Preparation and use of Coppersensor-1, a synthetic fluorophore for live-cell copper imaging

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Coppersensor-1 (CS1) is a small-molecule, membrane-permeable fluorescent dye for imaging labile copper pools in biological samples, including live cells. This probe, comprising a boron dipyrromethene (BODIPY) chromophore coupled to a thioether-rich receptor, has a picomolar affinity for Cu^+ with high selectivity over competing cellular metal ions. CS1 fluorescence increases up to 10-fold on binding to Cu^+. In this protocol we describe the synthesis of CS1 and how to use this chemical tool to investigate intracellular levels of labile copper in cultured cells. The preparation of CS1 is anticipated to take 4–5 d, and imaging assays can be performed in 1–2 d with cultured cells.

INTRODUCTION

Copper is an essential nutrient for living organisms[1,2], and cells tightly regulate this redox cofactor for proteins that control aerobic respiration, iron transport, oxidative stress protection, hormone production, neurotransmitter processing and angiogenesis[3–7]. On the other hand, mismanagement of cellular copper stores is implicated in severe diseases such as cancer[8,9] and cardiovascular disorders[10], as well as neurogenerative diseases including Alzheimer's disease[11–16], Menkes and Wilson diseases[17,18], familial amyotrophic lateral sclerosis[19–22], and prion diseases[23,24]. Despite the importance of copper homeostasis to situations of health and disease, mechanisms of copper accumulation, trafficking, and function remain incompletely characterized. Fluorescence imaging of labile, subcellular copper pools with selective copper-responsive dyes offers a potentially powerful technique for elucidating many of these pathways with spatial and temporal resolution; analogous small-molecule reagents have greatly aided researchers of calcium biology[25], and a pyrazoline dye has been applied successfully for detecting exchangeable copper in fixed cells with ultraviolet excitation[26]. The present protocol describes a recipe-style preparation of CS1 (**Fig. 1**), a BODIPY-based fluorophore for selective and sensitive detection of copper(I) ions in aqueous solution, as well as its application for imaging ionic copper pools in living cells[27]. CS1 is composed of a BODIPY chromophore possessing visible-wavelength excitation and emission profiles to minimize cellular autofluorescence and photodamage coupled to a thioether-rich receptor to achieve selective and stable binding of the Cu^+ ion in water over abundant cellular cations, including divalent magnesium, calcium, and zinc ions. Molecular recognition of Cu^+ by CS1 triggers up to a 10-fold increase in emission intensity for the probe. The sensor is membrane-permeable and can be used to report changes in labile copper levels within living cells using standard fluorescence microscopy. Control experiments with a chelator must be performed to establish that any observed fluorescence signals are due to copper binding. In addition, cell experiments are repeated at least in triplicate on separate days to verify results. Potential advantages of this CS1 fluorescence method over traditional atomic absorption and radioactivity assays include the ability to resolve labile copper pools with spatial resolution and redox selectivity, whereas potential limitations include the inability to assess total copper content and the current availability of only one emission color and Cu^+ binding affinity.

MATERIALS

REAGENTS

- 3,6,12,15-Tetrathia-9-monoazaheptadecane **2** prepared as described elsewhere[28]
- Copper chelator *N*-ethyl-3,6,12,15-tetrathia-9-monoazaheptadecane **4** prepared as described elsewhere[27]
- 2,4-Dimethyl-3-ethylpyrrole (kryptopyrrole; Sigma-Aldrich)
- Chloroacetyl chloride (Acros)
- Triethylamine (EM Science)
- Boron trifluoride diethyl etherate (Sigma-Aldrich)
- Potassium carbonate
- Potassium iodide
- Acetonitrile (distilled from CaH_2)
- Sodium sulfate
- Dichloromethane (Fisher)
- Toluene (Fisher)
- Hexanes (Fisher)
- Silica gel 60
- DMSO HPLC grade (EMD)
- Millipore-purified water
- $CuCl_2$ (anhydrous; Sigma-Aldrich)
- DMEM (Invitrogen)
- Glutamine (Sigma-Aldrich)
- Poly-L-lysine (Sigma-Aldrich)

EQUIPMENT

- Petri dishes (35 mm)
- Hotplate magnetic stirrer with contact thermometer
- Oil bath
- Dual nitrogen-vacuum manifold with vacuum pump
- Rotary evaporator
- Three-neck round-bottom flask (1,000 ml)
- Two-neck round-bottom flask (50 ml)
- Rubber septa
- Water-cooled condensers
- Air-inlet adapters
- Graduated cylinders
- Disposable glass Pasteur pipettes
- Pipette bulbs
- Single-neck round-bottom flasks (200–300 ml)
- Separatory funnels (250–1,000 ml)
- Powder funnels
- Fluted filter paper
- Columns for chromatography
- Fluorescence microscope

REAGENT SETUP

Cells for imaging Cells for imaging are grown on glass coverslips in medium. Cells typically used include mammalian cell lines HEK293T, COS-7 and HeLa. A representative example is given here. Briefly, HEK293T cells are cultured in DMEM supplemented with 10% (vol/vol) FBS (Invitrogen) and glutamine (2 mM). One day before imaging, cells are passaged and plated on 18-mm glass coverslips coated with poly-L-lysine (50 μg ml^{-1}). Adherent cells for imaging are grown to 50–80% confluency.

EQUIPMENT SETUP

Petri dishes Petri dishes were used in our laboratory in conjunction with water-immersion microscope objectives; this protocol can be readily adapted to coverslip holders and inverted microscopes with oil-based objectives.

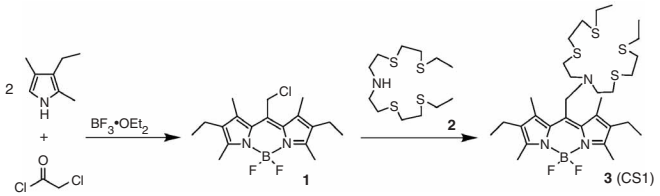

Figure 1 | Synthesis of CS1 (compound **3**).

PROCEDURE

Synthesis of 8-chloromethyl-2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene (compound 1)

1| Dry the three-neck round-bottom flask, a magnetic stirbar, condenser, and air-inlet adapter overnight in an electric oven at 130 °C.

2| After cooling the glassware to room temperature (20–25 °C), assemble the apparatus used for the reaction consisting of the 1,000-ml three-neck round-bottom flask and stirbar, condenser, septum, and air-inlet adapter.

3| To the flask add 200 ml of dry dichloromethane and then 7.38 g of 2,4-dimethyl-3-ethylpyrrole.

4| Next add 3.39 g of chloroacetyl chloride dropwise to the reaction.

5| Heat the resulting solution under a nitrogen atmosphere to 50 °C for 1 h.

6| Cool the reaction to room temperature, and remove the solvent under vacuum.

7| Add 400 ml of toluene, 20 ml of dichloromethane and 17 ml of triethylamine, and stir at room temperature for 15 min.

8| Add 19 ml of boron trifluoride diethyl etherate, and heat the resulting mixture to 50 °C for 1 h.

9| Cool the reaction to room temperature, and remove the solvent under vacuum.

10| Redissolve the residue in 200 ml of dichloromethane, pour the solution into a separatory funnel and wash the organic extract three times with water (100 ml each wash).

11| Separate the organic layer, dry over sodium sulfate, gravity-filter through fluted filter paper and remove the solvent by rotary evaporation.

12| Flash column chromatography on silica gel (2 × 24-inch column) by using 1:1 dichloromethane to hexanes as an eluant gives 5.8 g of compound **1** as an orange solid. Yield: 55% from chloroacetyl chloride. $R_f = 0.7$ (1:1 dichloromethane to hexanes). The identity and purity of the compound can be established by proton nuclear magnetic resonance (^{1}H NMR) and mass spectrometry (MS). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.82 (2H, s), 2.50 (6H, s), 2.45 (6H, s), 2.40 (4H, q, $J = 7.5$ Hz), 1.05 (6H, t, $J = 7.5$ Hz). FAB-MS: calculated for [M^+] 352, found 352.

Synthesis of CS1 (3)

13| Dry the 50-ml two-neck round-bottom flask, stirbar, condenser, and air-inlet adapter overnight in an electric oven at 130 °C.

14| After cooling the glassware to room temperature, assemble the apparatus used for the reaction consisting of the 50-ml two-neck round-bottom flask and stirbar, condenser, septum, and air-inlet adapter.

15| To the flask, add 20 ml of dry acetonitrile, and then 71 mg of compound **1** from step 12, 125 mg of 3,6,12,15-tetrathia-9-monoazaheptadecane (**2**), 73 mg of potassium iodide and 61 mg potassium carbonate.

16| Heat the stirred dark-red solution at reflux under a nitrogen atmosphere for 18 h.

17| Let the reaction cool to room temperature, and evaporate to dryness using a rotary evaporator.

18| Redissolve the residue in 100 ml dichloromethane, and transfer to an Erlenmeyer flask.

19| Pour the solution into a separatory funnel, and wash the organic extract three times with water (50 ml each wash).

20| Separate the organic layer, dry over sodium sulfate, gravity-filter through fluted filter paper and remove the solvent by rotary evaporation.

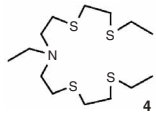

Figure 2 | Structure of the copper chelator *N*-ethyl-3,6,12,15-tetrathia-9-monoazaheptadecane (compound **4**).

21| Flash column chromatography on silica gel (1 × 12-inch column) using dichloromethane as an eluant gives 26 mg of CS1 (**3**) as a red solid. Yield: 22% from compound **1**. $R_f = 0.6$ (dichloromethane). The identity and purity of the compound can be established by ^{1}H NMR and MS. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.02 (2H, s), 2.87 (4H, t, $J = 7.6$ Hz), 2.52–2.65 (16H, m), 2.50 (s, 6H), 2.40 (s, 6H), 2.38 (4H, q, $J = 7.6$ Hz), 1.24 (6H, t, $J = 7.6$ Hz) 1.05 (6H, t, $J = 7.6$ Hz). FAB-MS: calculated for [MH^+] 630, found 630.

Preparation of reagent stock solutions for imaging experiments

22| Prepare a 1 mM stock solution of CS1 (MW 630 g/mol) in DMSO by dissolving 0.63 mg of solid CS1 per milliliter of DMSO solvent.
■ **PAUSE POINT** Stock solutions of CS1 can be stored for months at room temperature in the dark. Solutions can also be stored frozen and thawed when needed, but repeated freeze/thaw cycles may lead to decomposition.

23| Prepare a 10 mM stock solution of $CuCl_2$ (MW 134 g/mol) by dissolving 1.34 mg of solid $CuCl_2$ per milliliter of Millipore water solvent.

24| Prepare a 100 mM stock solution of *N*-ethyl-3,6,12,15-tetrathia-9-monoazaheptadecane (**4**, MW 342 g/mol, **Fig. 2**) in DMSO by dissolving 34.2 mg of the liquid chelator per milliliter of DMSO solvent.
■ **PAUSE POINT** Stock solutions of the chelator can be stored for months at room temperature in the dark under a nitrogen atmosphere.

CS1 labeling of live cells

25| Remove cells from incubator, and transfer one coverslip into a 35-mm Petri dish containing 3 ml PBS buffer.
Should you wish to supplement the cells with copper, add an aliquot of the $CuCl_2$ stock solution to the growth medium and return the cells to the incubator for 1–8 h before continuing to Step 26. To add a competing chelator to copper-supplemented cells, remove cells from the incubator and add 15 µl of a 100 mM compound **4** stock solution to give a final chelator concentration of 500 µM. Mix thoroughly, and incubate in the dark for 5 min at 25 °C, before continuing to Step 26.
▲ **CRITICAL STEP** In the case of, for example, HEK293T cells growing in 1 ml of medium, add 10 µl of the 10 mM $CuCl_2$ stock solution to give a final copper concentration of 100 µM. A 5- to 10-fold excess of chelator over copper supplementation gives optimal results.

26| To the Petri dish, add 15 µl of a 1 mM CS1 stock solution to give a final dye concentration of 5 µM. Mix thoroughly.
▲ **CRITICAL STEP** Higher concentrations of dye may result in high levels of background fluorescence.

27| Incubate for 5–20 min in the dark at 25° or 37 °C.

Imaging the CS1-labeled cells

28| CS1 can be imaged using any type of fluorescence microscope, including epifluorescence, confocal and multiphoton. For standard confocal experiments, best results were obtained with 543 nm excitation to match the absorption maximum of the apo and Cu^+-bound probe.

● **TIMING**
With appropriate precursors in hand, the synthesis and purification of BODIPY (**1**) and CS1 (**3**) is anticipated to require 4–5 d. Imaging experiments with cells will require 1–2 d with cells in culture.

ANTICIPATED RESULTS

The approaches described here have been found useful for detecting labile pools of copper in a variety of cell types, including mammalian cell line and primary culture sources. For example, the ability of CS1 to detect various levels of intracellular copper within living cells is demonstrated here using live HEK293T cells (**Fig. 3**).

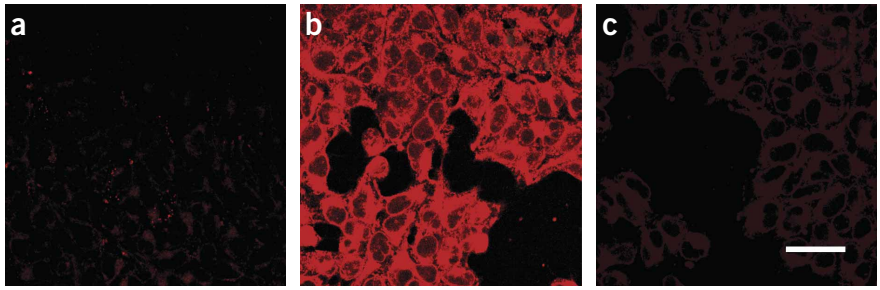

Figure 3 | Live-cell copper imaging with CS1. (**a**) HEK293T labeled with 5 µM CS1 for 5 min at 25 °C. (**b**) HEK293T grown with 100 µM $CuCl_2$ supplement for 6 h at 37 °C and stained with CS1 using the same conditions as in **a**. (**c**) Copper-supplemented HEK293T treated with 500 µM of the competing Cu^+ chelator **4** for 5 min at 25 °C and stained with CS1 using the same conditions as in **a** and **b**. Scale bar = 38 µm.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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