17\) These criteria immediately seem to present a conflict: how can an ion simultaneously be tightly bound and enjoy rapid exchange between the many sites that require it? The situation is not unlike how runners hold and transfer a baton during a relay race. Each runner acts as a chaperone of the baton and tightly controls it during his or her leg of the race. In a sense, the runner-baton complex is thermodynamically stable. But at precise moments during the race – when the baton is transferred from one runner to the next – the runner must readily pass on the baton without it ever becoming completely free and unbound. Thus, there should be high kinetic lability of the baton to ensure a smooth hand-off between the runners.

Copper-buffering proteins, chaperones and cuproenzymes continually act out a more complex version of this relay on the molecular level; however, a misstep by one of these proteins can have far-reaching consequences in terms of organismal health.\(^15\)\(^,\)\(^18\)\(^,\)\(^19\) Indeed, the importance of healthy copper homeostasis is underscored by the severity of diseases that have been linked to altered copper handling. For instance, Menkes disease, a genetic X-linked disorder that is characterized by impaired copper efflux, often results in impeded fetal development and mental retardation.\(^20\) Patients with Menkes disease rarely live past their third year of life. Other neurodegenerative diseases, including Alzheimer’s disease\(^22\)\(^,\)\(^23\) Parkinson’s disease\(^24\)\(^-\)\(^26\) and amyloid lateral sclerosis\(^25\)\(^,\)\(^27\), have etiologies associated with dysfunctions in copper homeostasis, which further emphasizes the need to examine the role of this metal in these diseases.

In higher eukaryotes and humans, and particularly in specialized cell types associated with brain, heart, intestine, and liver tissue, the molecular specifics of how kinetically labile copper pools are regulated at the subcellular level and the consequences of copper misregulation in aging and disease remain insufficiently understood. Biochemical and genetic studies have established a broad understanding of how cells acquire, maintain, redirect and release copper ions, while also identifying key proteins involved in these activities.\(^13\)\(^,\)\(^15\)\(^,\)\(^17\)\(^,\)\(^18\)\(^,\)\(^27\)\(^,\)\(^28\) The precise role of the copper ion, however, is more difficult to determine, owing mainly to a dearth of methods for directly following the fate of cellular copper stores.\(^29\)\(^,\)\(^30\)

**Traditional Methods for Detecting Biological Copper.** The choice of analytical method to examine copper in biological specimens is largely dependent on the type of information that is desired by the researcher. If only the amount or concentration of copper needs
to be determined, inductively coupled plasmon atomic absorption spectroscopy (ICP-AES), or mass spectrometry (ICP-MS), provides excellent sensitivity, though the user sacrifices spatial and speciation information. Histochemical stains can provide spatial information with limited oxidation state fidelity, but this approach requires extensive sample preparation (including sample dehydration) and is limited to fixed cellular and tissue samples. Finally, $^{67}$Cu detection yields an excellent signal to noise ratio and very low detection limits; however, the radioactive copper must be loaded into cells, which can alter the system under study. With a half-life of 13.7 hours, the $^{67}$Cu isotope limits experimental design, though for certain studies, it remains a popular choice due to its exquisite sensitivity. The aforementioned techniques have all found wide application in the environmental and biological chemistry of copper, and the reader is directed to several excellent reviews that cover the application of these techniques in more detail.

In this review, we wish to focus primarily on techniques and compounds that allow for dynamic, real-time monitoring of copper ions, ideally in living cells. To this end, we will focus on three modalities: 1) fluorescent sensors, especially those that have been used in confocal microscopy experiments; 2) Cu-responsive magnetic resonance imaging (MRI) contrast agents; and 3) X-ray fluorescence microscopy (XRFM).

**Cu-responsive Fluorophores for Molecular Imaging.** The field of fluorophore development and utilization has exploded in the past several decades. Publications resulting from the development and application of fluorescent molecules or devices have enjoyed exponential growth (Figure 1-1) since the early 1990s. The widespread availability of powerful fluorescence microscopy technologies has encouraged the development of increasingly sophisticated, well-characterized fluorescent probes, labels and sensors, especially in the field of ion detection. Only recently has this technology been extended beyond Ca$^{2+}$- and Zn$^{2+}$-sensors and been applied to Cu$^{+}/^{2+}$ responsive compounds that can operate in biological specimens.

**Design Criteria for Cu-responsive Fluorophores.** There are several different strategies to design a compound that responds selectively to copper with a robust fluorescent signal enhancement. Most fluorescent sensors utilize a modulation of electron-transfer or charge-transfer characteristics to translate a binding event into a fluorescence modulation. One general strategy for cation detection relies on a photoinduced electron transfer (PeT) process (Figure 1-2). This category of probes contains several conserved design components. An electron-rich atom, typically an amine, is located in close physical proximity to a fluorophore and almost always comprises a part of the ligand that binds the metal. Excitation of the fluorophore promotes an electron to its singlet excited state; however, if the electronics of the amine are appropriately positioned, the amine acts as an effective quencher and inhibits relaxation of the electron back to its ground state. This minimizes the probability that a photon will be emitted and inhibits the fluorescence process. Upon coordination of a metal ion to the amine, the lone pair of the amine falls in energy; this relieves the quenching effect of the amine and restores the fluorescence of the molecule. Utilization of PeT is a practical and broad strategy for the detection of metal ions and has been used in the design of Ca$^{2+}$,

Small-molecule Fluorescent Probes for Cu$^+$. Live-cell imaging with copper-specific fluorescent sensors offers a potentially powerful method for studying copper cell biology but presents distinct challenges beyond other metals for detection. For example, Cu$^+$, which is the major oxidation state within the reducing environment of the cytosol, is an effective fluorescence quencher by electron or energy transfer, and can readily disproportionate to Cu$^{2+}$ and Cu$^0$ metal...
in water. Cu^{2+} also possesses quenching capabilities, particularly in aqueous media, because of its redox activity and unfilled d-shell.

For these reasons, fluorescence detection of copper in biological environments with small-molecule sensors remains a difficult task. To date, only three chelation-based fluorescent probes and one chemodosimeter have been utilized for visualizing labile Cu^{+} in cellular systems. The first Cu^{+}-responsive probe suitable for study in cellular environments is CTAP-1.\textsuperscript{39} This pyrazoline dye platform is appended to an azatetrahydroacen receptor for selective Cu^{+} coordination. Upon UV excitation at 365 nm, the sensor undergoes up to a 4.6-fold fluorescence turn-on in the presence of Cu^{+} with good selectivity over competing cellular ions. Fixed NIH 3T3 fibroblast cells grown under elevated levels of copper show greater fluorescent staining with CTAP-1 compared to cells grown in basal media. Complementary experiments with organelle-specific co-stains and X-ray fluorescence (XRF) microscopy provide a coherent picture of labile copper localization confined largely to the Golgi apparatus and mitochondria in these cell types (Figure 1-3).

In parallel with this study, our laboratory presented the first type of Cu^{+}-specific fluorescent probe with excitation and emission in the visible region and demonstrated its utility for live-cell copper imaging.\textsuperscript{40, 41} Coppersensor-1 (CS1) combines a BODIPY reporter and thioether-rich receptor to provide high selectivity and sensitivity for Cu^{+} over other biologically relevant metal ions, including alkali and alkaline earth metal ions (Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+}, and Ca\textsuperscript{2+}) as well as other competing d-block ions (Zn\textsuperscript{2+} and Cu\textsuperscript{2+}); a 10-fold turn-on response; and picomolar affinity for labile Cu\textsuperscript{+} (\(K_d = 3.6\) pM) in aqueous solution. Moreover, confocal microscopy experiments in live HEK 293T cells establish that CS1 can respond to changes in labile intracellular copper concentrations within living samples. Treatment with a cell-permeable, Cu\textsuperscript{+}-specific chelator immediately reduces intracellular fluorescence intensities to control cell levels, indicating that CS1 can monitor changes in cellular copper in real-time. CS1 has been used in several independent laboratories to study the anti-bacterial and anti-fungal role of copper surfaces, as well as to study the metal ion differences in healthy and diseased brain tissue.\textsuperscript{42-44} We have improved upon this platform by altering the BODIPY electron-transfer properties and realized a brighter derivative, which has a femtomolar binding affinity for Cu\textsuperscript{+}. Coppersensor-3 (CS3) has recently been used to visualize mobilized Cu stores in rat hippocampal neurons.

Although intensity-based reagents are of practical utility, external influences that lead to variations in probe concentration and environment can complicate measurements in biological samples. These potential artifacts can be minimized by ratiometric imaging, which relies on probes that have two distinct measurable signals in the presence or absence of analyte. To this end, a reported ratiometric Cu\textsuperscript{+}-responsive fluorophore can respond to changes in intracellular Cu levels and the increased population of labile Cu in ascorbate-treated rat brain and human kidney cells (Figure 1-4).\textsuperscript{45} Ratio-Coppersensor-1 (RCS1) exhibits two emission maxima of near equal intensity upon excitation at 480 nm. Introduction of Cu\textsuperscript{+} prompts an increase of the longer wavelength emission with no change in the higher energy peak. A tight (40 pM) 1:1 RCS1:Cu\textsuperscript{+} complex is responsible for the fluorescence response. Owing to its NS\textsubscript{4} open-chain receptor, the selectivity for Cu\textsuperscript{+} is excellent and no significant interference is seen in the presence physiologically relevant concentrations of alkali, alkaline earth and d-block metals, including Cu\textsuperscript{2+}. The fluorescence increase in the presence of Cu\textsuperscript{+} can be reversed in the presence of Cu\textsuperscript{+} chelators, indicating that binding is reversible and rapid. Cellular studies in HEK 293T cells established that RCS1 can report changes in intracellular copper levels. Furthermore, RCS1 is
capable of reporting increases in labile, endogenous copper that are liberated upon incubation with the physiological reductant, ascorbate.

**Protein-based Probes for Cu\(^{+}\)-Detection.** Advancements in protein engineering have provided chemical biologists with the tools to develop Cu\(^{+}\)-responsive fluorescent proteins. These genetically encoded fusion proteins are typically designed to have a Cu\(^{+}\)-binding domain (usually from copper chaperone or Cu-binding transcription factor) that link a CFP and YFP fluorescent protein together. When the binding domain coordinates to Cu\(^{+}\), the distance between the two fluorescent proteins changes and the fluorescence ratio is altered. This change in fluorescence can be used to identify Cu\(^{+}\) changes inside of cells. The most recent reports indicate that these reagents will find broad utility as sensitive metal probes for intracellular Cu\(^{+}\).

A contribution by the Merkx laboratory reported their attempt to use the dimerization of copper domains from the Atox1 copper chaperone and the ATP7b copper efflux protein to initiate a change in a FRET response.\(^{46}\) Though the construct did respond to Cu(I), the construct-metal association was disrupted by low concentrations of glutathione or DTT. Surprisingly, this engineered protein worked quite well for the detection of Zn(II), and was reported in the context of a ratiometric genetically-encoded probe for Zn(II) imaging. A true genetically-encoded Cu(I)-probe was realized last year by Wegner, *et al*.\(^{47}\) This construct used the yeast transcription factor Amt1 to link CFP and YFP fluorescent proteins together. Amt1-FRET exhibits a very high affinity for Cu(I) \((K_d = 2.5 \times 10^{-18} \text{M})\) and it quickly responds to nanomolar changes in exogenous Cu concentrations with a robust fluorescence ratio change. A later report cleverly used the binding domains from Ace1, which activates Cu detoxification genes under conditions of excess copper, and Mac1, which activates genes under conditions of copper starvation, to generate FRET pairs in an analogous fashion to Amt1-FRET.\(^{48}\) The resulting \(K_d\) measurements allowed the authors to propose a Cu\(^{+}\)-availability window in yeast cells of \(8.9 \times 10^{-17} \text{M}\) to \(5.1 \times 10^{-21} \text{M}\).

**Reaction-based Probes for the Detection of Cu\(^{+}\).** In recent years, chemodosimeters – compounds that undergo an irreversible chemical reaction to provide a change in absorption or fluorescence – have gained in popularity. Common approaches include using an analyte of interest to catalyze a chemical transformation that alters the absorption and emission properties of a reporter compound. These chemical transformations can be as simple as hydrolysis and liberation of a tethered fluorescence quencher, or as complex as a metal-catalyzed cyclization that provides extended conjugation and a corresponding emission shift.

The Cu(I)-catalyzed conversion of an azide and alkyne into a triazole is a cornerstone of click chemistry and is perhaps the most well-known example of a Cu(I)-catalyzed synthetic transformation.\(^{49}\) Researchers from the Fahrni lab have shown that when an alkyne-substituted coumarin undergoes a Cu(I)-catalyzed triazole formation with an azide, a bright fluorescent output is observed.\(^{50}\) DFT and spectroscopic experiments indicated that this chemical transformation inverts the energy orders of the emissive \(^1(\pi, \pi^*)\) and the non-emissive \(^3(n, \pi^*)\) states giving rise to a product that is significantly more emissive than the starting coumarin. Though this report was framed in the context of a fluorogenic probe for the Cu(I)-catalyzed click reaction, it also can be regarded, in a sense, as an early Cu(I)-chemodosimeter. A later report from the Hulme group utilized Cu(I) in the same manner to catalyze the bond formation between a luminescent Eu\(^{3+}\) complex and a dansyl sensitizer.\(^{51}\) The reaction is efficiently catalyzed in the presence of low micromolar concentrations of glutathione (Figure 1-5), though its utility in a cellular context has not yet been demonstrated.

The detection of Cu\(^{+}\) in a cellular environment is difficult, owing to a high concentration of endogenous chelating ligands. A chemodosimeter that is capable of selectively detecting Cu\(^{+}\)
in physiologically relevant concentrations of glutathione (2 mM) provides a complementary approach to chelation-based methods of intracellular Cu\(^{+}\) detection.\(^5\) In this report from Yamamoto, a tris[(2-pyridyl)-methyl]amine ligand was tethered through a benzyl ether linkage to a reduced fluorescein platform. In the presence of Cu(I), the benzyl ether linkage is oxidatively cleaved to release a green, fluorescent product (Figure 1-6). The authors propose that a complex is formed between Cu(I) and the pro-fluorophore; after Cu(I) is coordinated, oxygen reacts with the copper complex to generate a reactive copper-oxygen intermediate. This species oxidatively cleaves its own benzyl ether bond to unveil a dihydrofluorescein derivative, which rapidly oxidizes to its highly fluorescent counterpart. Although this process is irreversible, its capability to detect Cu(I) without significant signal attenuation by glutathione and its demonstrated utility in living cells make it a significant advancement in Cu-fluorophore development. Future studies that elucidate the structure of the Cu(I) complex and the mechanism of cleavage should provide valuable insights for the development of second generation probes.

**Cu\(^{2+}\)-responsive Fluorescent Probes.** The detection of biological Cu\(^{2+}\) remains an open challenge for researchers. Because of the paramagnetic nature of Cu\(^{2+}\), this metal ion engages in a rapid quenching of fluorophores and, consequently, the reports concerned with Cu\(^{2+}\) responsive fluorophores fall almost exclusively into two categories: 1) turn-off probes in which the Cu\(^{2+}\)-fluorophore complex is less emissive than the apo form of the fluorophore, and 2) turn-on probes that perform well only in organic solvents where quenching via electron transfer processes is less facile. Examples of reversible, turn-on fluorescent probes for Cu\(^{2+}\) that operate in aqueous solution are rare, indeed. Fortunately, Cu\(^{2+}\) has several characteristics, such as a high Lewis acidity and the capability to act as an oxidant, that make chemodosimeters a suitable alternative to chelation-based detection strategies. We will cover several different approaches for monitoring Cu\(^{2+}\) ions, from a turn-off fluorescent probe used in cell culture studies, to a turn-on fluorescent probe used in aqueous solution, to several chemodosimeters that show impressive, though irreversible, responses to Cu\(^{2+}\). It should be noted, however, that a selective turn-on fluorophore with a reversible response to Cu\(^{2+}\) that can be used in biological studies remains a goal that has yet to be achieved.

**Turn-off Fluorophores for Cu\(^{2+}\) Detection.** The aforementioned quenching effects of divalent copper have complicated Cu\(^{2+}\)-fluorophore development; however, this quenching effect can, in principle, be utilized to indicate the presence of Cu\(^{2+}\). Practically speaking, less precise spatial and temporal information is gleaned from this type of device as compared to the information from a turn-on fluorophore, simply because it is more difficult to draw relevant conclusions from a dark spot with a bright background — as is the case with turn-off probes — than from a bright spot with a dark background. Nevertheless, a recent report has shown fluorescence quenching can be used to provide an indication of the Cu\(^{2+}\) status of a cell.\(^5\) In this study, an amide-linked picolyl arm forms a pocket where Cu\(^{2+}\) can bind and quench the fluorescence of a coumarin derivative. The receptor unit is shown to be selective for Cu\(^{2+}\) over fourteen other metals, while a Job’s plot and X-ray crystal structure establishes that a 1:1 binding between the metal and the compound is responsible for the quenching. A thorough derivative study showed the indispensable nature of the picolyl arm and quantum mechanical calculations establish that significant non-radiative deactivation of the excited state occurs by charge transfer from the excited coumarin moiety to the Cu\(^{2+}\) center. Cellular studies in LLC-MK2 cells suggest that the compound can respond to increasing levels of cellular Cu\(^{2+}\), as indicated by the decreased fluorescence from Cu\(^{2+}\)-treated cells.
A Turn-on Sensor for Cu$^{2+}$ Detection Based on Naphthalimide Excimer-Monomer Switching. One strategy for circumventing the quenching effects of Cu$^{2+}$ is to use a physical, conformational change to alter the emissive properties of a compound. Xu et al linked two naphthalimide units with a piperazine ring and the resulting compound exhibits a weak excimer emission. When this compound binds Cu$^{2+}$, the two flat, aromatic rings of the naphthalimide come together and form a “static excimer” as indicated by an increase in excimer intensity. Another equivalent of Cu$^{2+}$ prompts conversion to the monomeric form, which has a distinctly different emission from the excimer. This three-state mechanism (dynamic excimer, static excimer, monomer) proposed by the authors is supported by the observation that a 1:2 complex is responsible for the monomeric emission. Notably, in mixed aqueous solution, Cu$^{2+}$ is the only metal ion that can initiate a change in the monomeric emission feature, which indicates good selectivity over other metal ions. Perhaps the only complication in this device is the possibility of a “static excimer” form, which has bound 1 equivalent of Cu$^{2+}$ but has not yet converted to its differently emissive monomeric form. This three-state system could potentially complicate measurements, but the initial report is promising as a way to avoid the fluorescence quenching attributes of Cu$^{2+}$.

Chemodosimeters for Cu$^{2+}$. The potent Lewis acidity of Cu$^{2+}$ is another characteristic that can be exploited in chemodosimeter design. An early report of Cu$^{2+}$-catalyzed hydrolysis has set the benchmark for sensitivity and selectivity. Dujols et al demonstrated that a hydrazide functionalized non-fluorescent rhodamine can selectively and rapidly undergo Cu$^{2+}$-mediated oxidative hydrolysis to liberate a highly fluorescent product. The limits of detection for Cu$^{2+}$ are impressive – 10 nM Cu(II) was effectively detected within several minutes – and the selectivity for Cu$^{2+}$ is excellent over twenty-six different metals, including alkali, alkaline earth, d- and f-block elements. Though published over twelve years ago, this initial report of coupling Cu$^{2+}$-mediated hydrolysis to a rapid, robust fluorescence increase has remained a initial demonstration. No reported attempts have been made to study the compound in biological samples, but the chemodosimeter’s impressive performance in vitro should make it amenable to select biological and environmental investigations.

A slew of compounds that draw from Czarnik’s work have been published in the past decade; we will cover the most recent demonstrations here. All of these compounds exhibit several conserved features: a rhodamine scaffold, an acyl hydrazide linkage, and a hydrazone bond to a chelating unit (Figure 1-7). In all cases, coordination of a Cu$^{2+}$ ion causes the five-membered ring to open – this allows a fully conjugated, and highly absorptive and emissive rhodamine derivative to form. These sensors are both colorimetric (often a striking color change is seen immediately upon adding Cu$^{2+}$), but they also exhibit a significant increase in fluorescent quantum yield. It is necessary to recognize that all of these compounds use a hydrazone linkage as the key bond between the fluorophore and chelating unit. These tend to be pH-sensitive so the long-term hydrolytic stability of these types of reporters must be vigorously investigated.

Another compound uses the Cu$^{2+}$-catalyzed deprotection of a thioacetal to achieve a fluorescent turn-on response to Cu$^{2+}$. In this case, Cu$^{2+}$ is proposed to coordinate to the sulfur atom of a cyclic thioacetal and facilitate the hydrolysis of the “protecting group” to unveil an aldehyde. The significant change in electron-withdrawal character between these two groups gives rise to an internal charge transfer (ICT) type response to Cu$^{2+}$ and provides a selective fluorescent read-out of the presence of Cu$^{2+}$.

A sophisticated DNA based approach has also been reported. The authors use a Cu$^{2+}$-dependent DNA-cleaving DNAzyme to indicate the presence of Cu$^{2+}$ in solution.
strands that form a complex were used: one strand was modified with a fluorescein-based fluorophore at the 3’ end, and the other was modified with a fluorescence quencher at the 5’ end. When the two strands of DNA bind in a complementary manner to one another, the fluorescence from the fluorescein is quenched by the 5’-fluorescence quencher. When Cu$^{2+}$ is present, the metal binds to the DNA substrate and the stands are irreversibly cleaved by the DNAzyme. The two strands diffuse, the quenching effect of the quencher is relieved, and the fluorescence is restored. Further studies elucidated that Cu$^+$ is significantly faster than Cu$^{2+}$, so ascorbate is required in the assay.

The field of Cu$^+$-responsive fluorophores for cellular imaging applications has been elevated to the point to which several practical options exist for a variety of imaging needs. On the other hand, Cu$^{2+}$-responsive fluorophores have historically lagged behind Cu$^+$-fluorophores, though this is no fault of the researchers, but rather of the paramagnetic, fluorescence-quenching capabilities of the d$^9$ Cu$^{2+}$ ion that complicate fluorophore design. We are optimistic about recent reports that cleverly circumvent these issues and are eager for the realization of a widely applicable Cu$^{2+}$-responsive fluorophore for imaging purposes in complex biological systems.

**Nanoparticle-Based Detection Strategies for Cu$^{2+}$.** Nanoparticles have been used for the detection of various analytes including metal ions. The primary advantage of nanoparticles over small-molecule fluorophores lies in their remarkably high absorption coefficients (on the order of 10$^6$ M$^{-1}$ cm$^{-1}$). Depending on the size of the nanoparticles can also be emissive fluorescent species, or act as exceptionally good fluorescence quenchers. Strategies to detect metal ions have utilized all three of these characteristics; we will highlight two successful approaches below.

Mirkin’s group has used alkyne and azide functionalized DNA to decorate 30 nm gold particles.$^{68}$ In the presence of a triazole ligand, sodium ascorbate and Cu$^{2+}$, a Cu(I)-catalyzed coupling between the azide and alkyne is performed that links the two nanoparticles together. By measuring the melting temperature at a specific timepoint during the reaction, the aggregate measure of melting temperature provides an indication of how much Cu(II) is present. The more Cu(II), the more Cu(I) catalyst can be generated to covalently link the nanoparticles together, and the higher the melting temperature. This reaction-based detection scheme is selective for copper over fourteen other metals.

The quenching effects of paramagnetic Cu$^{2+}$ can also be extended to quenching the fluorescence of gold nanoparticles. The Guo group used 2.1 nm glutathione-capped fluorescent nanoparticles as a platform to detect Cu(II). These decorated nanoparticles are capable of detecting down to 8 nM Cu(II) with excellent selectivity. Furthermore, the addition of EDTA can fully reverse the quenching effect and restore the original fluorescence of the system. These characteristics provide a significant advantage over irreversible, reaction-based systems.

**Cu-Responsive Magnetic Resonance Imaging Agents.** Magnetic resonance imaging is a powerful method to non-invasively image soft tissue, including muscles, ligaments, internal organs and the spinal cord. It is especially useful for imaging the brain, and is used as both a diagnostic technique in clinical work and as an experimental technique in neurology research. MRI operates on the same principle as an NMR spectrometer: a strong magnetic field interacts with protons (in the case of MRI, with the protons from water molecules) and the nuclear spin of these protons align themselves in either a parallel or anti-parallel orientation to the applied magnetic field. After the magnetic field is removed, the spins revert to their original state through a combination of longitudinal ($T_1$) and transverse ($T_2$) relaxation pathways. The different rates at which the protons relax provide the contrast seen in the image — it is these rate
differences termed, *relaxivity rates*, that are key to the contrast seen in an MR image. Water protons in different tissues, tumors, etc. will relax at different rates and the generated MRI image is essentially a map of these observed relaxivity differences.

The contrast, and accordingly, the resolution of an MRI can be increased with MRI contrast agents. These are typically gadolinium chelates that interact with protons to alter the relaxivity rates of nearby nuclei. The details of how a paramagnetic metal ion can influence the relaxation rate of nearby nuclei has been described in detail by Solomon, Bloembergen, and Morgan.\textsuperscript{62, 63} but suffice to say, because the protons undergo a more pronounced relaxivity change, the difference in contrast is larger, and the resulting MR image is sharper than an image taken in the absence of a contrast agent.

Analyte-responsive MRI agents, on the other hand, modulate their ability to influence nearby nuclei but *only in the presence of a specific analyte*.\textsuperscript{44} The differing relaxivity values can then provide an indication that the analyte of interest is interacting with the agent. This category of bioresponsive MRI agents was first introduced by Tom Meade with his report of Egad, a reporter for β-galactosidase activity, and the field has since been expanded to K\textsuperscript{+}, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Fe\textsuperscript{2+}, Zn\textsuperscript{2+} and Cu\textsuperscript{2+/2+} responsive MRI contrast agents. We will cover the smattering of examples of Cu\textsuperscript{2+/-}-responsive MRI agents below.

An iminodiacetate derivatized DO3A scaffold serves as an effective Cu\textsuperscript{2+}-responsive MRI agent and exhibits a 41\% increase ($r_1 = 3.76$ in the apo form to 5.29 mM\textsuperscript{-1}s\textsuperscript{-1}) upon binding Cu\textsuperscript{2+}.\textsuperscript{64} CopperGad-1 (CG1), the first Cu\textsuperscript{2+}-responsive MRI contrast agent reported, is designed to cap the gadolinium center with its iminodiacetate arms and deny access of water molecules to the paramagnetic core. When Cu\textsuperscript{2+} coordinates to the iminodiacetate cap, the gadolinium center is exposed and effectively alters the relaxivity values of nearby nuclei. Selectivity for Cu\textsuperscript{2+} over other first-row transition metals is good; however, some interference is seen in the presence of 10-fold excess of Zn\textsuperscript{2+}.

A related sensor that uses an amide-linked quinoline moiety as a chelating agent improves upon this concept.\textsuperscript{65} The complex, Gd-QDOTAMA, operates in the same manner as CG1 — that is, when the capping quinoline group binds to Cu\textsuperscript{2+}, a water can coordinate to the Gd\textsuperscript{3+} and the complex can effectively influence the relaxivity of the bound water protons. Gd-QDOTAMA shows a 71\% change (4.27 mM\textsuperscript{-1}s\textsuperscript{-1} to 7.29 mM\textsuperscript{-1}s\textsuperscript{-1}) in relaxivity upon addition of equimolar amounts of Cu\textsuperscript{2+} and good selectivity for Cu\textsuperscript{2+} over K\textsuperscript{+}, Na\textsuperscript{+}, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Fe\textsuperscript{2+}, Fe\textsuperscript{3+}, Cu\textsuperscript{+} and even Zn\textsuperscript{2+}. The selectivity for Zn\textsuperscript{2+} is especially notable since it is difficult to engineer enough selectivity for Cu\textsuperscript{2+} over Zn\textsuperscript{2+} to compensate for the excess Zn\textsuperscript{2+} present in physiological samples. The selectivity and signal enhancement are showed in a series of $T_1$-weighted phantom images (Figure 1-8). Although the complex shows some sensitivity toward anions, it provides an excellent starting point for future generations of Cu\textsuperscript{2+/-}-responsive MRI probes.

MRI agents that respond to the cuprous ion have also been described.\textsuperscript{66} A second-generation of CopperGad (CG) compounds with a variety of ligand sets confers greater selectivity for copper over other metals (Figure 1-9). Instead of the iminodiacetate ligand found on CG1, representatives of the second CG generation have a pendant pyridine elaborated with a variety of substituents, including thioether arms, an NS\textsubscript{3} macrocycle, or a phenyl ring. Due to the soft donor sets, these compounds tend to bind Cu\textsuperscript{+} with with nanomolar to picomolar affinities and exhibit very large relaxivity changes upon binding metal. NMRD experiments agree well with deuterium measurements and establish that either one or two waters interact with the gadolinium center. Phantom images at clinical field strengths show that the CG series can respond to a range of physiologically relevant Cu\textsuperscript{+} concentrations.
The first bioresponsive MRI contrast agent was reported a little over ten years ago, and the intervening decade has seen a rapid expansion of this technology to small molecule analytes and metal ions. To the best of our knowledge, none of these agents have been approved for clinical use; however, we expect that MR imaging studies in mammals represent the next phase for this promising class of compounds.

**X-ray Fluorescence Microscopy (XRFM) For Cu Imaging.** X-ray fluorescence microscopy (XRFM) occupies a unique niche among copper-imaging modalities. Unlike the previous techniques, which report the presence of copper through an interaction of a small molecule with the metal, XRFM utilizes hard X-rays to eject a core shell electron from an atom. The vacancy is filled from a higher orbital and this transition results in the emission of a photon of energy equal to the difference between the two electron shell levels. Because each element has a characteristic emission energy, the emitted photons can be collected, measured and a spatial elemental map at sub-micron resolution can be generated. This technique provides information about the entire Cu pool, whereas the small-molecule fluorescent probes are oxidation state specific. MicroXANES can be used in combination with XRF if one seeks oxidation state information. We have briefly mentioned the use of XRFM to corroborate results from cellular molecular imaging studies; however, it is also a suitable stand-alone technique to study copper biology.

**XRFM Reveals Significant Copper Reorganization During Angiogenesis.** Finney and coworkers have reported the use of XRFM to understand the role of copper in angiogenesis. A model system for angiogenesis of human microvascular endothelial cells (HMVEC) was induced to form vascular networks. Elemental XRF imaging revealed that copper was initially localized to the perinuclear area, and after the cells were prompted to undergo angiogenesis, the cellular copper redistributed first to growing fliopedia, and then across the membrane and into the extracellular space (Figure 1). Subsequent studies showed that although extracellular copper chelation did not abrogate capillary formation, the generation of a mature network formation was significantly impaired. Taken together, this study is an impressive demonstration of the capability of XRF to elucidate the roles of metal nutrients and provide a coherent picture of copper dynamics at a subcellular level.

One primary advantage of XRF over other techniques is the capability to simultaneously measure several different metals so that relative amounts and localizations can be determined. A study of metal differences in the brain of Parkinson’s patients (PD) and healthy brains does just that. Rapid-scanning X-ray fluorescence microscopy suggests that the PD brain has significantly depleted levels of the three most several physiologically abundant metals (Fe, Cu, and Zn). Generally, high iron levels correspond to low zinc levels. Not only could a gross quantification of metals be made, but localized differences could also be determined. The PD brain showed a dramatic 40% increase iron content in the medial substantia nigra than a normal, healthy brain of similar age. This study represents a significant advancement and standardization of metal distribution visualization compared to traditional histology techniques. Notably, the large sample sizes (brain slices) could still be accurately and efficiently mapped.

XRF is a powerful technique that allows experimenters to extract valuable spatial data about the entire metallome in a single experiment. It is particularly appealing since the data are quantifiable as a metal ion concentration. The use of XRF also mitigates the concern that an added reagent, whether it is a small-molecule fluorescent probe or a responsive MRI agent, may perturb the system under study. The appeal of XRF suffers only because of its limited availability (beamtime at a third-generation synchotron is required) and fairly long acquisition
times per sample compared to traditional confocal microscopy. The information obtained, however, is well worth the time. As advancements in optics and beamlines improve and the technique becomes more commonplace, we are confident that the use of XRF will rapidly gain in popularity, and we look forward to the discoveries that it will facilitate.

**Concluding Remarks**

The field of fluorophores, especially that of analyte-responsive fluorophores, has matured in the past several decades and accordingly, the current probes for Cu$^+$ provide researchers with several options for small-molecule fluorophores. Though significant advances have been made in the field of turn-on fluorophores for Cu$^+$, there is still a need for probes that can be directed toward subcellular locations, and sensors with different wavelengths, a range of binding affinities and more desirable optical properties. In the context of biological imaging, Cu$^{2+}$-responsive fluorophores remain less developed, though it is clear that many research groups are directing significant resources toward designing and testing lead compounds. Metal-responsive MR imaging and XRFM are in their relative infancy compared to the field of fluorescent probes, but this may be, in part, due to a lack of widespread access to the appropriate technology. Their contribution to understanding the roles of copper and other metal ions in biology has been impressive, and we expect that the popularity and availability of these techniques to increase accordingly. Taken together, these imaging modalities will continue to provide novel insights into the roles of copper in biology.
Figure 1-1. A plot of publications referencing “fluorophore” per year as reported by Scifinder Scholar.
Figure 1-2. Generalized theory behind the development of Cu-fluorophores based on a photoinduced electron transfer principle. A) A fluorescent molecule with a tethered amine is in its ground state. B) The molecule absorbs a photon of light and a fluorophore electron from the HOMO to the LUMO. The amine lone pair is at an appropriate energy to inhibit the relaxation down to the fluorophore ground state and the emission is largely prevented. C) Upon coordination to the amine, the lone pair lowers in energy (decreases its reduction potential) and the quenching effect is relieved. The excited electron can relax back down to the ground state with concomitant emission of a photon.
Figure 1-3. Elemental copper maps in an NIH 3T3 fibroblast as reported by the small-molecule fluorescent reporter CTAP-1 and by X-ray fluorescence microscopy. The XRF sulfur map also corresponds well to the Cu map, indicating that it is a common elemental component in intracellular Cu coordination.
Figure 1-4. Ratio confocal microscopy images of (a) control and (b) ascorbate-supplemented HEK 293T cells with RCS1 showing that extracellular Cu⁺-chelation does not abrogate the effect of ascorbate on mobilizing intracellular Cu⁺-stores. (c) The fluorescence ratio is attenuated upon introduction of a Cu⁺-specific, cell-permeable chelator.
Figure 1-5. A [Cu(I)-GS] complex can catalyze the formation of a covalent bond between a dansyl sensitizer and a luminescent Eu\textsuperscript{3+} complex. The resulting triazole conjugate displays a 10-fold greater luminescence than the unsensitized complex.
Figure 1-6. The proposed mechanism of Cu(I)-detection by FluTPA1. The parent compound coordinates to Cu(I) and the resulting Cu(I)-complex prompts the oxidative cleavage of the benzyl ether linkage to unveil a dehydrofluorescein derivative. Oxidation to the fully conjugated and fluorescent compound provides an emissive read-out that Cu(I) is present.
Figure 1.7. Selected Cu$^{2+}$-sensors that use Cu$^{2+}$ chelation to convert a pro-fluorophore to its fluorescent parent rhodamine.
Figure 1-8. Schematic binding mode of Gd-QDOTAMA with Cu$^{2+}$ (left) and (right) $T_1$-weighted phantom images of (A) probe only or with; (B) 0.45 mM MgCl$_2$; (C) 0.45 mM ZnCl$_2$; (D) 0.45 mM CaCl$_2$; (E) 0.45 mM CuCl$_2$. 
Figure 1-9. A series of Cu^{2+/+}-responsive MR contrast reagents with a range of binding affinities, Cu-oxidation state preferences, and relaxivity values.
Figure 1-10. X-ray fluorescence image of human microvascular endothelial cells (HMVEC) that show Cu accumulation at the tips of growing filopodia.
References


Chapter 2:

Turn-on Fluorescent Copper Sensors by Electron Transfer Tuning: Confocal and X-ray Fluorescence Imaging of Endogenous Copper Stores in a Cell Model of Menkes Disease

Portions of this work were performed in collaboration with the following persons: Spectroscopic characterizations and synthesis were assisted by Drs. Li Zeng and Jiyoun Lee; XRF data collection were assisted by Ms. Sheel Dodani, and Drs. Evan Miller, Lydia Finney and Stefan Vogt.
Abstract

We report the syntheses, properties, and live-cell imaging applications of the Coppersensor (CS) family, a new class of fluorescent indicators for copper in biological systems. The CS reagents combine BODIPY reporters with thioether-rich recognition units to provide selective and sensitive optical detectors for Cu⁺ in water over competing metal ions, including abundant cellular alkali and alkaline earth cations Na⁺, K⁺, Ca²⁺, Mg²⁺ as well as d-block metal ions including Zn²⁺ and Cu²⁺. A targeted synthetic survey of the BODIPY platform provides a basis for tuning optical brightness and turn-on responses to copper; binding of Cu⁺ to the CS3 probe triggers a 75-fold fluorescence increase with a quantum efficiency of Φ = 0.40 for the metalated form. Confocal microscopy experiments establish the value of the membrane-permeable CS dyes for biological investigations by endogenous cellular copper stores in a genetic model of Menkes disease. These results are complemented by X-ray fluorescence microscopy to provide a coherent picture of copper distributions in healthy and diseased cells.
**Introduction**

All living organisms require metal ions for their growth, development, and survival, but disruptions in metal ion homeostasis at the cellular level are a major contributor to aging, disease, and death.1, 2 In this context, a rich frontier of inorganic physiology is offered by the brain, which uses the body’s highest levels of metals and oxygen consumption to drive specialized functions ranging from cognition to sensory and motor control.3-6 On the other hand, this combination of high metal content and oxidative demand also makes the brain particularly susceptible to oxidative stress, and diseases associated with aging and neurodegeneration have a strong connection to oxidative imbalance.7, 8 As a result, the contributions of redox-active transition metal centers to the inorganic chemistry of the brain are receiving increasing attention.

In this regard, copper is an essential element in the brain for structural and catalytic functions due to its potent redox activity.4, 9-11 However, unregulated copper pools can mediate aberrant oxidative stress reactions and aggravate disease states in neurodegenerative disorders, including Menkes and Wilson’s diseases,12-17 amyotrophic lateral sclerosis,18, 19 Alzheimer’s disease,8, 20-22 and prion diseases.23, 24 To balance the beneficial roles of copper with the deleterious effects of unchecked copper-dependent reaction cascades, cells exert stringent control over intracellular copper stores through a carefully orchestrated array of uptake, trafficking, storage, and efflux mechanisms.25-28 These homeostatic processes minimize thermodynamically free copper ions while rigorously maintaining kinetically labile copper stores for rapid deployment and utilization.29 Elegant studies of copper physiology, mainly in lower prokaryotic and eukaryotic organisms,28, 30-32 speak to the potential wealth of chemistry yet to be discovered in humans and higher organisms, particularly in more complex and specialized tissues such as the brain.

The foregoing open questions demand new methodologies to monitor bioavailable copper pools in living systems. Small-molecule fluorescent reporters provide one such way to study copper biology through molecular imaging.33-36 In principle, appropriate metal ion selectivities and sensitivities can be engineered into probes and modulation of electron-transfer properties can optimize optical emission properties for use in confocal microscopy – an ideal experimental technique for monitoring changes in copper mobility and distribution with spatial and temporal resolution. Cu⁺, which is stabilized in the reducing cytosol of eukaryotic cells, poses challenges that go beyond other metal ions for fluorescence visualization, including the need for redox specificity over Cu²⁺, the facile disproportionation of Cu⁺ to Cu²⁺ and Cu⁰ in water, and the quenching capabilities of Cu⁺ by electron or energy transfer.37 Accordingly, Cu⁺-responsive fluorescent sensors for aqueous use remain rare.33-35, 38

**Results and Discussion**

**Design and Synthesis of Copper-Responsive Fluorescent Sensors.** Our general strategy for achieving fluorescence Cu⁺ detection in physiological, aqueous environments relies on photoinduced electron transfer (PeT) sensing in an integrated fluorophore-receptor platform. In the absence of metal ion analyte, the relatively electron-rich receptor quenches fluorophore emission by back electron transfer to generate a non-fluorescent charge-transfer state. Metal ion binding disfavors formation of this charge-transfer state and alleviates PET quenching, resulting in a turn-on of fluorophore emission.39-41 PET switching provides a robust mechanism for metal ion detection, particularly for redox-inactive cations (e.g., Na⁺, K⁺, Ca²⁺, Mg²⁺, and Zn²⁺), but is difficult to apply to Cu⁺ owing to additional charge-transfer pathways that become available because of its redox activity. A more detailed framework for correlating off-on fluorescence...
switching comes from viewing PET quenching through application of a modified Rehm-Weller equation:

$$\Delta G_{\text{PET}} = E_{1/2}(D^*/D) - E_{1/2}(A/A^-) - \Delta E_{00} - C \quad (1)$$

where $E_{1/2}(D^*/D)$ represents the ground state oxidation potential of the amine donor, $E_{1/2}(A/A^-)$ is the ground state reduction potential of the fluorophore, $\Delta E_{00}$ corresponds to the energy of excitation, and $C$ represents an electrostatic term for the charge separation state. This approach is inspired by elegant work, most notably by Nagano and co-workers, on viewing fluorophore scaffolds as donor-acceptor units.

With this electron-transfer framework in mind, our efforts have focused on developing Cu$^+$-responsive fluorescent indicators with properties amenable to live-cell imaging applications. We chose the BODIPY reporter platform owing to its favorable photophysical characteristics (high quantum yields in aqueous solution, large extinction coefficients with visible light excitation and emission) and its demonstrated use as a readily functionalized, cell-permeable, biocompatible fluorophore. Tethering open-chain, sulfur-rich receptors provides not only a soft Lewis basic ligand environment suitable for the soft Lewis acid Cu$^+$, but also stabilizes positive Cu$^+$ reduction potentials to minimize charge-transfer contributions. The electronic characteristics of the BODIPY scaffold can be tuned through two different chemical approaches: (i) by changing the identity, and accordingly, the electron-donating or withdrawing ability of the substituents decorating the carbon dipyrromethene backbone (modulation of $E_{1/2}(A/A^-)$ and $\Delta E_{00}$ terms), or (ii) through manipulation of substituents appended to the boron atom (modulation of $E_{1/2}(A/A^-)$ term only).

**Spectroscopic Properties and Responses to Copper.** Scheme 2-1 outlines the synthetic routes to a series of four Cu$^+$-responsive CS indicators, CS0-CS3, that possess varying electronic properties. The synthesis of CS0 begins with condensation of 2,4-dimethylpyrrole with chloroacetyl chloride, followed by treatment with triethylamine and BF$_3$•OEt$_2$ to deliver chloromethyl BODIPY 1 as a red, crystalline solid in 61% yield. Nucleophilic displacement with the tetrathiaza receptor 5 provides CS0 (6) as an orange film in 20% yield. An analogous approach beginning with kryptopyrrole furnishes 2 in 55% yield, which is then coupled to 5 to produce CS1 as dark red crystals in 21% yield. Sequential treatment of 1 and 2 with aluminum trichloride and methanol affords the corresponding chloromethyl BODIPY compounds 3 and 4, respectively. Consistent with the preparation of CS0 and CS1, the intermediates 3 and 4 are smoothly converted to the final sensors CS2 and CS3, respectively, upon nucleophilic displacement with tetrathiaza receptor 5 in ca. 40% yields.

Spectroscopic evaluation of the CS dyes under simulated physiological conditions (20 mM HEPES, pH 7) show characteristic BODIPY absorption and emission elements in the visible region. Table 2-1 provides a summary of properties, including the range of driving forces accessed by the series of compounds presented and Figure 2-1 shows the cyclic voltammetry (CV) traces. The apo CS0 dye exhibits one major absorption peak at 515 nm ($\epsilon = 3.1 \times 10^4$ M$^{-1}$ cm$^{-1}$) with a weak fluorescence band centered at 535 nm ($\Phi = 0.01$). Upon addition of Cu$^+$, the absorption band maintains its position at 515 nm ($\epsilon = 4.3 \times 10^4$ M$^{-1}$ cm$^{-1}$) with a modest fluorescence intensity increase of ca. 1.2-fold. Spectroscopic analysis of CS1, an analog based on a more electron-rich parent BODIPY fluorophore, shows a prominent absorption at 540 nm ($\epsilon = 3.0 \times 10^4$ M$^{-1}$cm$^{-1}$) with faint emission ($\Phi = 0.016$) centered at 566 nm. Addition of one
equivalent of Cu\(^{+}\) induces a 10-fold increase in fluorescence (\(\Phi = 0.13\)) with a slight blue shift in its emission maximum to 561 nm (Figure 2-2).

Based on electron-transfer considerations, we reasoned that the improved Cu\(^{+}\) turn-on response of CS1 compared to CS0 was a result of increased electron density on the fluorophore for the former, making it harder to reduce. To pursue this idea further, we synthesized two additional derivatives to decrease the overall driving force for PET quenching in hopes of obtaining larger fluorescence turn-on responses to Cu\(^{+}\). Specifically, we converted the BODIPY BF\(_2\)-chelates to their more electron-rich 4,4-di-alkoxy derivatives. CS2 maintains the same organic pyrrole framework as CS0, but has a calculated \(\Delta \Delta G_{\text{PET}}\) of 0.137 eV that suggests it should be easier to quench than CS1, and exhibit a reduced fluorescence turn-on response to Cu\(^{+}\), relative to CS1. In line with these observations, apo CS2 possesses CS0-like absorption (515 nm, \(\epsilon = 3.4 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}\)) and emission (530 nm, \(\Phi = 0.009\)) maxima and triggers a 6-fold turn-on increase in fluorescence intensity upon introduction of one equivalent of Cu\(^{+}\) (\(\Phi = 0.057\), Figure 2-3). Diazopy CS3 is the most electron-rich compound in the series and achieves the largest turn-on response to Cu\(^{+}\). Spectroscopic analysis of apo CS3 shows a major peak centered at 550 nm (\(\epsilon = 3.1 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}\)) with a shoulder at 511 nm. Maximal emission occurs at 560 nm with weak fluorescence (\(\Phi = 0.006\), Figure 3). Addition of Cu\(^{+}\) leads to a slight blue shift in the absorption band to 540 nm (4.6 \(\times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}\)) and a large 75-fold increase in fluorescence intensity (\(\Phi = 0.40\)) with a corresponding hypsochromic shift in emission maximum to 548 nm (Figure 2-4). Binding analysis using the method of continuous variations (Job’s plot) indicates that 1:1 Cu\(^{+}\)-dye complexes are responsible for the enhanced fluorescence responses observed for the CS indicators (Figure 2-5). Apparent dissociation constant values (\(K_d\)) span the range of 3 pM to 90 fM (Figure 2-6), offering a set of tools with varying Cu\(^{+}\) affinities.

The turn-on fluorescence responses of the CS fluorophores show high metal ion and oxidation state selectivity for Cu\(^{+}\) over biologically relevant metal ions. Figure 2-7 displays the relative fluorescence responses of CS1 to a panel of competing cations. Figures 2-8 and 2-9 present analogous data collected for CS2 and CS3, respectively. Owing to their soft sulfur donor sets, the CS dyes are selective for soft Cu\(^{+}\) over hard Mg\(^{2+}\) and Ca\(^{2+}\) ions at millimolar cellular concentrations; these divalent metal ions do not give false emission positives or interfere with the turn-on Cu\(^{+}\) responses. The CS indicators are also selective for Cu\(^{+}\) over a 25-fold excess of the first-row transition metal ions Mn\(^{3+}\), Fe\(^{3+}\), Co\(^{2+}\), Ni\(^{2+}\), and Cu\(^{2+}\). Of note is the observed oxidation-state specificity for Cu\(^{+}\) over Cu\(^{2+}\). Finally, the emission profiles of the apo or Cu\(^{+}\)-bound CS probes or their turn-on responses to Cu\(^{+}\) are unaffected by millimolar concentrations of Zn\(^{2+}\), indicating robust selectivity for d\(^{10}\) Cu\(^{+}\) over d\(^{10}\) Zn\(^{2+}\).

**CS3 is Capable of Imaging Labile Pools of Copper in Living Cells at Basal and Copper-Depleted Levels.** The two previously reported turn-on fluorescent probes for live-cell Cu\(^{+}\) detection, CTAP-1 and CS1, are capable of detecting changes in labile intracellular copper levels, but their relatively low quantum efficiencies limits their use to visualizing differences under situations of acute or prolonged copper overload.\(^{33, 34}\) We reasoned that CS3, with its improved brightness and turn-on response to Cu\(^{+}\), would provide the new ability to report pools of intracellular, exchangeable Cu\(^{+}\) at basal levels. We therefore sought to test whether this new 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.\(^{61}\) Accordingly, human embryonic kidney (HEK 293T) cells were grown either in
normal media or in media containing 200 µM BCS for 48 h to make them copper depleted, stained with 2 µM CS3 for 10 min, and subsequently imaged by confocal microscopy (Figure 2-10). Cells grown in normal control media exhibit markedly higher fluorescence signals compared to cells grown in the presence of BCS (Figure 2-10A, B), indicating that CS3 can respond to changes in basal, endogenous levels of exchangeable Cu⁺ as well as sense differences between copper-depleted and copper-normal conditions. Furthermore, treatment of HEK 293T cells with the cell-permeable Cu⁺-chelator tris((ethylthio)ethylamine) (TEMEA) could rapidly diminish the fluorescence intensity from CS3-stained cells (Figure 2-10C), indicating that CS3 could also monitor dynamic Cu⁺-stores in living cells.

We next sought to determine if CS3 could image endogenous, basal copper stores in disease model cells lines. To this end, we employed the mottled murine cell line – a mouse cell line that is deficient in the ATP7a protein and used as a mouse model for Menkes disease. Specifically, the Mo dun mutant fibroblasts and the Mo*/cell lines were used as wild-type, control fibroblasts. Previous studies have established that cells with dysfunctional ATP7a acquire large excesses of copper compared to wild-type cells, presumably because the machinery for copper efflux is compromised. We reasoned that with CS3, we could examine the fate of these expanded metal stores and see if any marked temporal differences existed between the two cell lines. To this end, we stained wild-type and mutant cells with CS3 and examined the signal distribution and intensity. Notably, wild-type cells had significantly less fluorescence signal than ATP7a mutant cells, which tracks well with ICP-MS data that shows a hyperaccumulation of copper in these mutant fibroblasts (Figure 2-11). However, a decreased intensity from a chelation-based fluorophore can have two different causes: there can be a lower absolute level of metal, or there can be a lower level of kinetically labile, exchangeable metal. Though these represent limiting cases, and a gradient of interpretations can be made between those extremes, we sought to investigate how well a spatial map from CS3-stained cells correlated with spatial map that directly interrogated the presence of the metal.

To study copper in these cells using an independent technique as well as verify our CS3-based molecular imaging results, we performed X-ray fluorescence microscopy (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. 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. 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.

First, Mo dun fibroblasts that had been grown in the presence of copper (50 µM, 48 h) were stained with CS3, imaged live by confocal microscopy and then fixed and subjected to XRFM scanning (Figure 2-12). The spatial maps generated by CS3 fluorescence or by XRFM show excellent correlation, despite some morphological alterations due to drying prior to XRFM analysis. This suggests two things: that CS3 can provide accurate, spatial information of intracellular stores and that the majority of the cellular copper is indeed, Cu⁺. We also noticed, however, that a significant amount of signal stemmed from the nucleus region of the cell – an observation that we could not make with CS3, since it does not penetrate the nucleus.
To investigate this further, we collected a complete XRFM dataset of wild-type and mutant fibroblasts grown in the presence and absence of copper. Wild-type cells grown under both of these conditions show a similar perinuclear elemental copper distribution, though cells grown in exogenous copper have elevated levels of copper (Figure 2-13). These observations are in line with ICP-MS studies. Mo<sub>lap</sub> cells grown in basal media show, like wild-type cells, a perinuclear distribution of copper, whereas cells grown in the presence of copper show a remarkable 20-fold increase in intracellular copper levels. (Figure 2-14). More strikingly, a significant amount of copper is concentrated in the nucleus region as indicated by the colocalization with the zinc and phosphorus channels. The observation that significant levels of copper is concentrated in the nucleus of these mutant cells suggests that copper-mediated DNA damage may provide an insight into the etiology and treatments for Menkes disease.

**Concluding Remarks**

Through a targeted synthetic survey of BODIPY fluorophores, we have described the Coppersensor series of probes that range in turn-on from 1.2- to 70-fold fluorescence enhancement upon binding Cu<sup>+</sup>. A comprehensive electrochemical and spectroscopic evaluation shows that the most electron-rich derivative, Coppersensor-3, has the most ideal properties for cellular imaging. A combination of CS3 molecular imaging and X-ray fluorescence microscopy provides a coherent picture of cellular copper distributions under conditions of Cu-depleted, basal and Cu-overload conditions, and suggests that in ATP7a-mutant cells, significant copper nuclear accumulation occurs. Taken together, this study highlights the power of using multiple imaging modalities. Our results suggest that the development of nuclear-targeted chelation therapeutics may provide a potential strategy for abrogating the effects of disrupted copper handling in patients with Menkes disease.

**Experimental Section**

**Synthetic Materials and Methods.** All reactions were carried out under a dry N<sub>2</sub> 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<sub>1</sub><sup>50</sup> Coppersensor-1 (CS1, 7), receptor<sub>5</sub><sup>25</sup> and [tris(ethylthio)ethyl]amine] (TEMEA)<sup>68</sup> were synthesized according to literature procedures. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. <sup>1</sup>H NMR spectra were collected in CDCl<sub>3</sub> (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<sub>3</sub> 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-4,4-dimethoxy-1,3,5,7-tetramethyl-4-bora-3α-diaza-s-indacene** (3). AlCl<sub>3</sub> (45 mg, 0.33 mmol) was added to a solution of 1 (66 mg, 0.22 mmol) in dichloromethane (3.0 mL) and stirred vigorously for 5 minutes. Methanol (1.5 mL) was added and the reaction was allowed to stir for an additional 5 minutes. The mixture was diluted with ethyl acetate (50 mL), washed with water (2 x 20 mL) and brine (20 mL) and the organic layer was separated and dried over Na<sub>2</sub>SO<sub>4</sub>. Purification by column chromatography (silica gel, ethyl acetate) delivered 2 as a dark red solid (50 mg, 71%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz): δ 6.08 (2H, s), 4.82 (2H, s), 2.84 (6H, s), 2.55 (6H, s), 2.49 (6H, s). ESI-MS calculated for [M<sup>+</sup>] 320, found 320.
8-Chloromethyl-2,6-diethyl-4,4-dimethoxy-1,3,5,7-tetramethyl-4-bora-3a-diaza-s-indacene (4). 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 minutes. Methanol (5 mL) was added and the reaction was allowed to stir for an additional 5 minutes. 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 2 as a dark red solid (246 mg, 65%). ¹H NMR (CDCl₃, 300 MHz): δ 4.87 (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). HRFAB-MS calculated for [M⁺] 376.2089, found 376.2088.

8-[N,N-Bis(3',6'-dithiaoctyl)-aminomethyl]-4,4-dimethoxy-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (Coppersensor-2, CS2, 8). A 25-mL Schlenk tube was charged with 3 (17 mg, 0.05 mmol), 5 (22 mg, 0.07 mmol), KI (11 mg, 0.07 mmol), and K₂CO₃ (12 mg, 0.09 mmol). CH₂CN (0.7 mL) was added via syringe and the resulting solution was stirred at 50 °C for 2 h under a nitrogen atmosphere. The mixture was concentrated and the residue was dissolved in dichloromethane (20 mL) and washed with water (2 × 10 mL). The organic phase was dried over Na₂SO₄, concentrated to dryness and purified by column chromatography (silica gel, ethyl acetate) to provide 8 as an orange-red oil (22 mg, 69%). ¹H NMR (CDCl₃, 300 MHz): δ 6.06 (s, 2H), 4.00 (s, 2H), 2.89 (4H, m), 2.87 (6H, s), 2.68 – 2.51 (22H, m), 2.50 (6H, s), 1.19 (6H, t, J = 4.5 Hz). FAB-MS: calculated for [MH⁺] 598, found 598.

8-[N,N-Bis(3',6'-dithiaoctyl)-aminomethyl]-2,6-diethyl-4,4-dimethoxy-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (Coppersensor-3, CS3, 9). A 25-mL Schlenk tube was charged with 4 (52 mg, 0.14 mmol), 5 (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 9 as a orange-red oil (50 mg, 55%). ¹H 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 (Walnut Creek, CA) and fluorescence spectra were recorded using a Photon 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 (1.4-mL or 3.5-mL volume, Starna, Atascadero, CA). Fluorescence quantum yields were determined by reference to rhodamine 101 inner salt in methanol (Φ = 1.0)⁶⁰ for CS1 and CS3 or rhodamine B in water (Φ = 0.31)⁷⁰ for CS0 and CS2. The binding affinities of Cu⁺ to the CS dyes were measured using thiourea as a competitive ligand to provide buffered Cu⁺ solutions. Stability constants for thiourea binding were taken from the literature: β₁₂ = 2.0 × 10¹², β₁₃ = 2.0 × 10¹⁴, β₁₄ = 3.4 × 10¹⁵.⁷¹ Cu(I) was delivered in the form of [Cu(MeCN)₄][PF₆] from an acetonitrile stock solution (2 mM). Measurements were carried out in 20 mM HEPES, pH 7. Excitation was provided at 540 nm, and collected emission was integrated from 550 to 700 nm. The apparent
dissociation constants ($K_d$) were determined using the following equation: \((F - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}}) = [\text{Cu}^+]/(K_d + [\text{Cu}^+])\), where \(F\) is the observed fluorescence, \(F_{\text{max}}\) is the fluorescence for the \(\text{Cu}^+:\text{CS}\) complex, and \(F_{\text{min}}\) is the fluorescence for the free CS dye.

**Cell Culture.** HEK 293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Carslad, CA) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen), glutamine (2 mM) and penicillin/streptomycin (50 µg/mL, Invitrogen). One day before imaging, cells were passed and plated on 18-mm glass coverslips coated with poly-L-lysine (50 µg/mL, Sigma, St. Louis, MO). \(\text{Mo}^{\text{XY}}\) and \(\text{Mo}^{\text{lap}}\) fibroblasts were a gift from Chris Vulpe. Wild-type and mutant fibroblasts were cultured in Eagle’s Minimum Essential Medium (EMEM, ATCC) and Ham’s F12 Nutrient Mixture (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS). Three days before imaging, cells were passed and plated on 18-mm glass coverslips coated with poly-L-lysine. Immediately before the experiments, cells were washed with PBS buffer, incubated with the probe in DMEM, washed and image. Experiments to assess copper uptake were performed in the same media supplemented with the additives at the indicated concentrations. Image analysis was performed in Image J.

**Live-Cell Imaging.** Confocal fluorescence imaging was performed with a Zeiss LSM 510 NLO Axiovert 200 laser scanning microscope and a 40x water-immersion objective lens. Excitation of CS-loaded cells at 543 nm was carried out with a HeNe laser, and emission was collected using a 560 nm longpass filter. CS3 (1-2 µM) was incubated with live cells samples for 15 min at 24 °C. Additions of the intracellular copper chelator TEMEA were performed directly on the microscope stage by bath application to the media.

**XRFM Samples.** \(\text{Mo}^{\text{XY}}\) and \(\text{Mo}^{\text{lap}}\) fibroblasts were plated on poly-L-lysine coated silicon nitride windows (2 × 2 mm; thickness 500 nm) manufactured by Silson (Bilsworth, U.K.). After the treatments indicated in the text or supporting information, cells were fixed in 4% paraformaldehyde 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, IL) as previously described. Analysis was performed with the MAPS software suite.
Scheme 2-1. Synthetic routes to the Coppersensor (CS) family of copper-responsive fluorescent indicators.
Figure 2-1. Electrochemical characterization of CS sensors. All measurements were performed in MeCN with 0.2 M NBu₄PF₆ as electrolyte and referenced to ferrocene.
Table 2-1. Spectroscopic and Thermodynamic Data for CS Chemosensors

<table>
<thead>
<tr>
<th></th>
<th>Excitation (λ/ nm, ε/ x 10^4 M⁻¹cm⁻¹)</th>
<th>Emission (λ/ nm, Φ)</th>
<th>K_d (M)</th>
<th>E_{1/2}(A/A*) (V)</th>
<th>ΔΔG_{pet} (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo dye</td>
<td>Cu⁺-bound</td>
<td>Apo dye</td>
<td>Cu⁺-bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS0 (6)</td>
<td>515, 3.1</td>
<td>535, 4.3</td>
<td>515, 0.01</td>
<td>535, 0.01</td>
<td>n.d.</td>
</tr>
<tr>
<td>CS1 (7)</td>
<td>540, 3.0</td>
<td>540, 4.0</td>
<td>566, 0.016</td>
<td>561, 0.13</td>
<td>3.6(0.3) x 10^{-12}</td>
</tr>
<tr>
<td>CS2 (8)</td>
<td>515, 3.4</td>
<td>494, 4.8</td>
<td>523, 0.01</td>
<td>524, 0.057</td>
<td>2.8(0.3) x 10^{-14}</td>
</tr>
<tr>
<td>CS3 (9)</td>
<td>550, 3.1</td>
<td>540, 4.6</td>
<td>560, 0.006</td>
<td>548, 0.40</td>
<td>8.8(0.3) x 10^{-14}</td>
</tr>
</tbody>
</table>

All spectroscopic measurements were performed in 20 mM HEPES buffer pH 7. Quantum yields are based on a rhodamine 101 standard (Φ = 1.0 in MeOH) for CS1 and CS3, or a rhodamine B standard (Φ = 0.31 in water) for CS0 and CS2. Reduction potentials are relative to Fc⁺/Fc couple. Driving force for quenching is relative to CS0.
Figure 2-2. Fluorescence responses of 2 mM CS1 to Cu⁺. Spectra shown are for buffered [Cu⁺] of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 µM. Spectra were acquired in 20 mM HEPES, pH 7, with excitation at 540 nm.
Figure 2-3. Fluorescence responses of 1.5 µM CS2 to Cu\(^{+}\). Spectra shown are for buffered [Cu\(^{+}\)] of 0, 0.3, 0.5, 0.8, 1.0, 1.3, and 1.5 µM. Spectra were acquired in 20 mM HEPES, pH 7, with excitation at 510 nm.
**Figure 2-4.** Fluorescence responses of 4 µM CS3 to Cu⁺. Spectra shown are for buffered [Cu⁺] of 0, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 µM. Spectra were acquired in 20 mM HEPES, pH 7, with excitation at 530 nm.
Figure 2-5. 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.
Figure 2-6. Normalized fluorescence response of 4 µM CS3 to thiourea buffered Cu⁺ solutions for $K_d$ 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 3000 fM, respectively. The observed $K_d$ value is $8.8(3) \times 10^{-14}$ M.
Figure 2-7. Fluorescence responses of CS1 to various metal ions. Bars represent the final integrated fluorescence response ($F_f$) over the initial integrated emission ($F_i$). Spectra were acquired in 20 mM HEPES, pH 7. 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 2 µM solution of CS1. Black bars represent the subsequent addition of 10 µM Cu$^{+}$ to the solution. Excitation was provided at 540 nm, and the collected emission was integrated over 550 to 650 nm.
Figure 2-8. Fluorescence responses of CS2 to various metal ions. Bars represent the final integrated fluorescence response (F_f) over the initial integrated emission (F_i). Spectra were acquired in 20 mM HEPES, pH 7. White bars represent the addition of an excess of the appropriate metal ion (2 mM for Ca^{2+}, Mg^{2+}, and Zn^{2+}, 50 mM for all other cations) to a 1.5 µM solution of CS2. Black bars represent the subsequent addition of 2 µM Cu^{+} to the solution. Excitation was provided at 510 nm, and the collected emission was integrated over 520 to 700 nm.
Figure 2-9. Fluorescence responses of CS3 to various metal ions. Bars represent the final integrated fluorescence response ($F_f$) over the initial integrated emission ($F_i$). Spectra were acquired in 20 mM HEPES, pH 7. White bars represent the addition of an excess of the appropriate metal ion (2 mM for $\text{Ca}^{2+}$, $\text{Mg}^{2+}$ and $\text{Zn}^{2+}$, 50 µM for all other cations) to a 4 µM solution of CS3. Black bars represent the subsequent addition of 4 µM $\text{Cu}^{2+}$ to the solution. Excitation was provided at 530 nm, and the collected emission was integrated over 540 to 700 nm.
Figure 2-10. Molecular imaging of endogenous basal Cu in human embryonic kidney (HEK 293T) cells with CS3. Cells were either grown in basal media (A), in the presence of the extracellular Cu-chelator BCS (200 µM) for 48 h (B) or treated with 1 mM of the intracellular Cu⁺-chelator tris((ethylthio)ethylamine) (TEMEA) for 5 minutes (C). (D) Graph showing the quantification of mean fluorescence intensity of each condition normalized to the control condition (n = 5-8 fields of cells per condition). Error bars represent s.e.m. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared to control cells.
Figure 2-11. (A) Wild-type fibroblasts ($Mo^{XY}$) and (B) mutant ($Mo^{dap}$) stained with 2 µM CS3 for 10 min at 37 ºC and then imaged by confocal microscopy. Consistent with ICP-MS results indicating that ATP7a-mutant cells have impaired copper efflux capabilities, these cells have higher levels of copper than wild-type fibroblasts.
Figure 2-12. (A) Elemental Cu map of a Mo^<sup>sup</sup> fibroblast as visualized by XRF imaging and (B) the same cell stained with CS3 and imaged by confocal microscopy to reveal the Cu^+ distribution. There is a good correlation between the spatial information generated by XRF and by CS3 imaging, which suggests that CS3 can provide reliable spatial information in a cellular system.
Figure 2-13. Elemental maps of phosphorus, copper, and Zn distributions in a $Mo^{3+}$ fibroblast grown in the absence (basal) or presence of 50 µM CuCl$_2$ (copper supplemented). Wild-type fibroblasts grown with exogenous copper salts show an increased intracellular Cu content compared to cells grown in basal media. The Cu is distributed, in both cases, in a perinuclear fashion. The P and Zn channels can be used to identify the nucleus.
Figure 2-14. Elemental maps of phosphorus, copper, and zinc distributions in a \( Mo^{dap} \) fibroblast grown in the absence (basal) or presence of 50 \( \mu \)M CuCl\(_2\) (copper supplemented). ATP7a mutant fibroblasts grown with exogenous copper salts show a very large (ca. 20-fold) increase in intracellular Cu content compared to cells grown in basal media. Intriguingly, the excess copper seems localized to the nuclear region of the cell, which provides a possible explanation for the increased DNA damage seen in Menkes patients.
References


62. Masson, W., Hughes, H., Papworth, D., Boyd, Y. & Horn, N. Abnormalities of copper accumulation in cell lines established from nine different alleles of mottled are the same as those found in Menkes disease. *Journal of Medical Genetics* 1997, 34, 729-732.

Chapter 3:
Visualizing Ascorbate-Triggered Release of Labile Copper within Living Cells using a Ratiometric Fluorescent Sensor

Portions of this work were published in the following scientific journal:

Portions of this work were performed in collaboration with the following persons:
Spectroscopic characterizations and synthesis were assisted by Dr. Li Zeng.
Abstract

We present the synthesis, properties, and biological applications of Ratio-Coppersensor-1 (RCS1), a new water-soluble fluorescent sensor for ratiometric imaging of copper in living cells. RCS1 combines an asymmetric BODIPY reporter and thioether-based ligand receptor to provide high selectivity and sensitivity for Cu\(^+\) over other biologically relevant metal ions, including Cu\(^{2+}\) and Zn\(^{2+}\), a ca. 20-fold fluorescence ratio change upon Cu\(^+\) binding, and visible excitation and emission profiles compatible with standard fluorescence microscopy filter sets. Live-cell confocal microscopy experiments show that RCS1 is membrane-permeable and can sense changes in the levels of labile Cu\(^+\) pools within living cells by ratiometric imaging, including expansion of endogenous stores of exchangeable intracellular Cu\(^+\) triggered by ascorbate stimulation in kidney and brain cells.
Introduction

The potent redox activity of copper is essential yet toxic to living organisms.\(^1\)-\(^4\) Accordingly, cells tightly regulate copper to harness this capacity for beneficial purposes, as disruption of copper homeostasis is linked to oxidative or nitrosative stress through the aberrant production and consumption of reactive oxygen and nitrogen species (ROS/RNS), respectively.\(^5\)\(^-\)\(^7\) Because spatial and temporal fluxes in cellular copper pools can have disparate physiological or pathological consequences, new methods for monitoring copper in living cells can help elucidate its complex contributions to healthy and disease states.\(^8\)\(^-\)\(^10\) Molecular imaging with copper-responsive indicators provides an attractive approach to achieving this goal, and synthetic sensors\(^11\),\(^12\) and dosimeters\(^13\)\(^-\)\(^15\) that give a turn-on emission increase to Cu\(^+\) or Cu\(^{2+}\) in water have been reported recently.\(^16\)

Despite these advances, fluorescent copper indicators that have been used successfully in biological experiments are rare,\(^11\),\(^12\),\(^17\)\(^-\)\(^19\) as creating molecules that possess an appropriate combination of chemical selectivity, optical sensitivity, and biological compatibility remains a challenging task. In addition, although intensity-based reagents are of practical utility, external influences that lead to variations in probe concentration and environment can complicate measurements in biological samples. These potential artifacts can be minimized by ratiometric imaging, which relies on probes that have two distinct measurable signals in the presence or absence of analyte.\(^20\) Here, we present Ratio-Coppersensor-1 (RCS1), a new type of ratiometric fluorescent reporter for copper. RCS1 possesses high selectivity for Cu\(^+\) over competing metal ions at cellular concentrations and a ca. 20-fold fluorescence ratio change upon Cu\(^+\) binding. Confocal microscopy experiments establish the ability of RCS1 to report changes in Cu levels, including mobilization of endogenous Cu\(^+\) stores by ascorbate.

Results and Discussion

Design and Synthesis of a Ratiometric Copper Probe. Our strategy for ratiometric sensing of cellular copper is based on modulating an asymmetric BODIPY fluorophore platform. A related fluorescent sensor for detecting K\(^+\) in mixed aqueous-acetonitrile media has been reported.\(^21\) RCS1 is synthesized in five steps as outlined in Scheme 1. Dipyrrromethane 2, available by the method of Laha et al., is chlorinated with NCS in 56% yield. Oxidation to the dipyrrmethene, followed by insertion of the BF\(_2\) unit provides BODIPY 5. Substitution of one of the chlorine atoms with a methoxy group proceeds in quantitative yield, and displacement of a chloride with the receptor provides RCS1 as a dark, pungent red oil. The oil can be prompted to crystallize through slow evaporation of a hexane solution.

Spectroscopic Properties and Responses to Copper. In HEPES buffer at pH 7.0, RCS1 exhibits a major absorption peak centered at 550 nm (\(\varepsilon = 4.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\)) with a shoulder at 523 nm (\(\varepsilon = 2.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\)) (Figure 3-1). Upon excitation at 480 nm, RCS1 displays two emission maxima of near equal intensity centered at 505 nm (\(\Phi = 0.002\)) and 570 nm (\(\Phi = 0.003\)) (Figure 3-2). Addition of one equivalent of Cu\(^+\) induces a hypsochromic shift of the dominant absorption and emission bands to 548 nm (\(\varepsilon = 4.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\)) and 556 nm, respectively, with a concomitant 20-fold fluorescence increase (\(\Phi = 0.05\)). Notably, the emission intensity at 505 nm is unchanged, rendering the compound useful for ratiometric applications. Binding analysis using the method of continuous variations suggests that a 1:1 RCS1:Cu\(^+\) complex is responsible for the ratiometric fluorescence response (Figures 3-3, 3-4), and the apparent \(K_d\) for Cu\(^+\) complexation to RCS1 in HEPES buffer at pH 7.0 is 4.0(3) \(\times\) \(10^{-11}\) M (Figure 3-5). Owing to its thioether-rich receptor, the ratiometric fluorescence response of RCS1 is
selective for Cu⁺ over various biologically relevant metal ions (Figure 3-6). The emission profiles of apo or Cu⁺-bound RCS1 are unchanged in the presence of 1 mM Na⁺, K⁺, Ca²⁺, or Mg²⁺, and first-row d-block metal ions, including 50 mM Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, and 1 mM Zn²⁺ do not interfere or give false positives within 10% of the full turn-on to Cu⁺. The fluorescence increase in the presence of Cu⁺ can be reversed upon addition of an excess of Cu⁺ chelator such as TEMEA. Control experiments establish that the ratio is largely independent of changes in probe concentration or pH (Figures 3-7, 3-8).

**Fluorescence Detection of Copper in Living Cells in Situations of Copper Overload.**

We next sought to assess whether RCS1 could report changes in the levels of labile Cu in living cells by ratiometric fluorescence imaging. Experiments in HEK 293T cells established that we could observe changes in fluorescence ratios in cells treated with exogenous Cu sources or Cu chelators (Figure 3-9). Cells treated with 100 µM CuCl₂ for 6 h, followed by staining with 2 µM RCS1 for 10 minutes, show an increase in intracellular fluorescence ratio as compared to cells in without exogenous CuCl₂. Furthermore, the fluorescence ratio Cu-treated cells can be decreased upon addition of a cell-permeable Cu⁺-specific chelator. Taken together, these results indicate that RCS1 can respond reversibly to intracellular copper levels.

**Fluorescence Detection of Copper Mobilized by Ascorbate.** We then examined whether this indicator could visualize changes in endogenous pools of exchangeable intracellular copper. In this context, ascorbate has been reported to facilitate the ceruloplasmin-dependent uptake and distribution of Cu in K562 cells.²² We reasoned that application of this reductant would shift Cu²⁺ redox equilibrium and increase the kinetically labile Cu⁺ pool. Ratio images of live C6 rat glioma cells labeled with RCS1 reveal that ascorbate-treated samples possess an elevated level of labile Cu⁺ compared to untreated samples (Figure 3-10). Control experiments with a cell-permeable Cu⁺ chelator, tris((ethylthio)ethyl)amine (TEMEA), confirms that the observed fluorescence ratio changes are due to Cu⁺ binding. Furthermore, co-incubation of C6 cells with ascorbate and bathocuproine disulfonate (BCS), a cell-impermeable chelator, has no effect on ascorbate-induced increases in the labile Cu⁺ pool, showing that the transient increases in labile Cu⁺ detected by RCS1 originate from intracellular stores. Analogous studies in HEK 293T cells show similar results (Figures 3-11, 3-12).

**Concluding Remarks**

In closing, we have presented a new type of ratiometric Cu⁺-specific fluorescent sensor for molecular imaging in living systems. RCS1 features high selectivity for Cu⁺ over other metal ions and a ca. 20-fold fluorescence ratio change with visible excitation and emission profiles. Experiments with live cells show that RCS1 is capable of detecting changes in levels of intracellular Cu⁺ upon exogenous copper addition, as well as sensing ascorbate-stimulated expansions of endogenous Cu⁺ pools. We are applying RCS1 and developing improved versions for studying the intracellular redox biology of copper, with a particular interest in brain and immune systems.

**Experimental Section**

**Synthetic Materials and Methods.** All reactions were carried out under an atmosphere of dry nitrogen and stirred magnetically. 2,2’-(Mesitylmethylene)bis(1H-pyrrole) (2) was synthesized according to the method of Laha et al. Receptor 7 was synthesized according to our previously published procedure. Tris((ethylthio)ethyl)amine (TEMEA) was synthesized according to a previously published procedure. 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. 1H NMR spectra were collected in CDCl₃ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C 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 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.

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 N₂ 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 Na₂SO₄. Column chromatography (silica gel, 1:1 hexanes/dichloromethane) provided 3 as a brown oil (740 mg, 56%). 1H NMR (CDCl₃, 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 N₂. 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 NaHCO₃ (20 mL) was added. The layers were separated, the organic phase washed with water (20 mL), dried over Na₂SO₄, and concentrated to a red-brown solid. Column chromatography (silica gel, 4:1 hexanes/dichloromethane) afforded 4 as reddish-brown needles (500 mg, 70%). 1H NMR (CDCl₃, 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 NEt₃ (2.1 mL, 15.1 mmol) in one portion. The resulting mixture was stirred for 1 h at room temperature. BF₃•OEt₂ (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 Na₂SO₄ and concentrated to dryness. Purification by column chromatography (silica gel, 1:1 dichloromethane/hexanes) gave 5 (525 mg, 92%) as an orange, crystalline solid. 1H NMR (CDCl₃, 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 Na₂SO₄ 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%). 1H NMR (CDCl3, 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). CH2CN (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%). 1H NMR (CDCl3, 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 spectrophotometer (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 [NH4]2[Fe][SO4]2·6H2O. Fluorescence quantum yields were determined by reference to rhodamine 101 inner salt in methanol (Φ = 1.0).23 The binding affinity of Cu+ to RCS1 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 H2O. Cu(I) was delivered in the form of [Cu(MeCN)]6[PF6]4 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: β12 = 2.5 × 1012, β13 = 2.0 × 1014, β14 = 3.4 × 1015.24 Excitation was provided at 480 nm and the emission was integrated over 490-525 and 525-650 nm. The apparent dissociation constant (Kd) was determined using the following equation: 

\[
\frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} = \frac{[\text{Cu}^+] / (K_d + [\text{Cu}^+])}{F_{\text{max}}}.
\]

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 CuCl₂ 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 and Schemes

Scheme 3-1. Synthesis of Ratio-Coppersensor-1 (RCS1).
Figure 3-1. 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 3-2. Fluorescence response of 2 mM RCS1 to Cu$^{+}$. Spectra shown are for [Cu$^{+}$] of 0, 0.3, 0.5, 0.8, 1.0, 1.3, 1.5, 1.8, and 2.0 μM. Spectra were acquired in 20 mM HEPES, pH 7, with excitation at 480 nm.
Figure 3-3. 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 3-4. 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 3-5. 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 $4.0(3) \times 10^{-11}$ M.
Figure 3-6. Fluorescence responses of RCS1 to various metal ions. Bars represent the final integrated ratiometric fluorescence response from 525-650 nm ($F_{560}$) over the initial integrated emission from 490-525 nm ($F_{490}$). Initial spectra were acquired in 20 mM HEPES, pH 7. White bars represent the addition of an excess of the appropriate metal ion (1 mM for Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, and Zn$^{2+}$, 50 µM for all other cations) to a 2 µM solution of RCS1. Black bars represent the subsequent addition of 2 mM Cu$^+$ to the solution. Excitation was provided at 480 nm and the emission was integrated over 490-525 nm and 525-650 nm.
Figure 3-7. Effect of concentration on the fluorescence ratio from apo RCS1. 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 3-8.** Effect of pH on the fluorescence ratio from a 2 µM solution of RCS1. Bars represent the ratio generated from the integrated emission from 530 – 650 nm ($F_{550}$) over the integrated emission from 490 – 530 nm ($F_{490}$). Data were collected in 20 mM HEPES at the indicated pH.
Figure 3-9. 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 mm pinhole, 2.51 ms 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 ($I_{564-639}/I_{522-554}$) 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 3-10. (a) Confocal fluorescence ratio images of live C6 rat glioma cells grown in basal media and stained with 2 μM RCS1 for 10 min at 37 °C. (b) C6 cells treated with 1 mM ascorbate for 4 h and stained with 2 μM RCS1 for 10 min at 37 °C. (c) C6 cells from condition (b) treated with 1 mM TEMEA for 5 min by direct addition to the Petri dish on the microscope stage. (d) C6 cells incubated with 200 μM BCS for 4 h and stained with 2 μM RCS1 for 10 min at 37 °C. (e) C6 cells treated with 200 μM BCS and 1 mM ascorbate for 4 h, then stained with 2 μM RCS1 for 10 min at 37 °C. (f) Bar graph representing the integrated intensity from 564-639 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 3-11. (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 mm pinhole, 2.51 ms 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_{564-639} / I_{522-554}) 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 3-12. 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 mm pinhole, 2.51 ms 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 ($I_{564-639}/I_{522-554}$) 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 3-13. 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. 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 3-14. Time course study of fluorescence intensity and fluorescence ratio changes in the presence of changing dye concentrations. HEK 293T cells were incubated in DMEM with 2 µM RCS1 and I scans were acquired at different time points during the dye uptake process. (a) I scans showing the increase in intracellular fluorescence during the intracellular accumulation of RCS1 from a 2 µM RCS1 solution 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 I 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 ($F_{522-554}$). The ratio remains constant despite changes in intracellular RCS1 levels.
Figure 3-15. 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 3-16. 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 mm pinhole, 3.20 ms 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 mm pinhole, 3.20 ms pixel time) with emission collected from 672 to 715 nm; (c) RCS1 (543 nm laser excitation, 75.3% laser power, 730 detector gain, 376 mm pinhole, 3.20 ms 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 3-1. Stability constants (logK) of metal chelators for Cu(I) and Cu(II) complexes

<table>
<thead>
<tr>
<th>Chelator/Name</th>
<th>logK$_{\text{Cu(I)L}}$</th>
<th>logK$_{\text{Cu(II)L}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bathocuproine Disulfonate (BCS)$^a$</td>
<td>~20</td>
<td>7.5</td>
</tr>
<tr>
<td>Tris((ethylthio)ethyl)amine (TEMEA)$^b$</td>
<td>15.53</td>
<td>6.35</td>
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<tr>
<td>Ratio-Coppersensor-I (RCS1)$^c$</td>
<td>10.4</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

References


Chapter 4:

Structural and Spectroscopic Characterization of a Borondipyrromethene Fluorophore and its Complexes with Cu(I) and Hg(II).

Portions of this work were performed in collaboration with the following persons: X-ray crystal structures were solved by Dr. Han Sen Soo.
Abstract

We report the synthesis, spectroscopic, structural and solution phase characterization of a new Cu²⁺-responsive BODIPY fluorophore. This compound undergoes a 10-fold fluorescence increase and significant conformation rearrangement in the presence of Cu⁺. Variable-temperature NMR experiments and full-lineshape analysis suggest that RCS2 proceeds through more ordered transition state under conditions of self-exchange, and that at room temperature, the rate of self-exchange is on the order of $10^5$ sec⁻¹.
Introduction

The development of metal-responsive fluorophores has provided chemical biologists and environmental chemists with powerful analytical tools to monitor the presence and distribution of a wide variety of metal ions in biological and environmental samples. The spectroscopic and structural characterization of Zn$^{2+}$ and Ca$^{2+}$ fluorophores has rich literature precedence, which in turn, has prompted the rational design of compounds with more ideal properties. The design criteria for fluorescent metal sensors for other ions, however, has remained underdeveloped, partly because fluorescent sensor technology has only recently been extended to more exotic metals like Ag$^+$, Cd$^{2+}$, and Pb$^{2+}$ and redox-active metals like Cu$^{2+/+}$ and Fe$^{2+/3+}$.

We have previously reported the properties and live-cell imaging capabilities of Ratio-Coppersensor-1 (RCS1) - a BODIPY based fluorophore capable of detecting changes in intracellular Cu levels, including the ascorbate-mediated expansion of endogenous Cu$^+$-pools. We sought to investigate the solid-state and solution phase properties of this class of fluorophores, with the ultimate aim of improving the cellular imaging characteristics of these compounds.

Results and Discussion

Design and Synthesis of a Ratio-Coppersensor-2. Scheme 4-1 shows the abbreviated synthesis of RCS2 – a compound that is identical to RCS1 except for a macrocyclic NS$_4$ receptor instead of the open-chain analog in the RCS1 architecture. BODIPY 1 is available in five steps from commercially available materials and is the penultimate intermediate common to RCS1 and the work reported herein. Treatment of BODIPY 1 with the macrocyclic tetrathiaza receptor in acetonitrile and gentle heating provides RCS2 in 30% yield as a dark red oil after workup and chromatography. Allowing a concentrated solution of RCS2 in CH$_2$Cl$_2$:Et$_2$O solution to stand at room temperature yields crystalline RCS2 as ruby red blocks.

Spectroscopic Properties and Responses to Copper. RCS2 exhibits a characteristic BODIPY absorption spectrum in 20 mM HEPES buffer at pH 7 with a maximal absorption centered at 555 nm ($\varepsilon = 41,500$ M$^{-1}$ cm$^{-1}$) and a shoulder at 530 nm ($\varepsilon = 30,000$ M$^{-1}$ cm$^{-1}$) (Figure 4-1). With excitation at 488 nm, RCS2 exhibits dual-emission characteristics (Figure 4-2): a weak, broad emission centered at 514 nm, and a lower-energy transition positioned at 580 nm. Titration of a 2 µM RCS2 solution with Cu$^+$ triggers a 17 nm blue-shift in the lower-energy emission peak to 563 nm and causes a linear increase in fluorescence intensity that plateaus upon introduction of a stoichiometric amount of Cu$^+$. The quantum efficiency of the Cu$^+$-responsive emission increases from $\Phi = 0.002$ in its apo state to $\Phi = 0.012$ in its Cu$^+$-saturated form. Investigation of the binding stoichiometry with the method of continuous variations (Job’s plot) suggests a 1:1 binding stoichiometry between RCS2 and Cu$^+$. Competitive titration experiments in the presence of thiourea reveal that RCS2 forms a tight complex with Cu$^+$ with an apparent $K_d$ of $1.6(7) \times 10^{-11}$ M in HEPES buffer at pH 7 (Figure 4-3). The higher-energy transition at 512 nm remains unchanged in the presence or absence of Cu$^+$; notably, the spectral characteristics of apo RCS2 and the RCS2:Cu$^+$ complex strongly resemble the apo and Cu$^+$-bound forms of RCS1.

X-ray Crystal Structures of Apo and Holo RCS2. Diffusion of pentane into an ether solution of RCS2 provides X-ray quality crystals of the apo fluorophore (Figure 4-5). Examination of its molecular structure reveals that, by virtue of its methyl substituents, the mesityl group in the 8-position is almost perfectly orthogonal to the planar BODIPY core (85.6º). The nitrogen from the NS$_4$ receptor is oriented with its p-orbital positioned to donate its electrons.
into the π-system of the aromatic system of the fluorophore and the thioether donor atoms poised to coordinate a soft, thiophilic metal.

When RCS2 binds Cu⁺, minimal reorganization is observed in the mesityl and BODIPY portions of the molecule, as disclosed by its X-ray crystal structure (Figure 4-6). The four thioether donor atoms of the receptor bind the Cu²⁺-ion in a distorted tetrahedral fashion and clasp the metal in a cage under the fluorine atoms of the molecule. The nitrogen atom of the receptor is pushed slightly out of conjugation (24.2°) with the fluorophore π-system, but in this molecular snapshot, is not formally involved in coordination to the metal (3.42 Å).

**Variable-temperature NMR Studies of RCS2 and its Cu⁺ Complex.** Because the nitrogen is the only atom in the ligand that is electronically coupled to the BODIPY core, it is difficult to rationalize a modulation of the fluorescence characteristics in the absence of a Cu(I)-N interaction. We reasoned that since the receptor portion of the molecule has five donor atoms, and d¹⁰ Cu⁺ prefers 3- or 4-coordinate geometries, RCS2 may exhibit fluxional behavior in solution.

To interrogate the dynamic, solution phase behavior of RCS2, we examined the ¹H spectra of RCS2 in the absence and presence of Cu(I). In the absence of Cu(I), the methylene protons adjacent to the receptor nitrogen atom exhibit a clean triplet centered at ca. 4.1 ppm. Upon introduction of Cu⁺, this signal migrates 0.1 ppm upfield and overlaps with the singlet from the methoxy group. Furthermore, the aromatic protons on the BODIPY core undergo significant chemical shift perturbations and broadening, relative to the spectrum of apo RCS2. If only the sulfur atoms were involved, as suggested by the X-ray structure, this binding event would not induce spectral shifts of the BODIPY aromatic protons since no alterations to the electronically coupled nitrogen would be possible.

To verify that the nitrogen was not involved in coordination at lower temperatures, a variable-temperature NMR study was undertaken. At 323K, the receptor amine α-protons and the methoxy singlet essentially coalesce into a single peak. Upon cooling, the two signals resolve, and a clean separation of the peaks is observed at 243K. Notably, the receptor nitrogen α-protons of the Cu⁺-complex return to the chemical shift of the apo probe, suggesting that at low temperature, the Cu⁺ is complexed by only the sulfur atoms.

Additional VT-NMR data were acquired for a full lineshape analysis to extract the kinetic parameters of dynamic self-exchange. Construction of Eyring and Arrhenius plots provides activation parameters for this self-exchange process (Figure 4-6). Under these experimental conditions, RCS2 exhibits rapid self-exchange with a low barrier (ΔG‡ = 44.5 ± 1.1 kJ•mol⁻¹ and Eₐ = 34.9 kJ•mol⁻¹) and a negative degree of entropy (ΔS‡ = -42.6 ± 3.1 J•K⁻¹). These values agree very well with the activation parameters measured for a model phenyl-NS₄ system in CD₃CN. The entropy of activation of RCS2 is ca. 20 J•K⁻¹ more negative than the reported value for its phenyl NS₄ congener, which could suggest a more ordered transition state in the exchange mechanism; however, because this parameter is calculated by an extensive extrapolation of the measured data, small errors during the fitting process are amplified and such a precise conclusion is premature. Nevertheless, this study indicates that the BODIPY fluorophore does not substantially affect the rate or barrier to self-exchange compared to a simple phenyl ring and that the dynamics and activation parameters of the NS₄-macrocycle appear to be largely independent of the aryl substituent to which it is attached.

**Spectroscopic Characterization of RCS2 and its Hg(II) Complex.** Because the NS₄-type macrocycle has been used as receptor on several reported Hg²⁺-responsive fluorophores, including two examples from our laboratory,²⁰, ²¹ we briefly investigated the spectroscopic
characteristics of the RCS2 in complex with Hg^{2+}. The excitation and emission spectrums of the DF1:Hg^{2+} complex substantially differs from the RCS2:Cu^{+} complex (Figures 4-7, 4-8). Whereas the response of DF1 to Cu^{+} resulted in no alteration of the 514 nm emission but causes a significant hypsochromic shift and intensity enhancement of the 580 nm emission, Hg^{2+} prompts a fluorescence increase of the 514 nm emission, but does not substantially alter the 580 nm emission (Figure 4-9). Unlike the case of Cu^{+}, where only a stoichiometric amount of Cu^{+} is required to saturate an equimolar solution of RCS2, 250 equivalents of Hg^{2+} (500 µM) is necessary to saturate a 2 µM solution of DF1. A Hill plot provides a coefficient of 1.66, suggesting some degree of cooperativity in binding multiple metal ions. Consistent with these observations, RCS2 shows a relatively high \( K_d \) of 256 µM for the DF1:Hg^{2+} complex. Although crystals of the RCS2:Hg^{2+} complex were isolated from a solution of N,N-dimethylacetamide with ether, the crystallographic data suggested that organomercuration of the BODIPY fluorophore had occurred. Since the effect of Hg^{2+} on RCS2 fluorescence is reversible with EDTA, the minimal solid-state information is not representative of the solution state of RCS2 with Hg^{2+}. It is premature to conclude the precise mode of coordination or stoichiometry beyond what has been mentioned; however, it is clear that RCS2 is involved in complex association(s) with Hg^{2+}, and the resulting complex has fluorescence characteristics distinct from RCS2 in complex with Cu^{+}. This unusual and unexpected behavior warrants further study.

**Concluding Remarks**

To close, we have presented the synthesis, spectroscopic properties, X-ray crystal structures, solution phase dynamics and kinetic parameters of a Cu(I)-responsive fluorophore. These studies represent the first detailed structural analysis of a Cu(I)-responsive fluorophore in its unbound and bound states, and provide information regarding the conformational changes that occur upon coordination of the compound to Cu(I). In combination with VT-NMR studies, a coherent picture of the dynamics of this class of compounds is presented.

**Experimental Section**

**Synthetic Materials and Methods.** All reactions were carried out under an atmosphere of dry nitrogen and stirred magnetically. Compound 1 was synthesized according to our previously published procedure. The NS4 receptor was synthesized according to our previously published procedure. 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 °C on a Bruker AV-300 or AVQ-400 spectrometer. VT-NMR data was acquired on a Bruker DRX-500 spectrometer equipped with a 5 mm Z-gradient broad band probe. All spectra were acquired at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard \( \delta \) 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.

**Ratio-Coppersensor-2, RCS2, (I).** A 25-mL Schlenk tube was charged with 6 (36 mg, 0.1 mmol) and NS4 (72 mg, 0.67 mmol). CH\(_3\)CN (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) followed by 1:1 CHCl₃/hexanes furnished RCS2 as a dark red film (22 mg, 35%). ¹H NMR (CDCl₃, 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.

Crystallization of RCS2 in complex with Cu⁺. A diethyl ether solution of RCS2 (10 mg, 0.016 mmol) was treated with Cu(MeCN)₄PF₆ (12 mg, 0.032 mmol) and a precipitate immediately formed. Acetonitrile was added dropwise until the precipitate was solubilized. Diffusion of pentane into this solution provided crystalline red blocks that were suitable for single-molecule X-ray analysis.

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 spectrophotometer (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 [NH₄]₂[Fe][SO₄]₂•6H₂O. Fluorescence quantum yields were determined by reference to rhodamine 101 inner salt in methanol (Φ = 1.0). The binding affinity of Cu⁺ to RCS2 was measured using thiourea as a competitive ligand to provide a buffered Cu⁺ solution. Briefly, a 2 µM solution of RCS2 was made in 20 mM HEPES, pH 7.0 and buffered with a known concentration of thiourea from a 500 mM stock in MilliQ H₂O. Cu(I) was delivered in the form of [Cu(MeCN)₄][PF₆] 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 RCS2 for Cu⁺.

Stability constants for thiourea binding were taken from the literature: β₁₂ = 2.0 × 10¹², β₁₃ = 2.0 × 10¹⁴, β₁₄ = 3.4 × 10¹⁵. Excitation was provided at 480 nm and the emission was integrated over 490-525 and 525-650 nm. The apparent dissociation constant (Kᵣ) was determined using the following equation: (F - Fₘᵢₙ)/(Fₘₐₓ - Fₘᵢₙ) = [Cu⁺]/(Kᵣ + [Cu⁺]), where F is the observed fluorescence; Fₘₐₓ is the fluorescence for the Cu⁺:RCS2 complex; Fₘᵢₙ is the fluorescence for RCS2; and [Cu⁺] is the ‘free’ Cu⁺ available for complexation, which was calculated using the stability constants for thiourea and standard competition equilibrium expressions.

General Methods for X-ray Crystallography. Crystals were mounted on Kaptan or monofilament loops in Paratone-N hydrocarbon oil. All data collection was performed on a Bruker (formerly Siemens) SMART diffractometer/CCD area detector equipped with a low temperature apparatus. Data integration was performed using SAINT. Preliminary data analysis and absorption correction were performed with the Bruker APEX2 software package. Structure solution by direct methods was performed using SIR2004, and the resulting solution was refined using SHELX. Hydrogen atoms were included in calculated positions.

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Variable Temperature NMR Studies: RCS2 (19 mg, 0.03 mmol) as dissolved in 0.5 mL acetone-d$_6$ with Cu(MeCN)$_2$PF$_6$ (5 mg, 0.013 mmol). Spectra were acquired over the range of 248 – 323 K in 5 K increments. The sample was allowed to equilibrate for 5 minutes at each temperature to ensure that thermal equilibrium was reached. After the spectra were collected over the indicated temperature range, a room temperature spectrum was acquired to verify that no significant changes or decomposition had occurred during the course of the experiment. NMR data were converted from WinNMR with the gCVT utility program (included in the GNMR software package) to a gNMR V5.0 spectrum. The concentration was fixed and chemical shifts were allowed to vary during the iterative fitting of calculated to experimental spectra. A pseudo-first-order rate was included as a variable and a full-lineshape analysis was performed.
**Figures and Schemes**

![Chemical Structure](image)

**Scheme 4-1.** Synthesis of Ratio-Coppersensor-2 (RCS2).
Figure 4-1. Absorption spectra of RCS2 in HEPES buffer, pH 7 with increasing concentrations of Cu\(^{+}\). Spectra shown are a 4 \(\mu\)M solution of RCS2 with 0 (thick dashed line), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 \(\mu\)M Cu\(^{+}\) (thick solid line). Two isosbestic points are evident at 531 nm and 548 nm.
**Figure 4-2.** Fluorescence emission spectra of a 4 µM solution of RCS2 in HEPES buffer, pH 7 with increasing concentrations of Cu$.^{+}$ Spectra shown are for a 4 µM solution of RCS2 with 0 (dashed line), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µM Cu$.^{+}$ (thick solid line). The maximal emission of the apo state is 585 nm and upon introduction of Cu$.^{+}$, the peak emission shifts to 560 nm.
Figure 4-3. $K_d$ measurement a 2 $\mu$M solution of RCS2 with Cu$^+$ in 20 mM HEPES buffered solution, pH 7. Shown are values for 1.86, 3.81, 5.25, 12.1, 21.8, 50.3, and 211 pM. The apparent $K_d$ value is $1.6(7) \times 10^{-11}$ M.
Figure 4-4. Thermal ellipsoid plot of apo RCS2. Red, dark yellow, light yellow, blue, pink and gray ellipsoids represent oxygen, sulfur, fluorine, nitrogen, boron and carbon atoms, respectively. Hydrogens have been omitted for clarity.
Figure 4-5. Thermal ellipsoid plot of RCS2 in complex with Cu⁺. Red, dark yellow, light yellow, blue, pink, orange and gray ellipsoids represent oxygen, sulfur, fluorine, nitrogen, boron, copper, and carbon atoms, respectively. Hydrogen atoms, a non-coordinating acetonitrile molecule and a PF₆⁻ counterion have been omitted for clarity.
Figure 4-6. Select experimental (top traces) and GNMR simulated (bottom traces) $^1$H NMR spectra of RCS2 with 0.45 molar equivalents of Cu$^+$ in acetone-d$_6$ at (A) 253 K (B) 273 K (C) 283 K (D) 293 K (E) 303 K and (F) 313 K. (G) Eyring plot from the $k_{obs}$ values calculated from the VT-NMR data. The following activation parameters were determined from this analysis: $\Delta H^\ddagger = 32.6 \text{ kJ} \cdot \text{mol}^{-1}$, $\Delta S^\ddagger = -42.6 \pm 3.1 \text{ J} \cdot \text{K}^{-1}$ and $\Delta G^\ddagger = 44.5 \pm 1.1 \text{ kJ} \cdot \text{mol}^{-1}$. 
Figure 4-7. Excitation scan of a 2 μM RCS2 solution in HEPES buffer, pH 7 with increasing concentrations of Hg$^{2+}$. Spectra shown are for a 2 μM solution of RCS2 with 0 (dashed line), 25, 50, 100, 200, 300, 400, 500, 600 and 700 μM Hg$^{2+}$. The emission intensity was measured at 525 nm and the excitation scan covered 400 – 515 nm.
Figure 4-8. Fluorescence emission profile of a 2 µM RCS2 solution in HEPES buffer, pH 7 with increasing concentrations of Hg$^{2+}$. Spectra shown are for a 2 µM solution of RCS2 with 0 (dashed line), 25, 50, 100, 200, 300, 400, 500, 600, and 700 µM Hg$^{2+}$. The emission intensity at 580 nm is not significantly altered, however, the emission at 514 nm increases until 250 equiv. of Hg$^{2+}$ has been added.
**Figure 4-9.** Overlay of the fluorescence emission profiles of RCS2 to Cu$^+$ and Hg$^{2+}$. 
**Table 4-1.** Experimental details for the X-ray structures of RCS2 and its Cu⁺ complex

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References

9. Huang, S. et al. Highly sensitive and selective fluorescent chemosensor for Ag+ based on a coumarin-Se2N chelating conjugate. Chemical Communications ASAP,


Appendix A:

A Regioselective C-H Oxidation Provides Access to Elaborated BODIPY Fluorophores
Abstract
A variety of BODIPY scaffolds undergo regioselective C-H oxidation with Selectfluor to generate a carbocation, which is immediately trapped by a molecule of acetonitrile to form a nitrilium ion. This intermediate can rapidly react with oxygen, phosphorus, sulfur, nitrogen, and carbon-based nucleophiles to form the corresponding conjugates in high yield.
**Introduction**

The chemical modification and elaboration of parent BODIPY fluorophores represents an expanding field in synthetic methodology. Oftentimes, pyrroles bearing the desired functionality are carried through the condensation, oxidation and BF$_2$-insertion steps of typical BODIPY formations. These reaction conditions can limit the synthetic complexity of the final BODIPY, and require that the desired functionality to be introduced at the nascent stages of the synthesis. Reactions that rely on late-stage elaborations of core BODIPY units are exceptionally useful, and ideally, can provide access to a functionally diverse library from a select few BODIPY precursors.

**Results and Discussion**

In the course of other studies, we observed that peralkyl BODIPY 1 underwent rapid reaction with 1 equivalent of the electrophilic fluorinating reagent, Selectfluor. TLC analysis indicated that the starting material was completely consumed (< 2 minutes) and the formation of a new, immobile product that remained on the TLC baseline, regardless of elution solvent. Upon quenching the reaction mixture with a methanol bolus, $^1$H NMR confirmed the formation of methoxy BODIPY 2. This conversion represents an intriguing case of a regioselective C-H functionalization. A literature search indicates that Selectfluor has been used in a similar manner for the C-H functionalization of hexamethylbenzene; however, we have improved upon this procedure by demonstrating its regioselectivity when applied to BODIPY compounds, and have expanded its substrate scope to include the formation of carbon-carbon bonds.

**Optimization and substrate scope.** A brief optimization study indicated that treatment of a cold acetonitrile solution (-10 °C) with Selectfluor (5 minutes), followed by addition of a nucleophile consistently provided good yields of coupled products. Oxygen-based nucleophiles required a large excess to successfully couple (entry 2) whereas even sterically congested amines reacted smoothly with a slight excess of nucleophile. Phosphine (PPh$_3$) and thiol-based nucleophiles (methyl thioglycolate) also react cleanly to give the expected adduct in good yield. Notably, carbon-carbon bonds can also be formed. Of special note is that a ketone functionality can be directly introduced by reacting TMS-silyl enol ethers with the nitrilum ion in high yield (entries 5 and 6). To the best of our knowledge, this represents a novel approach to sp$^3$ carbon-carbon bond formation methodology.

**Mechanism.** Though Selectfluor is most often used as a fluorinating agent, it can also serve as a potent oxidant (0.44 V vs. NHE). We reasoned that Selectfluor was functioning as a 2 e$^-$ oxidant to generate a pseudo-benzylic carbocation intermediate; in acetonitrile solvent, this carbocation is immediately captured as a nitrilum ion. Treatment of the nitrilium ion with a nucleophile displaces the nitrile and allows a facile coupling to the carbon atom. (Scheme 1). This mechanism is supported, in part, by an isolated report in which the authors observed that a peralkyl BODIPY compound treated with DDQ in THF:H$_2$O underwent successive oxidations to the corresponding aldehyde compound. The scope of this transformation was limited to this single product. Further evidence is provided by the nucleophile studies. If, for instance, a bare carbocation were invoked as the reactive intermediate, no difference in rate or reactivity would be expected for alcoholic or amine nucleophiles. This is not the case. As noted above, alcohol nucleophiles require a large excess to successfully couple, whereas only a slight excess of sulfur, phosphorus and amine-based are required to form the products. This is more consistent with an S$_N$2-type
mechanism, where a leaving group is displaced, than an S_N1-type mechanism.

Having established the scope of the methodology, we next sought to apply it toward the synthesis of a BODIPYs for use in cellular labeling studies. To this end, we used Selectfluor to generate the nitrilium ion intermediate of BODIPY 1 and subsequently treated it with PPh_3 to make its corresponding triphenylphosphonium salt. Because of its lipophilic cation characteristics, we expected that it would accumulate almost exclusively in the mitochondria of actively respiring cells. An initial control experiment with BODIPY 1 showed significant accumulation in lipid droplets and no significant accumulation in mitochondria stained with MitoTracker Deep Red (Figure A-1). However, HeLa cells treated with 5 µM of the triphenylphosphonium BODIPY 4 show excellent, specific mitochondrial staining as confirmed by co-localization studies with the commercially available mitochondrial stain, MitoTracker Deep Red (Figure A-2).

CHO cells express sialic acid residues on their cell surface. It has been shown that a brief treatment of CHO cells with periodate will oxidatively cleave the vicinal diol to its corresponding aldehyde and effectively produce a cell that is decorated with aldehydes on its cell surface. Incubation of these chemically treated cells with hydrazine or alkoxyamine derivitized fluorophores, in the presence of millimolar concentrations of aniline, will effect a cell-surface bioconjugation reaction between the hydrazine/alkoxy amine fluorophore and cell-surface aldehydes. We hypothesized that if we could alter the solubility characteristics of the BODIPY through installation of a water-solubilizing group, and also include a hydrazine functionality for bioconjugation purposes, we could covalently label the membranes of periodate-treated cells.

BODIPY 1 was first treated with Selectfluor in acetonitrile, and the resultant nitrilium ion was quenched with a solution of tetrabutylammonium taurine. This elaboration served two purposes: 1) to install a water-solubilizing group (sulfonate) on the lipophilic BODIPY scaffold and 2) provide a synthetic handle for further elaboration. Reaction of the amino-BODIPY with succinic anhydride provided the acid-derivatized compound; the acid was then activated with HOBt/EDC and the corresponding ester was quenched with hydrazine to form the acyl hydrazide compound in an unoptimized yield of 15% over three steps.

We incubated periodate-treated CHO cells with a 300 µM solution of the functionalized BODIPY, and, taking cue from the Dawson laboratory, performed this coupling in the presence of 5 mM aniline to accelerate the reaction. After 90 minutes, the cells were fixed in 4% paraformaldehyde, washed three times with a 10% FBS solution, mounted and imaged by confocal microscopy. We were pleased to see that the cells were limned with bright fluorescence, indicating that the fluorescent compound had covalently linked to the aldehyde functionality expressed on the cell membrane. Control imaging experiments with BODIPY 1 or with compounds lacking the sulfonate group gave no observable fluorescent enhancement on the cell membrane.

Concluding Remarks

To close, we have presented the discovery of a regioselective C-H functionalization that proceeds in high yield, has a broad substrate scope and is capable of C-C bond formation. We have demonstrated the utility of this reaction by converting the same readily available BODIPY into two structurally distinct compounds that exhibit different subcellular compartmentalization. We expect that this methodology will find broad application in the functionalization of BODIPY fluorophores.
Experimental Section

Synthetic Materials and Methods. All reactions were carried out under an atmosphere of dry nitrogen and stirred magnetically. Compound 1 was synthesized according to our previously published procedure. The NS₄ receptor was synthesized according to our previously published procedure. 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. ¹H NMR spectra were collected in CDCl₃ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker AV-300 or AVQ-400 spectrometer. All spectra were acquired 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.

General method for BODIPY functionalization. A solution of 1 (31 mg g, 0.1 mmol) in dry MeCN (1 mL) was cooled to –10 ºC in an acetone/ice bath. Selectfluor (38 mg, 0.11 mmol) was suspended in 2 mL MeCN and sonicated until clear. The Selectfluor solution was added dropwise to the BODIPY solution and stirred for 2 minutes at –10 ºC, after which a solution of the nucleophile in MeCN (1 mL) was added. The reaction was allowed to proceed for 5 minutes, at which point the reaction was diluted with water (10 mL), extracted with CH₂Cl₂ (3 x 10 mL) and the organics combined and concentrated. Final compounds were isolated by column chromatography.

2,8-diethyl-5,5-difluoro-3-(methoxymethyl)-1,7,9,10-tetramethyl-5H-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide: A solution of 1 (31 mg, 0.1 mmol) in dry MeCN (1 mL) was cooled to –10 ºC in an acetone/ice bath. Selectfluor (38 mg, 0.11 mmol) was suspended in 2 mL MeCN and sonicated until clear. The Selectfluor solution was added dropwise to the BODIPY solution and stirred for 2 minutes at –10 ºC, after which MeOH (1 mL) was added. The reaction was allowed to proceed for 5 minutes, at which point the reaction was diluted with water (10 mL), extracted with CH₂Cl₂ (3 x 10 mL) and the organics combined and concentrated. Column chromatography (silica gel, 1:1 hexanes:CH₂Cl₂) provided an orange solid (25 mg, 0.072 mmol) in 72% yield. ¹H NMR (CDCl₃, 300 MHz): δ 4.73 (2H, s), 3.47 (3H, s), 2.63 (s, 3H), 2.53 (5H, m), 2.40 (2H, q, J = 5.7), 2.35 (6H, s), 1.03-1.115 (6H, m).

2,8-diethyl-5,5-difluoro-3-(((2-methoxy-2-oxoethyl)thio)methyl)-1,7,9,10-tetramethyl-5H-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide: A solution of 1 (10 mg g, 0.03 mmol) in dry MeCN (1 mL) was cooled to –10 ºC in an acetone/ice bath. Selectfluor (38 mg, 0.11 mmol) was suspended in 2 mL MeCN and sonicated until clear. The Selectfluor solution was added dropwise to the BODIPY solution and stirred for 25 minutes at –10 ºC, after which methyl thioglycolate (10 mg, 0.09 mmol) in MeCN (200 µL) was added. The reaction was allowed to proceed for 25 min, at which point the reaction was diluted with water (10 mL), extracted with CH₂Cl₂ (3 x 10 mL) and the organics combined and concentrated. Preparatory TLC (CH₂Cl₂) afforded a red-orange oil. ¹H NMR (CDCl₃, 400 MHz):
δ 4.14 (2H, s), 3.76 (3H, s), 3.30 (2H, s), 2.61 (3H, s), 2.53 (m, 5H), 2.33 (s, 3H), 1.05 (6H, m).

LC-MS Found: 403.1, expected [M−F] = 403.1.

2,8-diethyl-5,5-difluoro-1,7,9,10-tetramethyl-3-(3-oxobutyl)-5H-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide. A solution of 1 (16 mg, 0.05 mmol) in dry MeCN (3 mL) was cooled to −10 ºC in an acetone/ice bath. Selectfluor (19.5 mg, 0.055 mmol) was suspended in 3 mL MeCN and sonicated until clear. The Selectfluor solution was added dropwise to the BODIPY solution and stirred for 2 minutes at −10 ºC, after which trimethyl(prop-1-en-2-yl)oxy)silane (49 µL, 0.25 mmol, 85% purity) was added. The reaction was allowed to proceed for 40 min at −10 ºC, at which point the reaction was diluted with water (10 mL), extracted with CH2Cl2 (3 × 10 mL) and the organics combined, dried over Na2SO4 and concentrated. Flash column chromatography (silica gel, CH2Cl2–hexanes to 4:1 CH2Cl2:hexanes) provided a bright orange solid (15.8 mg, 0.042 mmol) in 84% yield.

1H NMR δ 3.11 (2H, t, J = 7.2), 2.80 (2H, t, J = 7.2), 2.60 (3H, s), 2.48 (3H, s), 2.39 (4H, m), 2.33 (6H, s), 2.17 (3H, s), 2.00 (3H, s), 1.03 (6H, m).

2,8-diethyl-5,5-difluoro-1,7,9,10-tetramethyl-3-(3-oxo-3-phenylpropyl)-5H-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide. A solution of 1 (16 mg, 0.05 mmol) in dry MeCN (3 mL) was cooled to −10 ºC in an acetone/ice bath. Selectfluor (19.5 mg, 0.055 mmol) was suspended in 3 mL MeCN and sonicated until clear. The Selectfluor solution was added dropwise to the BODIPY solution and stirred for 2 minutes at −10 ºC, after which trimethyl(1-phenylvinyl)oxy)silane (48 µg, 0.25 mmol) was added. The reaction was allowed to proceed for 30 min at −10 ºC, at which point the reaction was diluted with water (10 mL), extracted with CH2Cl2 (3 × 10 mL) and the organics were combined, dried over Na2SO4 and concentrated. Flash column chromatography (silica gel, 2:1 CH2Cl2:hexanes to 4:1 CH2Cl2:hexanes) gave a red waxy solid (18.0 mg, 0.042 mmol) in 83% yield. 1H NMR δ 8.00 (2H, d, J = 7.2), 7.72 (1H, m), 7.55 (2H, m), 7.45 (2H, m), 4.86 (2H, s), 4.37 – 3.33 (2H, m), 2.52 (3H, s), 2.49 (6H, s), 2.45 (4H, m), 1.09 (6H, m).

3-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)oxy)methyl)-2,8-diethyl-5,5-difluoro-1,7,9,10-tetramethyl-5H-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide: A solution of 1 (50 mg, 0.16 mmol) in dry MeCN (1 mL) was cooled to −10 ºC in an acetone/ice bath. Selectfluor (58 µg, 0.165 mmol) was suspended in 2 mL MeCN and sonicated until clear. The Selectfluor solution was added dropwise to the BODIPY solution and stirred for 2 min at −10 ºC, after which a solution of the FmocNHOH (410 µL, 1.6 mmol) in THF (1 mL) was added. The reaction was allowed to proceed for 5 minutes, at which point the reaction was diluted with water (10 mL), extracted with CH2Cl2 (3 × 10 mL) and the organics and concentrated. Column chromatography (silica gel, CH2Cl2 to 97:3 CH2Cl2:EtOAc) provided a red oil (27 µg, 0.043 mmol) in 30% yield. 1H NMR (CDCl3, 300 MHz): δ 7.76 (2H, d, J = 7.2), 7.68 (2H, d, J = 7.2), 7.39 (2H, t, J = 7.2), 7.32-7.27 (2H, m), 6.75 (1H, broad triplet), 5.07 (2H, s), 4.45 (2H, d, J = 7.6), 4.31 (1H, t, J = 7.6), 2.63 (3H, s), 2.52 (5H, m), 2.42 (2H, q, J = 7.6) 2.35 (6H, s), 1.06 (6H, m).
2,8-diethyl-3-((N-ethyl-4-hydrazinyl-4-oxobutanamido)methyl)-5,5-difluoro-1,7,9,10-tetramethyl-5H-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-iium-5-uide: A solution of 1 (10.6 mg, 0.03 mmol) in dry MeCN (1 mL) was cooled to – 10 ºC in an acetone/ice bath. Selectfluor (12.5 mg, 0.035 mmol) was suspended in 2 mL MeCN and sonicated until clear. The Selectfluor solution was added dropwise to the BODIPY solution and stirred for 2 minutes at -10 ºC, after which point a solution of ethylamine (2 mL, 2.0 M in THF) was added. The reaction was allowed to proceed for 5 minutes, at which point the reaction was diluted with water (10 mL), extracted with CH₂Cl₂ (3 × 10 mL) and the organics combined and concentrated to a red solid. The crude material was dissolved in THF (1 mL) and added to a Schlenk tube that had been charged with succinic anhydride (6 mg, 0.06 mmol). NEt₃ (10 µL) was added and the reaction was stirred for 8 h at room temperature. The reaction mixture was quenched with H₂O (5 mL) and extracted with CH₂Cl₂ (3 × 5 mL). The layers were separated, and the organics were combined and concentrated to a red solid. Preparatory TLC (5% MeOH, 95% CH₂Cl₂) provided 12.2 mg (80% over 2 steps) of a red solid. The entirety of the solid (12.2 mg, 0.027 mmol) was dissolved in MeCN (500 µL) and HOBt (5.5 mg, 0.04 mmol) and EDC (6.3 mg, 0.04 mg) were added. The reaction was stirred at room temperature and after 12 h. In a separate flask, N₂H₄ (1 mL, 1.0 M in THF) was prepared and the solution containing the activated ester was added dropwise. The reaction was stirred for 5 min at room temperature and the mixture was concentrated. Preparatory TLC (93:7 CH₂Cl₂:MeOH) provided an orange solid (5 mg, 0.01 mmol) in 39% yield. LC/MS = 476.4 [M+H] and 456.4 [M-F].

2,8-diethyl-5,5-difluoro-3-((4-hydrazinyl-4-oxo-N-(2-sulfonatoethyl)butanamido)methyl)-1,7,9,10-tetramethyl-5H-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-iium-5-uide (BODIPY Membrane): A solution of 1 (15 mg, 0.05 mmol) in dry MeCN (1 mL) was cooled to – 10 ºC in an acetone/ice bath. Selectfluor (20 mg, 0.055 mmol) was suspended in 2 mL MeCN and sonicated until clear. The Selectfluor solution was added dropwise to the BODIPY solution and stirred for 2 minutes at –10 ºC, after which the solution was added to a solution of tetrabutylammonium taurine (367 mg, 1.0 mol) in MeCN (1 mL). The reaction mixture immediately turned a dark red color. After stirring for 5 min at room temperature, the mixture was partitioned between CH₂Cl₂ (20 mL) and H₂O (10 mL). The layers were separated, and the organics were dried over Na₂SO₄ and concentrated to a red oil. The crude residue was dissolved in THF (1 mL) with NEt₃ (10 µL, 0.08 mmol), and succinic anhydride (7.5 mg, 0.08 mmol). After stirring for 24 h at room temperature, the reaction was concentrated to provide a red oil. This crude material (13 mg, 0.024 mmol) was dissolved in THF (1 mL) and stirred with HOBT (5 mg, 0.036 mmol), and EDC (7.0 mg, 0.036 mmol) for 24 h at room temperature. The solution of activated ester was added N₂H₄ (1 mL, 1.0 M in THF) and the reaction immediately became cloudy. The residue was concentrated to a dark red oil and was purified by preparatory TLC (85:15 CH₂Cl₂:MeOH) to yield a dark red residue (4.0 mg, 0.007 mmol) in 14% yield over three steps. LC-MS: Expected: 554; Found: 554 [M-H].
Preparation and Staining of Cell Cultures. Chinese hamster ovary (CHO) cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before imaging, cells were passaged and plated on 18-mm coverslips in DMEM without Phenol Red. To oxidize membrane surface sialic acid residues, the coverslip was treated with a 1 mM NaIO₄ solution in PBS (without CaCl₂ or MgCl₂) for 30 min at 4 ºC. The periodate solution was aspirated and the cells were washed with PBS (3 × 1 mL) and incubated with 300 µM of compound in 5% FBS/PBS and 5 mM aniline for 90 min. The cells were washed with PBS and fixed with PFA (15 min, 4 ºC) and washed in 0.1% Triton for 3 h. The coverslips were dried, mounted and imaged by confocal microscopy.
Figures and Schemes

Scheme A-1. Conversion of peralkyl BODIPY 1 to its methoxy substituted BODIPY.
**Table A-2.** Synthetic scope of the reported methodology.

![Chemical structure](image)

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<td>OTMS_Ph</td>
<td><img src="image" alt="Product 6" /></td>
<td>83%</td>
</tr>
</tbody>
</table>
Scheme A-3. Synthesis of BODIPY 7, a compound capable of labeling the membrane of periodate-treated CHO cells.
Figure A-1. Molecular imaging with peralkyl BODIPY 1. Incubation of HeLa cells with 5 µM BODIPY 1 for 10 min at 37 °C shows significant accumulation in lipid droplets (left) and no significant colocalization with MitoTracker Deep Red (right).
Figure A-2. Molecular imaging with triphenylphosphonium BODIPY 4. Incubation of HeLa cells with 5 µM BODIPY 4 for 10 min at 37 °C shows significant accumulation in the mitochondria (left panels) as indicated by the colocalization with MitoTracker Deep Red (right panels).
Figure A-3. Molecular imaging of periodate-treated CHO cells that have been incubated with the acyl hydrazide derivatized BODIPY 7. This compound covalently links to the aldehydes that decorate the cell surface of periodate-treated CHO cells.
Appendix B:

Synthesis and Characterization of a Mitochondrial-Targeted Cu\(^{+}\)-Responsive Fluorophore
**Experimental Section**

**Synthetic Materials and Methods.** All reactions were carried out under an atmosphere of dry nitrogen 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). 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. All spectra were acquired at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard $\delta$ 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.

**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$_4$]$_2$·6H$_2$O. Fluorescence quantum yields were determined by reference to rhodamine 101 inner salt in methanol ($\Phi = 1.0$). The binding affinity of Cu$^+$ to RhodCS1 was measured using thiourea as a competitive ligand to provide a buffered Cu$^+$ solution. Briefly, a 2 µM solution of RhodCS1 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$_2$O. Cu(I) was delivered in the form of [Cu(MeCN)$_4$][PF$_6$] from an acetonitrile stock solution (2 mM). Stability constants for thiourea binding were taken from the literature: $\beta_{12} = 2.0 \times 10^{12}$, $\beta_{13} = 2.0 \times 10^{14}$, $\beta_{14} = 3.4 \times 10^{15}$. 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: $(F - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}}) = [\text{Cu}^+] / (K_d + [\text{Cu}^+])$, where $F$ is the observed fluorescence; $F_{\text{max}}$ is the fluorescence for the Cu$^+$:RCS1 complex; $F_{\text{min}}$ is the fluorescence for RhodCS1; and $[\text{Cu}^+]$ is the ‘free’ Cu$^+$ available for complexation, which was calculated using the stability constants for thiourea and standard competition equilibrium expressions.

**RhodCS1:** A solution of 3-diethylamino phenol (33 mg, 0.2 mmol) and 2 (42 mg, 0.1 mmol)) in glacial acetic acid (1.5 mL) was heated to 65 ºC for 36 h. After the solution had cooled to room temperature, it was poured into 3 M NaOAc (10 mL) and extracted with CHCl$_3$ (3 × 20 mL). The organic phases were combined, washed with H$_2$O (2 × 20 mL), dried over Na$_2$SO$_4$, and evaporated to a yield a purple residue. The crude material was dissolved in CH$_2$Cl$_2$:MeOH (1:1, 10 mL) and p-chloranil (25 mg, 0.1 mmol) was added. The solution was sonicated for 15 min, after which the solution was concentrated to a dark purple oil. Column chromatography (silica gel, CHCl$_3$:MeOH, 9:1) gave 11 mg (31%) as a purple residue.
**Figure B-1.** Fluorescence responses of 5 µM RhodCS1. Spectra shown are for buffered [Cu⁺] of 0, 0.5, 1, 2, 3, 4, and 5 µM. Spectra were acquired in 20 mM HEPES, pH 7, with excitation at 540 nm.
**Figure B-2.** Fluorescence response of RhodCS1 to various metal ions. Bars represent the final integrated fluorescence response ($F_f$) over the initial integrated emission ($F_i$). Spectra were acquired in 20 mM HEPES, pH 7. White bars represent the addition of an excess of the appropriate metal ion (2 mM for Na$^+$, Mg$^{2+}$, K$^+$, Ca$^{2+}$ and Zn$^{2+}$; 50 µM for all other cations) to a 5 µM solution of RhodCS1. Black bars represent the subsequent of 10 µM Cu$^{2+}$ to the solution. Excitation was provided at 540 nm, and the collected emission was integrated from 550-700 nm.
Figure B-3. Normalized fluorescence response of 5 µM RhodCS1 to thiourea buffered Cu\(^+\) solutions for \(K_d\) value determination. Excitation was provided at 540 nm and the peak intensity was measured at 575 nm. Spectra were acquired in 20 mM HEPES, pH 7. The points shown are for free Cu\(^+\) buffered at 0, 0.635, 1.04, 1.93, 4.57, 19.5, 83.4, 369, and 10300 pM, respectively. The observed \(K_d\) value is 1.0(8) x 10\(^{-11}\) M.
Figure B-4. Normalized fluorescence response of 5 µM RhodCS1 to Cu⁺. Excitation was provided at 540 nm and emission intensity was measured at 577 nm. Spectra were acquired in 20 mM HEPES, pH 7. The break at 5 µM added Cu⁺ (1 equiv) is consistent with formation of a 1:1 Cu⁺:RhodCS1 complex.
Figure B-5. Job’s plot of RhodCS1 and Cu⁺. The total concentration of RhodCS1 and Cu⁺ were kept at a constant 6 µM. Excitation was provided at 540 nm and emission intensity was measured at 575 nm. Spectra were acquired in 20 mM HEPES, pH 7. The maximum fluorescence response at 0.5 mole fraction of RhodCS1 indicates formation of a 1:1 RhodCS1:Cu⁺ complex.
Figure B-6. Co-localization studies in HEK 293T cells stained with (A) 5 µM RhodCS1 and (B) 500 nM MitoTracker Deep Red.
Figure B-7. Confocal microscopy images of HEK 293T cells (A) grown in basal media and stained with 5 μM RhodCS1; (B) grown in media supplemented with 100 μM CuCl2 and stained with 5 μM RhodCS1; and (C) enlarged view of cells in panel (B).
Appendix C:

Synthesis and Characterization of a Water-Soluble Cu⁺-Chelator
Experimental Section

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). Pyridine 1 was synthesized according to literature procedures. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. 1H NMR spectra were collected in CDCl3 (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 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.

Sodium(6-((bis(2-(ethylthio)ethyl)amino)methyl)pyridin-2-yl)methanesulfonate (PyrNS2). Compound 1 (291 mg, 0.87 mmol) was dissolved in 20 mL 1:1 EtOH:H2O and Na2SO3 (328 mg, 2.61 mmol) was added. The reaction was heated at 40 ºC for 12 h, after which it was cooled to room temperature and concentrated by rotary evaporation. Column chromatography (silica, 85:15 CHCl3:MeOH) gave a white solid (100 mg, 0.25 mmol) in 25% yield. 1H NMR (CDCl3, 300 MHz): δ 7.33 (1H, s), 7.25 (1H, s), 4.29 (2H, s), 3.78 (2H, s), 2.72 (4H, s), 2.43 (2H, s), 2.41 (2H, s), 1.15 (6H, t, J = 6.9 Hz). LC-MS calculated for [M-H]=377.1, found 377.1.

Spectroscopic Materials and Methods. The binding affinities of Cu+ to PyrNS2 was measured using bathocuproine disulfonate (BCS) as a competitive ligand. A stability constants of logK = 20 was used for BCS. Cu(I) was delivered in the form of [Cu(MeCN)4][PF6] from an acetonitrile stock solution (2 mM). Measurements were carried out in 100 mM HEPES, pH 7.4. The apparent dissociation constants (Kd) were determined using the following equation: (A – Amin)/(Amax – Amin) = [Cu⁺]/(Kd + [Cu⁺]), where A is the observed absorption.
Scheme C-1. Synthesis of PyrNS2.
Figure C-1. Determination of the Kd of the PyrNS2-Zn$^{2+}$ complex. Aliquots of ZnCl$_2$ from a 1 M stock solution in MilliQ water were added to a solution of 50 µM of PyrNS2 in 100 mM NaCl, 20 mM HEPES at pH 7.4 and the intensity of the charge-transfer band at 260 nm was monitored. The apparent Kd of PyrNS2 with Zn$^{2+}$ is $3.5(8) \times 10^{-3}$ M.
Figure C-2. Determination of the $K_d$ of the PyrNS2-Cu$^+$ complex. Aliquots of BCS from a 50 µM stock solution in MilliQ water were added to a solution of 15 mM Cu$^+$ with 0.25 µM, 1 µM, or 2 µM PyrNS2 in 100 mM NaCl, 20 mM HEPES at pH 7.4 and the intensity of the charge-transfer band at 530 nm was monitored. The apparent $K_d$ of PyrNS2 with Cu$^+$ is $2.9(8) \times 10^{-19}$ M.
References

Appendix D:

Protocols
Simulating NMR data with the GNMR software suite.

1) Acquire a series of VT-NMR spectra.
2) Process these in the NMR facility with the WinNMR program.
3) Download all files to a computer with the GNMR program installed.
4) Use the gCVT.exe program to convert the WinNMR files to a GNMR spectrum. You will need to select “File of type: All files” to see the files.
   a. Select the source.
      i. Filter: WinNMR
      ii. AcqPar file: acqu
      iii. ProcPar file: proc
      iv. Spectrum file: 1r
      v. Imag spectrum: 1i
      vi. FID file: fid
   b. Select the target.
      i. Filter: gNMR V5.0 Spectrum
      ii. Click “Browse”. Type a filename to save it as.
      iii. Click “Go!” and the WinNMR file will be converted to a gNMR file (*.spg format).
5) Convert and save all of your VT NMR spectra this way. You will ultimately end up simulating each one in gNMR.
6) Open gNMR. Using two temperature dependent resonances (e.g. resonances that show line-broadening with respect to temperature), we will simulate a spectrum by typing in approximate chemical shifts.
   a. Type one chemical shift into the table provided.
   b. Open a new molecule (“Molecule”  “New”) and type in another chemical shift for the other temperature dependent shift.
   c. Click on the “Spectrum” button. A simulated spectrum will appear with resonances where you specified.
   d. Go to “Settings”, then “Spectrum Settings” and click on the “Iteration” tab.
   e. Choose “File” and select a converted experimental NMR file.
   f. Make sure “Full lineshape iteration” is checked. Click “Apply”.
7) You should now have an experimental data file on top of your simulated spectrum (in two separate windows).
8) Click on “Settings”  “Molecule” and enter a concentration for the particular resonance. Do the same for the other molecule. For instance, if you have a dynamic self-exchange at a total concentration of 60 mM and there is 30 mM of metal, there is 30 mM of metal complex and 30 mM free ligand.
9) Select “Molecule”  “Exchange”. We want to look at the interchange of species 1-1 with species 2-2. In the GNMR nomenclature, this is defined as 1-1  2-1; 2-1  1-1. Enter a rate. Change it from “Fixed” to “Variable”.
10) Go to the “Interate”  “Assignments”  “Window 1 (1H)” and choose which simulated resonances are associated with the experimental resonances.
11) Select “Iterate”  “Go”. A least-squares fitting will be performed, where the rate (variable) is altered while the concentration (and coupling, if applicable) is fixed.
Alternatively, one can select “Interactive rate” under the “Exchange” window and vary the rate manually.

12) A poor fit will be obvious. Either you’ll experience a barrage of error messages or the predicted spectrum will be very different from the experimental. Try altering the starting rate, or applying/lifting various constraints until a convergence can be met.

13) As a final note, I recommend working through the several tutorials in GNMR tutorial pdf. It will put the above instructions in context.
So You’re Going on a Beamrun…

This Appendix was written to help future generations of students collect high-quality X-ray fluorescence microscopy (XRFM) data at Argonne National Laboratories. It is compiled from knowledge I have actively obtained or by doing things incorrectly and learning the hard way. It is by no means exhaustive; collecting XRF data is a fickle process. Fortunately, the staff scientists are kind, generous with their time, and always accessible, even at 3 a.m. when the Linux system shuts down and the in-house computer programs need rebooting.

Booking a Flight

*General:* All flights are reserved through the UC Berkeley travel agent, Carlson Wagonlit (pronounced “Vagon-lee”).

1) Find your preferred flight (Southwest has direct flights to Chicago Midway from Oakland) and note the time and day of departure, as well as the flight number.

2) Call Carlson Wagonlit at 1.800.728.4918. Mention that you would like to book a flight from Oakland to Chicago Midway and provide the information requested by the travel agent. You will be asked for a date of birth to minimize the chances you are stopped by Homeland Security for additional screening.

3) The travel agent will email you a copy of a “CTS Form” as well as fare information and a booking number. Forward these to your Administrative Assistant who will fax the forms back to Carlson Wagonlit. This must be done within 24-hours or else the process has to be started over.

4) Once Carlson Wagonlit has the forms, the itinerary will be finalized.

Renting a Car

*General:* UC Berkeley has a contract with Enterprise Rent-A-Car with very favorable terms. In short, reservations made with the UC Berkeley contract code enjoy a 10% discount off of list price, in addition to free CDW and Supplemental liability coverage (so you and your insurance premiums are not on the hook if something should happen to the rental car).

1) Go to [www.enterprise.com](http://www.enterprise.com) and select the day and time for car pickup and return. In the box labeled **Optional:** Coupon, Customer, or Corporate Number type in: XZ23A01 (This is your system ID number). Hit “search” and on the next page use “UCB” as the 3-digit code. Continue to book the car. The contractual rates and conditions will automatically be applied.

Reserving Rooms at the Argonne Guest House (AGH)

*General:* Argonne National Laboratory has a dedicated dormitory for on-site users. This is the ideal place to stay (for convenience sake, that is) as it is within walking distance of the beamline.

1) Go to [http://www.anlgh.org/](http://www.anlgh.org/) and click on “Request Reservations”.
2) Fill out the form and submit.

3) You will receive an email stating that the rooms have been temporarily reserved but that you need to secure them by providing a credit card number.

4) Call them at 1.800.632.8990 and provide your credit card number to secure the reservation.

Sample Preparation for Correlative Microscopy Between Confocal and X-Ray Fluorescence Microscopy Techniques

*General:* Typically, the silicon nitride windows (Silson, UK) take at least one month to arrive from time that they are ordered. The windows do not degrade over time, so be sure to have plenty on hand if beamtime should unexpectedly be awarded. It is necessary to get a quote from the manufacturer and process that through the UC Berkeley ordering department. In general, cell culture conditions that are suitable for growing cells on PLL-coated coverslips translate very well to the silicon nitride windows. All cells should be plated on the perfectly smooth side, not the side with the depression.

1) Go to [www.silson.com](http://www.silson.com)

2) Use the “product finder” to see if the windows you want are available. Typical dimensions for our samples are 5.00 mm frame width, 2 x 2 mm membrane size, 200 nm membrane thickness and 200 µm frame thickness. Sometimes this isn’t listed, but just include these dimensions when you email the company for a quote.

3) Email the company for a quote, print it out and attach it to the order form.

4) Wait 1 month. The windows will come packaged in gel capsules. It is a delicate procedure to remove a window from the capsule. It helps to squeeze the capsule while pulling the window (by the frame!) with forceps.

5) Cells can be grown on the silicon nitride windows with little to no variation from typical cell culture conditions. Plate the cells as you would on PLL-coated glass coverslips and prepare for confocal microscopy.

*Samples can be prepared anytime prior to the beamrun as long as they are stored in a dark, dry place*

Confocal Microscopy with Silicon Nitride Windows

*General:* It is probably obvious that the 200 nm thick brittle membrane is extremely fragile – manipulating the windows is a skill in and of itself. In general, try to avoid picking up the window on the same axis as the membrane; instead, try to pick up the window on the corner so that if the forceps should slip, they slide over the metal frame instead of the membrane (Figure D-1). If you touch the membrane with the forceps, the membrane will shatter. Aspirate and mix
media and buffers carefully when the windows are in the wells since agitation can also destroy the membrane.

1) Prepare a 35-mm Petri dish with your staining solution and transfer the silicon nitride window into this vessel. Incubate and prepare the sample as you typically would for imaging live cells on a glass coverslip.

2) Arrange the dish under the dipping objective so that it is as straight as possible.

3) Draw a square on a piece of paper (6 × 6 in); this represents the membrane as seen through the microscope objective. This will serve as your “map” – a guide to where specific cells are located on the window.

4) Find a corner of the window that has distinct cellular morphologies and record an image. This will serve as a back-up technique for correctly orienting the window if it is rotated during processing.

5) Get an idea of how large the window appears under the objective and calibrate yourself to the hand-drawn map. Choose fields of cells that are not too overgrown and record on the map where they are approximately located. A field of view through the 40 x objective is roughly the size of a quarter on a 6 x 6 in. square. Image as you would typically image a coverslip.

6) After 6-8 fields of cells have been recorded (with or without higher-magnification images), remove the dish from the microscope stage. Take extreme care to not rotate it from the orientation in which you imaged it.

7) Remove the buffer / media and fix the cells in 4% PFA for 10 minutes at room temperature.

8) Wash with 20 mM PIPES/200 mM sucrose solution, pH 7 (2 x).

9) Aspirate the PIPES/sucrose wash and carefully dry the top left corner of the window with a Kimwipe.

10) Mark a small dot on the top left corner with an ethanol-resistant permanent marker. This will serve as your guide to orient the window at ANL in the same orientation as you imaged it at Berkeley.

11) Make sure the window is dry; wick away excess moisture with a Kimwipe. If residual sucrose solution is present, the window can stick to various substrates.

12) Place the window on a microscope slide with only the bottom portion of the metal frame touching the glass. Tape the window to the glass. If residual sucrose solution is present between the silicon nitride window frame and the glass microscope slide, the window may break when you try to remove it from the slide.
13) Repeat the staining, mapping, imaging, fixing and mounting procedure for all remaining samples. After the last sample is imaged, tape the slide to the bottom of an old tip box (20 µL tip boxes are good). Store in a dark, dry place until your flight to Argonne.

**Transporting Samples to Argonne.**

*General.* This has never been a problem – just be sure to keep the samples in your carry-on, preferably at the top of your bag. The tip box will provide a decent amount of protection. Security has never stopped me for having the samples with me; however, I always have my ANL ID and phone numbers of ANL contacts (Lydia Finney & Stefan Vogt) with me just in case.

1) Pick up the rental car at the Enterprise counter. Drive to Argonne. Be sure to have your ANL IDs in the cabin of the car since, as Evan and I have found out, the armed guards get concerned when a car stops 50 yards from the gate and two young men get out to start rummage through the trunk.

2) Drive to the Argonne Guest House. If you decide to drink some complimentary Choco-milk from the beverage dispenser in the lobby, keep this in mind: It may be free, but you’ll pay for it later.

3) Get some sleep and get to the beam at 8 am the next morning. Chances are that the beam will have to be re-aligned, calibrated, and possibly the energy tuned back to 10 keV, so this is a perfect opportunity to map some cells.

**Mapping Cells.**

*General:* This can be the most frustrating point of the whole trip, but hopefully, since we’ve worked out the common problems, it should be easier for anyone reading this. The idea is that we are going to find the same cells that were imaged at Berkeley, get their coordinates, and image them by XRF. Remember those maps you made and images you took? You’re going to need them.

1) Remove a window from the slide. If the window wasn’t completely dry before it was mounted on the slide, the sucrose solution will make it very sticky and difficult to remove. If this happens, I try to exert pressure along the axis of the window frame with a set of flat-tipped forceps.

2) Get a “stick” – a flat metal piece with two half-moons cut into it – this will hold the window in place.

3) Spray the stick with adhesive.

4) Carefully mount the window on the stick making sure that the dot you put on the window at Berkeley is in the same orientation as when you recorded the map. The exact positioning on the stick may take some trial-and-error; Figure 2 shows a placement that should be suitable so that the detector can move to all points of the sample window.
5) Put the stick in a sample holder and tighten it with an allen key.

6) Fire up the epifluorescent scope in the sample prep room and, using a pair of long tweezers, mount the sample holder onto the magnets of the uh, sample holder holder.

7) First, find the coordinates for the corners; record these. Then, it is a matter of finding the same cells that you imaged at Berkeley. Use the maps as a rough guidance and the confocal images to pinpoint the best looking cells. This is basically a game of matching shapes. It is easiest if the cells are distinct (e.g. neurons), and very difficult if it’s a sample with overgrown circular cells (e.g. macrophages).

8) When you find the same cell, record its X, Y and Z coordinates. Go back to the beam line; you’re going to start doing XRF.

**Collecting XRF data.**

*General.* You’re almost to the point where you can collect data. You just have to let 2-ID-D know that you’re closing the shutter, then you’ll open the hatch, be sure to place the sample in correctly – don’t break it! – retract the OSA and zone plate; put in the CCD camera; find the corners; solve a system of equations to calibrate the coordinates between the epifluorescent scope and the XRF; put the OSA and zone plate in; find the cells with low-resolution scans, appropriately crop them to the correct size; queue them in the batch scans and you’re ready to go! This is standard practice at beamline 2-ID-E and the staff scientists can walk you through it.

Repeat the mapping of cells and data collection for the duration of the beam run. Get on a plane and go home. Turn in your receipts from food and lodging for reimbursement.

The data will be fitted to the standards (collected at some point during the beamrun – be sure to ask about them) and you’ll be able to download the data in about a month.
Figure D-1. Proper method for picking up a silicon nitride window. Use quality forceps to pick up the window, ideally in a position so that if the forceps slip, they slide along the frame (purple) instead of onto the membrane (clear center square).
Figure D-2. Approximate placement of a silicon nitride window on a metal sample stick. There should be several millimeters of overhang to the right and the metal should not occlude the corners of the membrane.