A selective fluorescent sensor for imaging Cu^{2+} in living cells

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Copper ion is a biochemically essential yet toxic metal ion, connected to serious neurodegenerative diseases, and also has been identified as an environmental pollutant. For the effective detection of Cu^{2+} in biological and environmental systems, we have developed a new membrane-permeable Cu^{2+}-selective water-soluble BODIPY **1**, which was synthesized by nucleophilic disubstitution of novel 3,5-diiodo-BODIPY **4** with *N*,*N*-bis(2-hydroxyethyl)amines. BODIPY **1** shows a highly sensitive and selective fluorescence response to Cu^{2+} in aqueous solution. Fluorescence image experiments establish that **1** can be used to monitor intracellular Cu^{2+} within living cells.

Introduction

Copper ion is a biochemically essential yet toxic metal ion,[1] required as a cofactor for many fundamental biological processes, and catalyzes the production of highly reactive oxygen species,[2] connected to serious neurodegenerative diseases,[3] for example Alzheimer's disease.[3a] On the other hand, it has also been identified as an environmental pollutant,[1b,4] and can affect certain microorganisms at submicromolar concentrations. Effective detection of Cu^{2+} can facilitate the study of its physiological role *in vivo*, and monitor its presence in metal-contaminated sources, and has attracted much attention in environmental and biological analysis areas.[1d] The design and synthesis of chemosensors for highly sensitive and selective monitoring of Cu^{2+} are highly demanded.

Fluorescent chemosensors have advantages, such as sensitivity, specificity, simplicity and instantaneous response.[5] To be used for cellular imaging of ions, they also require long-wavelength excitation and emission, reducing scattering and securing low background emission. Fluorescent sensors that can permeate the plasma membrane have proven to be powerful and non-destructive tools for the study of intracellular metal ion distributions of calcium(II),[6] magnesium(II),[7] zinc(II),[8] cadmium(II),[9] mercury(II),[10] or copper(I),[11] yet suitable fluorescent sensors for sensitive *in vivo* measurements of intracellular copper(II) levels are lacking. Recently, a large number of fluorescent probes for Cu^{2+} has been reported based on different fluorophores,[1b,12] such as anthracene,[12a] coumarin,[12b,c] benzaldehyde hydrazone,[12d,e] naphthalimide,[12f] porphyrin,[12g] spiropyran,[12h] rhodamine[1b] and boron dipyrromethene (BODIPY).[12i] However, most of them have poor water-solubility and are often characterized in non-aqueous systems; this together with the requirement for short-wavelength excitation, and cross-sensitivities toward other metal cations, hinders their biological applications.

BODIPY dyes, which are widely applied as fluorescent sensors and labeling reagents,[13] have remarkable properties,[5c,14] such as high fluorescence quantum yields, high photophysical stability, and large absorption coefficients. Several positions are available for the functionalization of BODIPY chromophores. Most commonly, the derivation is carried out at the pyrrolic positions,[14,15] but functional groups such as ligands or biomolecules are often introduced *via* the 8-aryl group.[13a] Since the meso-aryl group and the chromophore are almost perpendicular to each other, electronic conjugation between the two moieties is weak. On the other hand, derivation at the pyrrolic position requires the preparation of phenyl or alkyl-substituted pyrrole building blocks, which is not always a straightforward synthesis. Moreover, it fails to introduce electron donating groups without aryl spacers at the 3,5-position due to the difficult synthesis of the corresponding pyrroles. Alternatively, the direct modification of BODIPY chromophores *via* nucleophilic mono- or di-substitution at 3,5-dichloro-BODIPY **2**, generated from the NCS chlorination of dipyrromethane **3** as shown in Scheme 1,[16] has been proved to be efficient. However, NCS chlorination is only suitable for dipyrromethanes without pyrrolic substituents. Thus only limited BODIPYs can be generated, and it is necessary to

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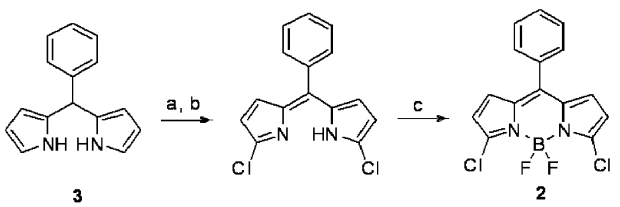


3 2

Scheme 1 Synthetic route for 3,5-dichloro-BODIPY **2**.[16] *Reaction conditions*: (a) NCS; (b) *p*-chloranil; (c) Et_3N, $BF_3{\cdot}Et_2O$.

develop a new methodology for the generation of 3,5-dihalogenated BODIPYs.

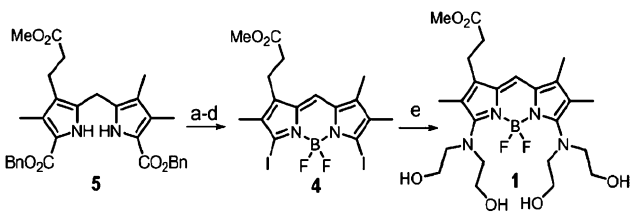
Herein, we report a new 3,5-diiodo-BODIPY **4**, as an alternative for BODIPY **2** mentioned above, which was generated by a new synthetic methodology as shown in Scheme 2. Its further functionalization with *N*,*N*-bis(2-hydroxyethyl)amines generates a new water-soluble BODIPY **1**, which has an increased Stokes shift and a 52 nm red-shifted emission band due to the introduction of electron-donating groups. BODIPY **1** shows a highly sensitive and selective fluorescence response to Cu^{2+} in aqueous solution (HEPES–DMSO = 9 : 1) (20 mM HEPES, buffer pH 7), and can be used to monitor intracellular Cu^{2+} within living cells.

Results and discussions

BODIPY **4** was synthesized in a one-pot, four-step procedure from **5**,[17] *via* catalytic hydrogenation, decarboxylative iodination, DDQ oxidation, and complexation with $BF_3{\cdot}Et_2O$. The catalytic hydrogenation used Pd/C (10%) as catalyst and was performed in dry THF at room temperature until dipyrromethane **5** was consumed. After filtration of the catalyst and evaporation of the solvent, the residue was dissolved in 2 M aqueous NH_3, and the solution was acidified with 2 M AcOH. The precipitate was collected through filtration and dissolved in methanol–water for the decarboxylative iodination using I_2 in MeOH at room temperature, and subsequent oxidation using DDQ in DCM at 0 °C. To the reaction mixture were added triethylamine and $BF_3{\cdot}OEt_2$. After work-up and purification using column chromatography (silica gel, DCM–hexane = 2 : 1, v/v), 3,5-diiodo-BODIPY **4** was obtained in 33% overall yield for four steps from **5**, and was characterized by NMR, elemental analysis and HRMS. HRMS gave a $[M - F]^+$ peak at *m*/*z* 552.9450.

BODIPY **4** has two iodines at 3,5-positions, and is a valuable synthetic precursor for the introduction of other functionalities. For example, it was used here for the nucleophilic substitution with *N*,*N*-bis(2-hydroxyethyl) amine (Scheme 2). This reaction proceeds smoothly in acetonitrile at 60 °C to generate BODIPY **1** in 39% yield. BODIPY **1** was characterized by NMR, elemental analysis and HRMS. HRMS typically gave a $[M - F]^+$ peak at *m*/*z* 507.3830.

Both the UV-Vis and fluorescence spectra of BODIPY **1** showed significant red-shifts of bands compared with those of BODIPY **4**, as shown in Fig. 1. In DMSO, BODIPY **4** showed



Scheme 2 Syntheses of BODIPY **4** and **1**. *Reaction conditions*: (a) Pd/C, H_2, THF; (b) $NaHCO_3$, I_2, MeOH–H_2O; (c) DDQ, CH_2Cl_2, 0 °C; (d) Et_3N, $BF_3{\cdot}Et_2O$; (e) *N*,*N*-bis(2-hydroxyethyl)amine, MeCN, 60 °C.

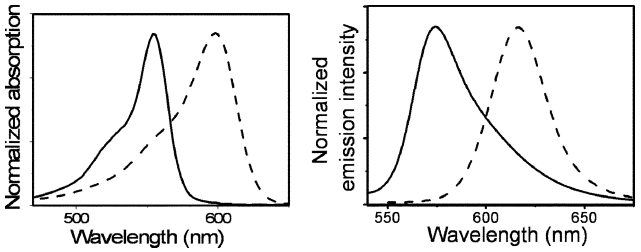


Fig. 1 Normalized absorption (left) and fluorescence emission (right) spectra of BODIPY **4** (solid line) and **1** (dashed line) in DMSO. Excitations were at 550 and 590 nm for BODIPYs **4** and **1**, respectively. The maximum emissions were observed at 565 and 617 nm for BODIPYs **4** and **1**, respectively.

an intense absorption and fluorescence emission at 554 nm ($\varepsilon = 1.1 \times 10^5\ M^{-1}\ cm^{-1}$) and 565 nm, respectively, while BODIPY **1** showed the corresponding bands at 598 nm ($\varepsilon = 2.7 \times 10^4\ M^{-1}\ cm^{-1}$) and 617 nm, respectively. Thus, we observed a 44 and a 52 nm red-shift of the longest wavelength absorption and the emission band for BODIPY **1**, respectively, along with increased Stokes shift (from 11 to 19 nm) due to the introduction of electron-donating *N*,*N*-bis(2-hydroxyethyl)amines.

Binding properties of BODIPY **1** toward selected metal ions were evaluated under simulated physiological conditions (HEPES–DMSO = 9 : 1, v/v), (20 mM HEPES, buffer pH 7). A DMSO stock solution of BODIPY **1** (20 μM) was prepared and used to prepare the sample solution (2 μM, HEPES–DMSO = 9 : 1, v/v) (20 mM HEPES, buffer pH 7) for the binding and titration experiments. The fluorescence reading was noted 60 s after adding each metal ion. There was no or only slight changes of fluorescence emission profiles of BODIPY **1** with the addition of even large excess amounts (200 equiv., 400 μM) of various biologically relevant metal ions, such as Na^+, K^+, Mn^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+},

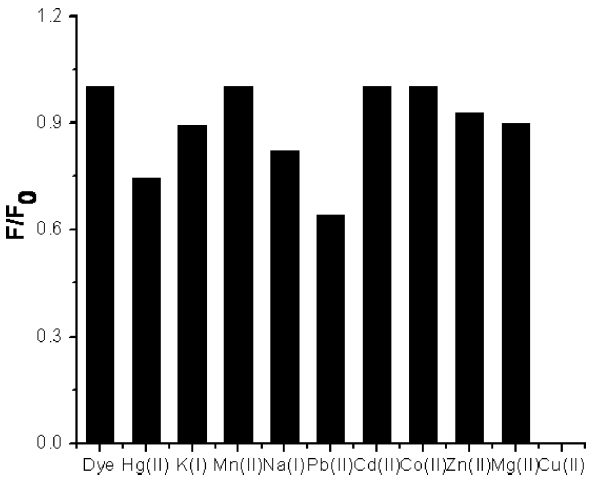


Fig. 2 Normalized integrated emission intensities (integrated emission intensity of the free dye = 1) of 2 μM BODIPY **1** in the presence of 200 equiv. of selected metal ions (400 μM) under excitation at 550 nm. Bars represent the final integrated fluorescence response (*F*) relative to the initial integrated emission (F_0). Initial spectra were acquired in aqueous solution (HEPES–DMSO = 9 : 1, v/v) (20 mM HEPES, buffer pH 7).

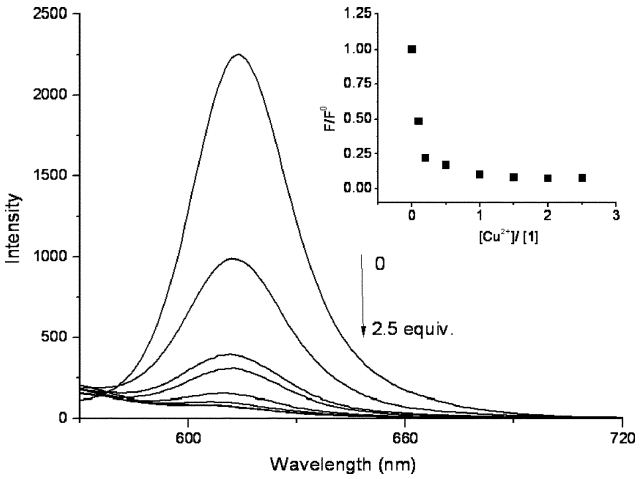


Fig. 3 Fluorescence response of 2 μM BODIPY **1** to Cu^{2+}. Spectra were acquired in aqueous solution (HEPES–DMSO = 9 : 1, v/v) (20 mM HEPES, buffer pH 7). Spectra shown were for buffered [Cu^{2+}] of 0, 0.2, 0.4, 1, 2, 3, 4, 5 μM. Inset: integrated fluorescence intensity ratio changes (F/F_0) of **1** upon gradual addition of Cu^{2+} ions. The excitation was 550 nm.

Mg^{2+} and Pb^{2+} as shown in Fig. 2. However, under identical conditions, fluorescence emission was totally shut down in the presence of 400 μM of Cu^{2+}.

We further studied the titration of BODIPY **1** with various amounts of Cu^{2+} in aqueous solution (HEPES–DMSO = 9 : 1, v/v), (20 mM HEPES, buffer pH 7) as shown in Fig. 3. Half of fluorescence emission of **1** was quenched upon addition of only 0.1 equiv. of Cu^{2+} (0.2 μM), and 90% of fluorescence emission was quenched with the addition of 1 equiv. of Cu^{2+} (2 μM).

Furthermore, competition experiments were performed between Cu^{2+} and selected metal ions as shown in Fig. 4. When 200 equiv. of selected metal ions (400 μM) was added into an aqueous solution of **1** (2 μM) containing

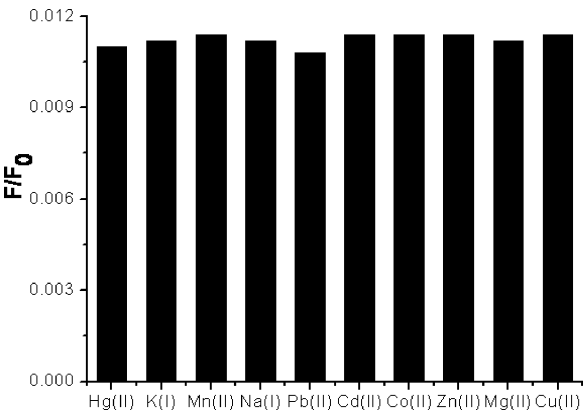


Fig. 4 The fluorescence response of BODIPY **1** containing Cu^{2+} to selected metal ions in aqueous solution (HEPES–DMSO = 9 : 1, v/v) (20 mM HEPES, buffer pH 7). Bars represented the final integrated fluorescence response (F) over the initial integrated emission of **1** (F_0). F = integrated fluorescence response after subsequent addition of 200 equiv. selected metal ions to an aqueous solution of **1** containing Cu^{2+}; F_0 = integrated emission of **1** when no metal ions are present; [**1**] = 2 μM, [Cu^{2+}] = 10 μM, [M^{n+}] = 400 μM; excitation at 550 nm.

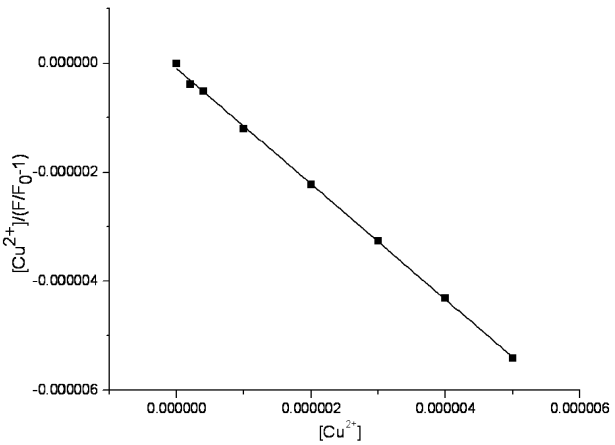


Fig. 5 Single reciprocal plot of compound **1** (2.0 μM) titrated with Cu^{2+} in aqueous solution (HEPES–DMSO = 9 : 1, v/v) (20 mM HEPES, buffer pH 7).

Cu^{2+} (10 μM), the emission spectra displayed a similar pattern to that with Cu^{2+} alone. Thus, BODIPY **1** showed excellent selectivities for Cu^{2+} over the other cellular abundant metal cations. The Scott plot of [Cu^{2+}]/(F/F_0 − 1) as a function of [Cu^{2+}] is shown in Fig. 5. The excellent linear relationship, according to literature,[18] indicates the formation of a 1 : 2 complex between BODIPY **1** and Cu^{2+}. The calculated association constant (K) is 1.03×10^7 L mol^{-1}. This large association constant indicates a strong interaction between BODIPY **1** and Cu^{2+}.

Further binding analysis was performed using the method of continuous variations (Job plot) as shown in Fig. 6. The total concentration of **1** and Cu^{2+} is fixed at 2 μM. A maximum and constant relative intensity occurs at [Cu^{2+}]/([Cu^{2+}] + [**1**]) = 0.66, which establishes the formation of a 1 : 2 complex of **1** and Cu^{2+} and is responsible for the observed fluorescence quenching. As expected, each Cu^{2+} ion binds to one *N*,*N*-bis(2-hydroxyethyl)amine unit.

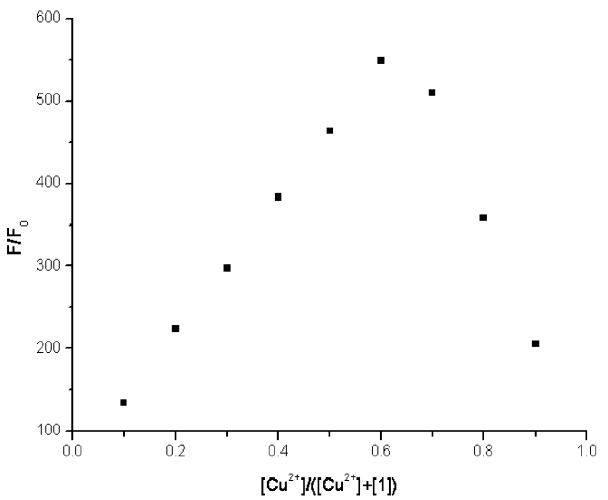


Fig. 6 Job plot of BODIPY **1** in DMSO–HEPES solution (HEPES–DMSO = 1 : 1, v/v) (20 mM HEPES, buffer pH 7). The total concentration **1** and Cu^{2+} ion was 2 μM; excitation at 550 nm. The Job plot indicated a 1 : 2 stoichiometry for **1**-Cu^{2+}.

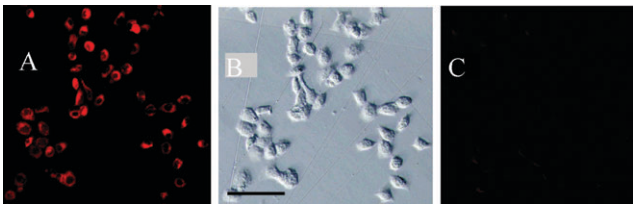


Fig. 7 Fluorescence images of live BEL 7402 cells. (A) Cells incubated with 10 μM BODIPY **1** for 3 h at 37 °C. (B) Bright-field image of live BEL 7402 cells incubated with **1** shown in panel A, confirming their viability. (C) Cells stained with 10 μM BODIPY **1** in the growth media for 3 h at 37 °C and subsequently supplemented with 100 μM $CuCl_2$ for 1 h at 37 °C. Scale bar = 100 μm.

To be used in biological systems, it is important that biosensing molecules can selectively monitor guest species in living cells.[9b,19] We next evaluated the ability of BODIPY **1** to operate within living cells. Because copper tends to accumulate in the liver and kidney cells, leading to its toxicity in animals, we primarily carried out an experiment using **1** on human liver cancer cell lines (BEL 7402). BEL 7402 cells were incubated with 10 μM **1** for 3 h at 37 °C, and washed with PBS to remove the remaining BODIPY **1**. The cells showed strong fluorescence as shown in Fig. 7A. Bright-field measurements after treating cells with BODIPY **1** confirmed that cells are viable throughout the imaging experiments as shown in Fig. 7B. Cells were stained with 10 μM BODIPY **1** in the growth medium for 3 h at 37 °C, then supplemented with 100 μM $CuCl_2$ and further incubated for 1 h at 37 °C. In this case, a marked quenching of intracellular fluorescence was observed, as shown in Fig. 7C. Thus BODIPY **1** is cell-permeable and can respond to intracellular changes of Cu^{2+} within living cells.

Conclusions

In summary, we have demonstrated a new synthetic route for 3,5-diiodo-BODIPY **4** through a one-pot four-step procedure, which provides an alternative approach to introducing multiple and distinct functional groups to the chromophore, offering many opportunities for developing BODIPY-based sensors for various ions. The nucleophilic disubstitution of BODIPY **4** with *N*,*N*-bis(2-hydroxyethyl)amines generates a new water-soluble BODIPY **1**. The introduction of electron-donating *N*,*N*-bis(2-hydroxyethyl)amines, leads to an appreciable red-shift in absorption and emission bands of the spectra. For the imaging of Cu^{2+} in aqueous solution, BODIPY **1** features excellent Cu^{2+} sensitivity and selectivity. The living cell imaging experiments further demonstrate its value for practical applications in biological systems.

Experimental

General

Reagents and solvents were purchased as reagent-grade and used without further purification unless otherwise stated. THF was freshly distilled from sodium benzophenone ketyl. All reactions were performed in oven-dried or flame-dried glassware under an inert atmosphere of argon unless otherwise stated, and were monitored by TLC using 0.25 mm silica gel plates with or without UV indicator (60F-254). Reactions involving light-sensitive compounds were carried out wrapped in foil. ^1H and ^{13}C NMR spectra were obtained on an AV-300 Bruker spectrometer at 298 K; chemical shifts (δ) are reported in δ [ppm] relative to $CDCl_3$ (7.26 ppm, ^1H); coupling constants J are given in [Hz] and the multiplicities are expressed as follows: s = singlet, d = doublet, t = triplet, m = multiplet. ESI-MS spectra were obtained on an Applied Biosystems QSTAR XL.

Syntheses

BODIPY **1** was synthesized *via* a nucleophilic substitution between 3,5-diiodo-BODIPY **4** and *N*,*N*-bis(hydroxyethyl)-amines. Dipyrromethane **5** was synthesized according to the literature.[17]

BODIPY **4** was synthesized from dipyrromethane **5** in a one-pot, four-step procedure *via* catalytic hydrogenation, decarboxylative iodination, DDQ oxidation, and complexation with $BF_3{\cdot}OEt_2$ as described below.

To a 100 mL round bottom flask was added Pd/C (644 mg, 0.6 mmol). After three freeze–thaw cycles, 15 mL freshly distilled THF was added, and hydrogen gas was applied through a balloon. It was left stirring for 20–30 min to activate the Pd/C catalyst before adding dipyrromethane **5** (1.6 g, 3.0 mmol) in THF (25 mL) through a syringe. The reaction system was stirred in the atmosphere of H_2 at room temperature in the dark for 6 h. After filtration of the catalyst and evaporation of the solvent, the residue was dissolved in 2 M aqueous NH_3 (300 mL), and acidified with AcOH to reach pH 5. The precipitate was collected by filtration to give 1,9-biscarboxylic acid dipyrromethane (1.0 g, 94% yield) as a greyish solid which was dissolved in H_2O (95 mL) and MeOH (30 mL) for the decarboxylative iodination. To this slurry was added $NaHCO_3$ (1.4 g, 16.0 mmol), sonicated to form clear solution, and iodine (1.2 g, 4.6 mmol) in MeOH (65 mL) was slowly added at room temperature. The resulting pink mixture became a brown slurry during the course of addition. After vigorous stirring at room temperature, the mixture was placed into an ice-bath and stirred for an additional 1.5 h at 0 °C. The product was filtered off and washed with H_2O, saturated aqueous $NaHCO_3$, H_2O and hexanes before drying in vacuum for 24 h to give the intermediate diiododipyrromethane (1.1 g, 80% yield) as a brown solid which was dissolved in DCM (110 mL) and cooled to 0 °C. To this solution was added DDQ (500 mg, 2.2 mmol) in one portion. The resulting solution was stirred at 0 °C for 10 min before adding triethylamine (2 mL) and $BF_3{\cdot}OEt_2$ (2 mL). After work-up and purification using column chromatography (silica gel, DCM–hexane = 2 : 1, v/v), BODIPY **4** was obtained as a reddish–brown powder (566 mg, 33% overall yield for four steps from dipyrromethane **5**). UV-Vis (in DMSO): λ = 554 nm, $\varepsilon = 1.1 \times 10^5\ M^{-1}cm^{-1}$. ^1H NMR (300 MHz, $CDCl_3$): δ 6.94 (s, 1H), 3.64 (s, 3H), 2.92–2.87 (m, 2H), 2.52–2.47 (m, 2H), 2.20 (s, 3H), 2.00 (s, 3H), 1.98 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.6, 139.4, 138.6, 137.4, 136.0, 133.5, 132.6, 119.0, 110.3, 107.3, 47.2, 34.6, 12.4, 12.3, 10.4, 8.8. Elemental analysis (%): Calcd for $C_{16}H_{17}BF_2I_2N_2O_2$: C 33.60,

H 3.00, N 4.90. Found: C 33.29, H 3.34, N 5.21. HRMS: Calcd for $C_{16}H_{17}BFI_2N_2O_2$ $[M - F]^+$: 552.9368, found 552.9450.

BODIPY **1** was synthesized *via* a nucleophilic substitution between BODIPY **4** and *N*,*N*-bis(2-hydroxyethyl)amine in acetonitrile at 60 °C under argon.[16c] To a dry Schlenk flask were added BODIPY **4** (112 mg, 0.2 mmol) and *N*,*N*-bis(2-hydroxyethyl) amine (87 mg, 0.8 mmol) and MeCN (20 mL). The reaction was performed at 60 °C under argon. After cooling the reaction mixture to room temperature, solvent was removed under vacuum. The crude product was purified using column chromatography (silica gel, EtOAc–Et_3N = 100 : 1, v/v), from which BODIPY **1** was obtained as a dark purple solid (30 mg, 39% yield). UV-Vis (in DMSO): λ = 598 nm, $\varepsilon = 2.7 \times 10^4$ $M^{-1}cm^{-1}$. ^1H NMR (250 MHz, $CDCl_3$): δ 6.56 (s, 1H), 5.75 (br, 2H), 4.04 (br, 2H), 3.83–3.69 (m, 8H), 3.57–3.54 (m, 2H), 3.39–3.37 (m, 2H), 3.16 (br, 2H), 2.83–2.78 (m, 2H), 2.57–2.46 (m, 7H), 2.07 (s, 3H), 1.93–1.92 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 181.3, 141.5, 139.4, 138.5, 137.4, 136.3, 135.9, 123.5, 111.9, 110.3, 60.4, 57.9, 55.8, 51.7, 47.4, 35.3, 19.9, 14.2, 10.4, 9.6. Elemental analysis (%): Calcd for $C_{24}H_{37}BFN_4O_6$: C 54.76, H 7.08, N 10.64. Found: C 54.38, H 6.91, N 10.92. HRMS: Calcd for $C_{24}H_{37}BFN_4O_6$, $[M - F]^+$: 507.3833, found 507.3830.

UV-visible and fluorescence study

UV-visible absorption spectra were recorded on a Hitachi U-3010 Spectrophotometer (190–1100 nm scan range). Fluorescence emission spectra were recorded on a Hitachi F-4600 FL Spectrophotometer. Stock solutions (10 mM) of metal salts were prepared in water or medium (for cell study), from which varying concentrations of sample metal salt solutions were prepared. A stock solution of **1** was prepared in DMSO (1 mM) and used to prepare 2 μM sample aqueous solutions (HEPES–DMSO = 9 : 1, v/v) (20 mM HEPES, buffer pH 7), and 10 μM medium solutions (medium–DMSO = 99 : 1, v/v) of **1**.

Excitation for fluorescence measurements was carried out at 550 nm for BODIPY **1**. The slit widths for both the excitation and emission were 2.5 nm. The increment of the spectra was 1 nm, and the integration time was 1 s. Fluorescence titration was studied in 2 μM aqueous solution (HEPES–DMSO = 9 : 1, v/v) (20 mM HEPES, buffer pH 7) of **1**. A continuous variation method was used for the titration experiments and the fluorescence was recorded 60 s after the addition.

Binding constant calculation

The emission intensity of fluorescence was integrated and was fitted using the following equation, as described by Connors.[18]

$$F/F_0 = \{1 + k_f/k_s)K[L]\}/(1 + K[L])$$

Here, F refers to the integrated fluorescence intensity of BODIPY **1** after adding Cu^{2+}, F_0 refers to the integrated fluorescence intensity of **1** before adding Cu^{2+}. k_f refers to the proportionality constant of the **1**-Cu^{2+} complex, k_s refers to the proportionality constant of **1**, and K refers to the binding constant. The dependence of F/F_0 on the concentration of Cu^{2+} were linearized, using the Scott plot analysis method as described in literature.[18] The titration were performed in triplicate, and there was less than 15% difference between the resulting binding constants. Among all ions investigated, **1** was highly selective to Cu^{2+} while insensitive to Na^+, K^+, Mn^{2+}, Mg^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+} and Pb^{2+}.

General procedure for Job plot

20 μM stock solution of BODIPY **1** was prepared in absolute DMSO and used to prepare 10 mL mixture aqueous solutions (HEPES–DMSO = 5 : 5, v/v) (20 mM HEPES, buffer pH 7) of **1** and Cu^{2+}. The total concentration of **1** and Cu^{2+} was fixed at 2 μM, while varying the ratio of $[Cu^{2+}]$: [**1**]. Varying amount of 20 μM stock solution of **1** and Cu^{2+} were added to the aqueous solution (HEPES–DMSO = 5 : 5, v/v) (20 mM HEPES, buffer pH 7) using a micropipette. The final volume of the mixture aqueous solution was adjusted to 10 mL using an aqueous solution (HEPES–DMSO = 5 : 5, v/v) (20 mM HEPES, buffer pH 7) and mixed using sonication. The fluorescence was recorded 60 s after the preparation of each solution.

Cell incubation

BEL 7402 cell line was used for fluorescence imaging. It was prepared from continuous culture in Dulbeccos modified Eagles medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum (HyClone), 100 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 0.25 μg mL^{-1} L-glutamine, at 37 °C in 5.0% CO_2 humidified air. When cells reached the logarithmic phase, the cell density was adjusted to 1.0×10^5 mL^{-1} in culture media. The cells were then used to inoculate in a six-compartment cell culture plate, with 2.0 mL of cell suspension in each dish. After cell adhesion, the culture medium was removed. The cell layer was rinsed twice with phosphate buffered saline (PBS), and subsequently 2.0 mL culture medium was added.

Fluorescence images with intracellular Cu^{2+}

An Olympus IX71 inverted fluorescence microscope was used for the fluorescence images. The excitation wavelength of the laser was 532 nm. BODIPY **1** (1 mM, 20 uL) was added to BEL 7402 cells in the six-compartment cell culture plate that contained 2.0 mL culture medium, and was incubated at 37 °C for 3 h. After removing the culture medium and washing with PBS twice, the fluorescence images of cells were taken, and confirmed the cell-membrane permeability of BODIPY **1**. Bright-field images confirmed the viability of the cells during the experiment. To one compartment of the culture plate was added 2 mL of culture medium and 10 equiv. of Cu^{2+} (10 mM, 20 uL), which was incubated at 37 °C for 1 h and the fluorescence image measured which confirmed the ability of BODIPY **1** to act as a sensor for Cu^{2+} within living cells. For all fluorescence images, the microscope settings, such as brightness, contrast and scan speed were held constant compared to the relative intensity of intracellular fluorescence of BODIPY **1**.

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References

1 (*a*) J. J. R. F. da silva and R. J. P. Williams, *The Biological Chemistry of the Elements*, Clarendon, Oxford, 1991; (*b*) R. Krämer, *Angew. Chem., Int. Ed.*, 1998, **37**, 772; (*c*) H. Tapiero, D. M. Townsend and K. D. Tew, *Biomed. Pharmacol.*, 2003, **57**, 386; (*d*) E. L. Que, D. W. Domailli and C. J. Chang, *Chem. Rev.*, 2008, **108**, 1517.

2 B. Halliwell and J. M. Gutteridge, *Biochem. J.*, 1984, **219**, 1.

3 (*a*) K. J. Barnham, C. L. Master and A. I. Bush, *Nat. Rev. Drug Discovery*, 2004, **3**, 205; (*b*) J. D. Gitlin, *Gastroenterology*, 2003, **125**, 1868; (*c*) J. F. B. Mercer, *Trends Mol. Med.*, 2001, **7**, 64; (*d*) G. Multhaup, A. Schlicksupp, L. Hesse, D. Beher, T. Ruppert, C. L. Masters and K. Beyreuther, *Science*, 1996, **271**, 1406; (*e*) D. J. Waggoner, T. B. Bartnikas and J. D. Gitlin, *Neurobiol. Dis.*, 1999, **6**, 221; (*f*) J. S. Valentine and P. J. Hart, *Proc. Acad. Sci. USA*, 2003, **100**, 3617; (*g*) L. I. Bruijin, T. M. Miller and D. W. Cleveland, *Annu. Rev. Neurosci.*, 2004, **27**, 723.

4 M. F. Yardim, T. Budinwa, E. Ekinci, N. Petrov, M. Razvigorava and V. Minkova, *Chemosphere*, 2003, **52**, 835.

5 (*a*) *Fluorescent Chemosensors for Ion and Molecular Recognition*, ed. A. W. Czarnik, American Chemical Society, Washington, DC, 1993; (*b*) A. W. Czarnik, *Trends in Organic Chemistry*, 1993, **4**, 123; (*c*) R. P. Haugland, *Handbook of Fluorescent Probes and Research Products*, Molecular Probes, Eugene, 9th ed., 2002.

6 *Calcium Signaling: A Practical Approach*, ed. A. Tepikin, Oxford University Press, Oxford, 2001.

7 R. E. London, *Annu. Rev. Physiol.*, 1991, **53**, 241.

8 (*a*) K. Kikuchi, K. Komatsu and T. Nagano, *Curr. Opin. Chem. Biol.*, 2004, **8**, 182; (*b*) N. C. Lim, H. C. Freake and C. Bruckner, *Chem.–Eur. J.*, 2004, **11**, 38; (*c*) S. Atilgan, T. Ozdemir and E. U. Akkaya, *Org. Lett.*, 2008, **10**, 4065.

9 (*a*) T. Cheng, Y. Xu, S. Zhang, W. Zhu, X. Qian and L. Duan, *J. Am. Chem. Soc.*, 2008, **130**, 16160; (*b*) X. Peng, J. Du, J. Fan, J. Wang, Y. Wu, J. Zhao, S. Sun and T. Xu, *J. Am. Chem. Soc.*, 2007, **129**, 1500; (*c*) L. Li, J. Han, B. Nguyen and K. Burgess, *J. Org. Chem.*, 2008, **73**, 1963.

10 X. L. Zhang, Y. Xiao and X. H. Qian, *Angew. Chem., Int. Ed.*, 2008, **47**, 8025.

11 (*a*) L. Zeng, E. W. Miller, A. Pralle, E. Y. Isacoff and C. J. Chang, *J. Am. Chem. Soc.*, 2006, **128**, 10; (*b*) L. C. Yang, R. McRae, M. M. Henary, R. Patel, B. Lai, S. Vogt and C. J. Fahrni, *Proc. Acad. Sci. USA*, 2005, **102**, 11179.

12 (*a*) R. Kramer, *Angew. Chem.*, 1998, **110**, 804; (*b*) H. S. Jung, P. S. Kwon, J. W. Lee, J. II. Kim, C. S. Hong, J. W. Kim, S. H. Yan, J. Y. Lee, J. H. Lee, T. H. Joo and J. S. Kim, *J. Am. Chem. Soc.*, 2009, **131**, 2008; (*c*) X. Zhang, Y. Shiraishi and T. Hirai, *Org. Lett.*, 2007, **9**, 5039; (*d*) Z. Xu, X. Qian and J. Cui, *Org. Lett.*, 2005, **7**, 3029; (*e*) Z. Xu, Y. Xiao, X. Qian, J. Cui and D. Cui, *Org. Lett.*, 2005, **7**, 889; (*f*) J. H. Huang, Y. F. Xu and X. H. Qian, *Org. Biomol. Chem.*, 2009, **7**, 1299; (*g*) Y. Q. Weng, F. Yue, Y. R. Zhong and B. H. Ye, *Inorg. Chem.*, 2007, **46**, 7749; (*h*) N. Shao, Y. Zhang, S. Cheung, R. Yang, W. Chan, T. Mo, K. Li and F. Liu, *Anal. Chem.*, 2005, **77**, 7294; (*i*) X. Qi, E. J. Jun, L. Xu, S. J. Kim, J. S. J. Hong and Y. J. Yoon, *J. Org. Chem.*, 2006, **71**, 2881; (*j*) M. Royzen, Z. Dai and J. W. Canary, *J. Am. Chem. Soc.*, 2005, **127**, 1612.

13 (*a*) A. Coskun and E. U. Akkaya, *J. Am. Chem. Soc.*, 2005, **127**, 10464; (*b*) K. Yamada, Y. Nomura, D. Citterio, N. Iwasawa and K. Suzuki, *J. Am. Chem. Soc.*, 2005, **127**, 6956; (*c*) W. Namkung, P. Padmawar, A. D. Mills and A. S. Verkman, *J. Am. Chem. Soc.*, 2008, **130**, 7794; (*d*) J. B. Wang and X. H. Qian, *Org. Lett.*, 2006, **8**, 3721; (*e*) M. Yuan, Y. Li, J. Li, C. Li, X. Liu, J. Lu, J. Xu, H. Liu, S. Wang and D. Zhu, *Org. Lett.*, 2007, **9**, 2313; (*f*) M. Yuan, W. Zhou, X. Liu, M. Zhu, J. Li, X. Yin, H. Zheng, Z. Zuo, C. Ouyang, H. Liu, Y. Li and D. J. Zhu, *J. Org. Chem.*, 2008, **73**, 5008; (*g*) S. H. Choi, K. Pang, K. Kim and D. G. Churchill, *Inorg. Chem.*, 2007, **46**, 10564; (*h*) T. W. Hundnall and F. P. Gabbai, *Chem. Commun.*, 2008, 4596; (*i*) M. Baruah, W. Qin, N. Basari, W. M. DeBorggaeve and N. Boens, *J. Org. Chem.*, 2005, **70**, 4152; (*j*) Y. Gabe, Y. Urano, K. Kikuchi, H. Kojima and T. Nagano, *J. Am. Chem. Soc.*, 2004, **126**, 3357; (*k*) C. Goze, G. Ulrich, L. Charbonniere, M. Cesario, T. Prange and R. Ziessel, *Chem.–Eur. J.*, 2003, **9**, 3748.

14 For recent reviews: (*a*) A. Loudet and K. Burgess, *Chem. Rev.*, 2007, **107**, 4891; (*b*) G. Ulrich, R. Ziessel and A. Harriman, *Angew. Chem., Int. Ed.*, 2008, **47**, 1184; (*c*) R. Ziessel, G. Ulrich and A. Harriman, *New J. Chem.*, 2001, **31**, 496.

15 (*a*) K. Umezawa, Y. Nakamura, H. Makino, D. Citterio and K. Suzuki, *J. Am. Chem. Soc.*, 2008, **130**, 1550; (*b*) K. Rurack, M. Kollmannsberger and J. Daub, *New J. Chem.*, 2001, **25**, 289; (*c*) K. Rurack, M. Kollmannsberger and J. Daub, *Angew. Chem., Int. Ed.*, 2001, **40**, 385.

16 (*a*) T. Rohand, W. Qin, N. Boens and W. Dehaen, *Eur. J. Org. Chem.*, 2006, 4658; (*b*) T. Rohand, W. Qin, N. Boens and W. Dehaen, *Chem. Commun.*, 2006, 266; (*c*) L. Li, B. Nguyen and K. Burgess, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 3112.

17 (*a*) P. A. Jacobi, L. D. Coutts, J. Guo, S. Hauck and S. H. Leung, *J. Org. Chem.*, 2000, **65**, 205; (*b*) P. A. Jacobi and J. Guo, *Tetrahedron Lett.*, 1995, **36**, 2717; (*c*) V. Karunaratne and D. Dolphin, *Tetrahedron Lett.*, 1996, **37**, 603.

18 (*a*) K. A. Connors, *Binding Constants: The Measurement of Molecular Complex Stability*, Wiley, New York, 1987; (*b*) K. B. Black, B. Andrioletti, A. C. Try, C. Ruiperez and J. L. Sessler, *J. Am. Chem. Soc.*, 1999, **121**, 10438.

19 M. C. Y. Chang, A. Pralle, E. Y. Isacoff and C. J. Chang, *J. Am. Chem. Soc.*, 2004, **126**, 15392.