

## Research Article

# A Coumarin-Based Fluorescence Probe for Selective Recognition of $\text{Cu}^{2+}$ Ions and Live Cell Imaging

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A new fluorescence probe L was rationally designed and synthesized for the recognition of  $\text{Cu}^{2+}$  ions by the combination of coumarin hydrazide and 2-acetylpyrazine. The photochemical properties and selectivity of L for  $\text{Cu}^{2+}$  ions in a  $\text{CH}_3\text{CN}/\text{HEPES}$  (3 : 2, v/v) buffer were investigated by UV-vis absorption and fluorescence emission spectra. A highly selective and sensitive response of L for  $\text{Cu}^{2+}$  ions over other competing metal ions was observed with limit of detection in  $3 \mu\text{M}$ . The coordination stoichiometry of L to  $\text{Cu}^{2+}$  ions was determined to be 1 : 1 by the UV-vis absorption spectrum, the fluorescence titrations, and density functional theory (DFT) calculations. Moreover, L was applied successfully for recognition of intracellular  $\text{Cu}^{2+}$  ions in living cells.

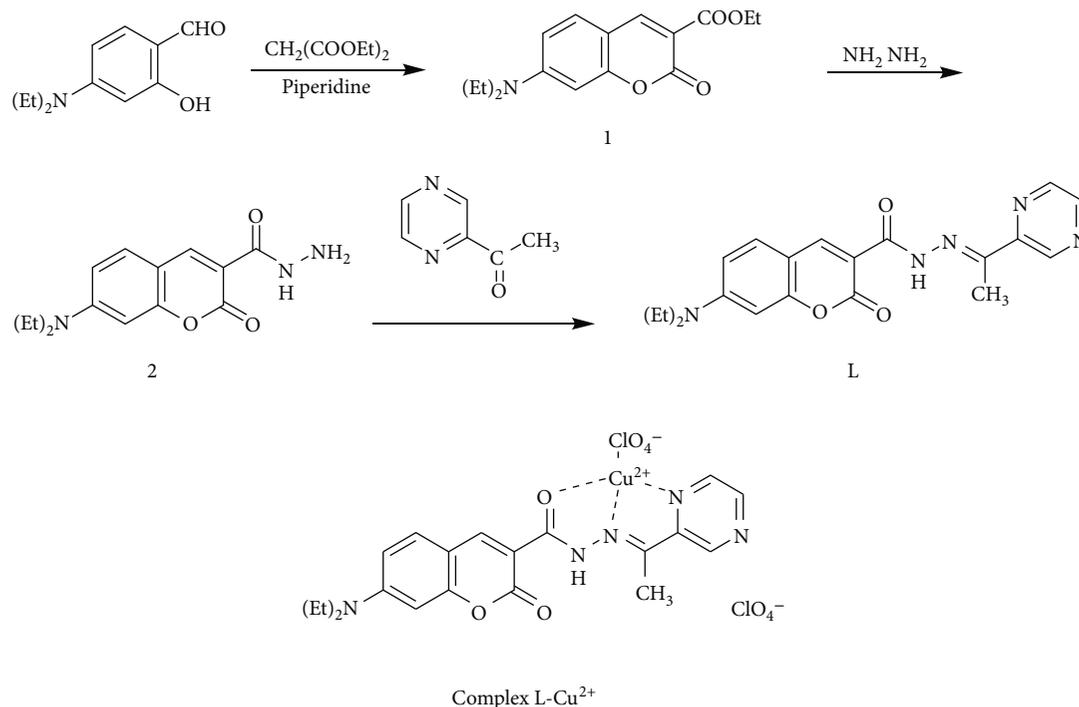
## 1. Introduction

The selective and sensitive detection for trace transition metal ions has received great attention in biological samples [1–3]. As a required element in humans, copper plays vital roles during various biochemical processes, such as cellular respiration, neurotransmission, and biosynthesis [4–7]. A copper balance is also maintained in the human body through daily diet and metabolism under normal conditions. However, excessive concentration of copper ions in the human body will cause a wide variety of diseases such as Menkes syndrome, prion disease, and Alzheimer's disease [8–11]. Therefore, it is significant to develop a highly sensitive method for selective detection of trace  $\text{Cu}^{2+}$  ions.

Fluorescence detection among various analytical methods is attracting more attention due to its high selectivity,

short response time, and great potential for live cell imaging [12–15]. By the virtue of these distinct advantages, fluorescence bioimaging technology has been applied successfully in living cells [16–18]. Typical fluorescent sensors have been documented involving inorganic composites, such as quantum dots @MOFs, or organic fluorophores such as photochromic diarylethene derivatives, coumarin derivatives, rhodamine B-based fluorescent probes, and naphthalimide-rhodamine B derivative [19–27]. Although a variety of fluorescent probes for the recognition of  $\text{Cu}^{2+}$  ions have been reported, it is still a challenge to directly contact metal ions with fluorophore for signal transduction.

Due to their strong fluorescence emission, efficient cell permeation, large Stokes shift, and a structure that can be easily modified, coumarin is an attractive starting material [28–33]. Besides, the carbonyl group of coumarin can take



SCHEME 1: Synthetic route of L and the presumable structure of complex L-Cu<sup>2+</sup>.

part in the coordination with metal ions. The introduction of a hydrazide chain at 3-position of coumarin could both increase the intramolecular charge transfer and provide coordination sites for metal ions via carbonyl oxygen and amide nitrogen [34–36]. In order to obtain a new efficient probe, we designed a coumarin derivative for selective recognition of Cu<sup>2+</sup> ions (Scheme 1). Electron donors of the N,N-diethyl group were introduced at the 7-position to modulate intramolecular charge transfer. L exhibited remarkable fluorescence quenching response to Cu<sup>2+</sup> ions due to the paramagnetic nature of Cu<sup>2+</sup> ions and/or photo-induced electron transfer effect. Density functional theory (DFT) calculations confirm the four-coordination configuration of Cu<sup>2+</sup> ions in complex L-Cu<sup>2+</sup>. Moreover, this probe was successfully applied for the recognition of Cu<sup>2+</sup> ions in living cells.

## 2. Results and Discussion

**2.1. Spectral Responses of L to Cu<sup>2+</sup>.** The specific bonding of L with Cu<sup>2+</sup> was first examined by UV-vis absorption spectroscopy in CH<sub>3</sub>CN/HEPES (3:2, v/v) buffer (pH = 7.4). As shown in Figure 1, the free L exhibited a maximum absorption at 442 nm. With the increase of Cu<sup>2+</sup> concentration in 0~7.5 equivalents, the maximum absorption intensity gradually decreased and the maximum absorption wavelength exhibited a red shift phenomenon. The former could be ascribed to the  $\pi - \pi^*$  transition of the coumarin chromophore, and the latter was attributed to a metal-to-ligand charge transfer (MLCT) caused by binding L and Cu<sup>2+</sup> ion. Based on the isosbestic point at 458 nm, a 1:1 stoichiometry for the L and Cu<sup>2+</sup> ion is confirmed by the data of Job's plot (Figure S1a) [37–40].

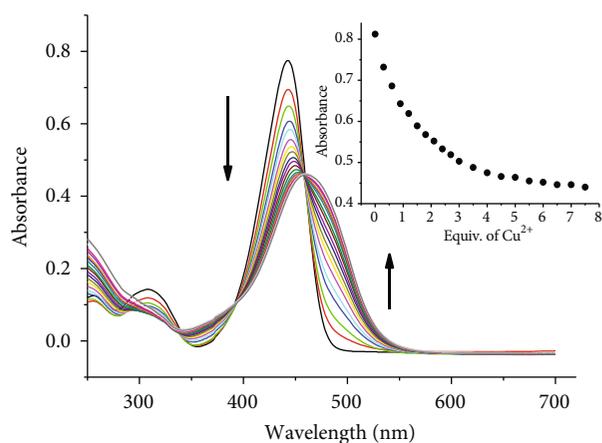


FIGURE 1: UV-vis absorption spectra of L (10  $\mu$ M) with addition of various concentrations of Cu<sup>2+</sup> ions (0~7.5 equivalents) in CH<sub>3</sub>CN/HEPES (3:2, v/v) buffer. Inset: absorbance at 442 nm versus the concentration of Cu<sup>2+</sup>.

Besides, the absorption spectra of L were measured in the presence of other potentially relevant metal cations to investigate the selectivity of L for Cu<sup>2+</sup> ions in CH<sub>3</sub>CN/HEPES (3:2, v/v) buffer solutions. Upon addition of 10 equivalents of relevant metal ions such as Pb<sup>2+</sup>, K<sup>+</sup>, Hg<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, Al<sup>3+</sup>, Ca<sup>2+</sup>, Cd<sup>2+</sup>, Fe<sup>3+</sup>, Ag<sup>+</sup>, Mg<sup>2+</sup>, Cr<sup>3+</sup>, Na<sup>+</sup>, Fe<sup>2+</sup>, and Zn<sup>2+</sup>, no obvious changes were observed on absorption intensity (Fig. S2). Besides, the addition of Ni<sup>2+</sup> ions caused a relatively slight absorbance change. In contrast, when 10 equiv. of Cu<sup>2+</sup> was added to the solution of L, a significant decrease in absorption intensity and a bathochromic shift in absorption wavelength appeared obviously, indicating

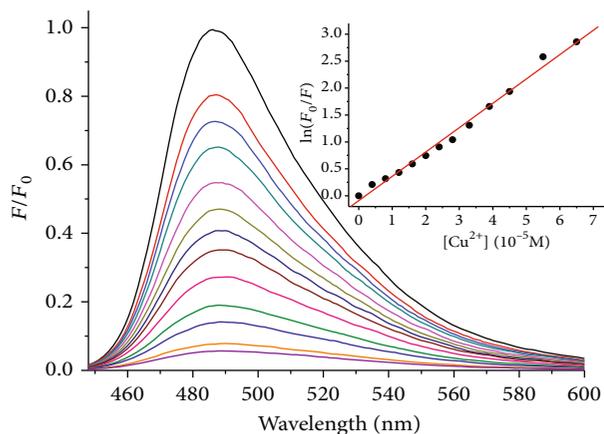


FIGURE 2: Fluorescence emission spectra of L (10  $\mu\text{M}$ ) upon addition of various concentrations of  $\text{Cu}^{2+}$  ions (0~65  $\mu\text{M}$ ) in  $\text{CH}_3\text{CN}/\text{HEPES}$  (3:2,  $v/v$ ) buffer solution. The excitation wavelength was 438 nm. Inset: fluorescence intensities at 487 nm of the L as a function of  $\text{Cu}^{2+}$  ion concentrations (0~65  $\mu\text{M}$ ).

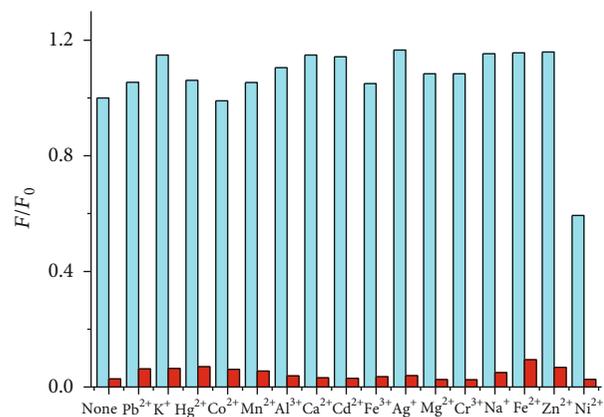


FIGURE 3: Fluorescence intensity changes ( $F/F_0$ ) of L upon the addition of various metal ions in  $\text{CH}_3\text{CN}/\text{HEPES}$  (3:2,  $v/v$ ) solutions. Cyan bars represented the fluorescence response of L to various metal ions. Red bars represented the subsequent addition of  $\text{Cu}^{2+}$  to the above solutions. Excitation and emission were recorded at 438 nm and 487 nm, respectively.

the excellent selectivity of L for  $\text{Cu}^{2+}$  over other metal ions. The results could be contributed to the strong coordination ability of  $\text{Cu}^{2+}$  and its larger association constant.

To calculate the fluorescent response ability of L, the fluorescence emission spectra of L (10  $\mu\text{M}$ ) were also investigated in the  $\text{CH}_3\text{CN}/\text{HEPES}$  (3:2,  $v/v$ ) buffer solution. L showed a maximum fluorescence emission at 487 nm upon excitation at 438 nm due to the presence of coumarin chromophore (Figure 2). With the increasing concentration of  $\text{Cu}^{2+}$  ions, the intensity of fluorescence emission at 487 nm decreased gradually. After the addition of 65  $\mu\text{M}$   $\text{Cu}^{2+}$  ions, the fluorescence emission was almost quenched. About 30-fold quenching of fluorescence emission intensity at 487 nm was observed compared with that of free L. The fluorescence quenching effect of L might be attributed to the paramagnetism of  $\text{Cu}^{2+}$  ions and/or photo-induced electron transfer process [35, 41].

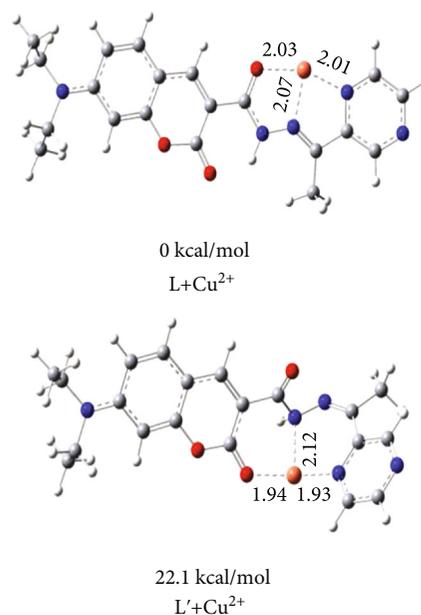


FIGURE 4: Optimized structures (and selected parameters) of possible complex  $\text{L}-\text{Cu}^{2+}$  and  $\text{L}'-\text{Cu}^{2+}$ .

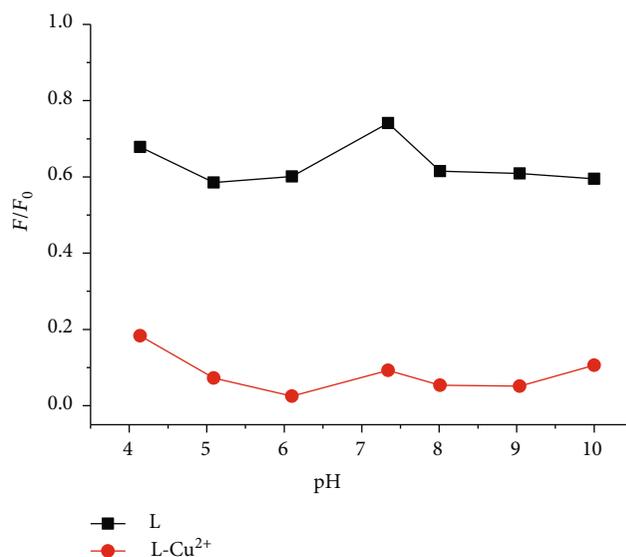


FIGURE 5: Variation of fluorescence intensity of L and its detection of  $\text{Cu}^{2+}$  ions with pH value at room temperature.

Moreover, the fluorescence emission intensity at 487 nm showed a good linear relationship ( $R^2 = 0.99$ ) against the concentration of  $\text{Cu}^{2+}$  ions in 1~65  $\mu\text{M}$  (Figure 2 inset). The quenching constant value of L with  $\text{Cu}^{2+}$  ions was determined from the titration plots. The corrected Stern-Volmer fitting indicates the value of  $4.5 \times 10^4 \text{ mol}^{-1} \cdot \text{L}$ . And Job's plot and the molar ratio of the fluorescence titrations also revealed a 1:1 binding stoichiometry (Fig. S1b). The detection limit of L for  $\text{Cu}^{2+}$  ions was estimated about 3  $\mu\text{M}$  based on  $\text{LOD} = 3\sigma/s$ , where  $\sigma$  and  $s$  represent the standard deviation of blank measurements and the slope between concentration of  $\text{Cu}^{2+}$  ions and fluorescence

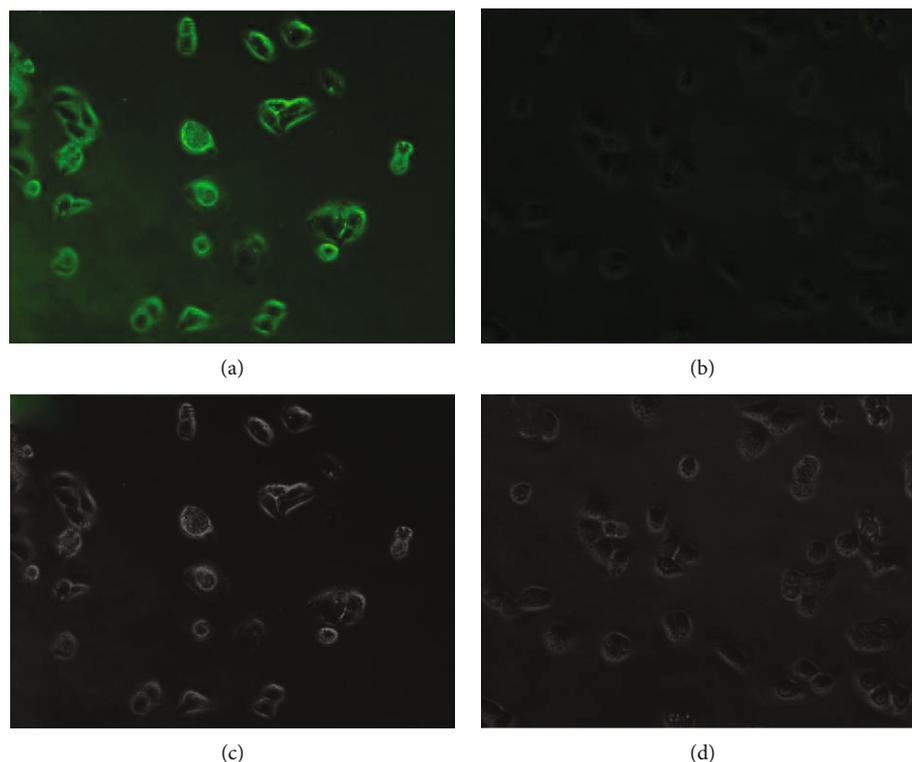


FIGURE 6: Inverted fluorescence imaging of HepG2 cells incubated with L (10  $\mu\text{M}$ ) for 2.5 hours (a) and then further incubated with 6 equivalents of  $\text{Cu}^{2+}$  ions for 30 minutes (b). The corresponding bright field images of HepG2 cells incubated with L (10  $\mu\text{M}$ ) (c) and then further incubated with 6 equivalents of  $\text{Cu}^{2+}$  ions (d).

intensity, respectively [42]. Thus, the probe L shows excellent sensitivity and low detection limit for the detection of  $\text{Cu}^{2+}$ .

The fluorescence changes of L in response to various relevant species in  $\text{CH}_3\text{CN}/\text{HEPES}$  (3:2,  $v/v$ ) solutions were also studied. As shown in Figure 3, only  $\text{Cu}^{2+}$  ions induced a significant decrease in the fluorescence intensity, whereas very weak fluorescence variations were observed for the other metal ions such as  $\text{Pb}^{2+}$ ,  $\text{K}^+$ ,  $\text{Hg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Ag}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Na}^+$ ,  $\text{Fe}^{2+}$ , and  $\text{Zn}^{2+}$  (10 equivalents). Furthermore,  $\text{Ni}^{2+}$  ions caused a relatively weak fluorescence quenching, which may be attributed to the low associated constant between L and  $\text{Ni}^{2+}$  ions. Such results indicated that L could be used for selective recognition of  $\text{Cu}^{2+}$  ions in the presence of other relevant species. Thus, the competing experiments were carried out to observe the selectivity for  $\text{Cu}^{2+}$  over other transition metal ions. The addition of  $\text{Cu}^{2+}$  ions still led to a fluorescence quenching of L even in the presence of competing species, suggesting that L had a good selectivity towards  $\text{Cu}^{2+}$  ions.

**2.2. Density Functional Theory (DFT) Calculations of  $\text{Cu}^{2+}$  Binding L.** From a mechanistic viewpoint, the unique selectivity for the  $\text{Cu}^{2+}$  ion was attributed to various factors, including the suitable coordination conformation of the Schiff-based receptor, the nitrogen (oxygen)-binding affinity character with the  $\text{Cu}^{2+}$  ion, and the deprotonation ability of L. The fluorescence quenching of L by  $\text{Cu}^{2+}$  ion could be

ascribed to the paramagnetism of  $\text{Cu}^{2+}$  ions and/or photo-induced electron transfer process. Density functional theory (DFT) calculations were used to determine the possible binding mode of  $\text{Cu}^{2+}$  to L. The geometry structures of L and metal complexes  $\text{L}-\text{Cu}^{2+}$  and  $\text{L}'-\text{Cu}^{2+}$  with all real frequency have been obtained using B3LYP/6-31G(d)/SDD level (the SDD basis set was used for transition metal Cu ion). The optimized geometries (and selected parameters) and electronic energies of metal complexes are shown in Figure 4. The result suggests that  $\text{L}-\text{Cu}^{2+}$  is more stable than  $\text{L}'-\text{Cu}^{2+}$  by 23.1 kcal/mol. Thus, the binding model between  $\text{Cu}^{2+}$  and L should be  $\text{L}-\text{Cu}^{2+}$ . Cartesian coordinates were shown in Scheme S1. The results indicated that copper ions coordinated with the ketonic oxygen atom, pyrazine nitrogen atom, and imine nitrogen (C=N) in L molecule. This coordination configuration always appeared in the other similar complexes [29, 43].

**2.3. Effect of pH on the Performance of L for  $\text{Cu}^{2+}$ .** Because the fluorescence property of L for the detection of  $\text{Cu}^{2+}$  ions may be affected by pH value, the fluorescence responses of L (10  $\mu\text{M}$ ) to pH value were investigated by fluorescence spectroscopy with and without  $\text{Cu}^{2+}$ , respectively. As shown in Figure 5, the fluorescence emission intensity of the free L and complex  $\text{L}-\text{Cu}^{2+}$  was almost independent of pH value in 4.0~10.0. Besides, the addition of  $\text{Cu}^{2+}$  causes a fluorescence quenching of L. Therefore, L could be applied for the recognition of  $\text{Cu}^{2+}$  over a wide pH range.

**2.4. Live Cell Imaging.** Live cell imaging with a fluorescence probe had important significance and application [44–46]. The recognizing ability of L toward  $\text{Cu}^{2+}$  ions was examined in living cells by using an inverted fluorescence microscope. The clear green fluorescence was observed from the intracellular region in human hepatocellular carcinoma HepG2 after incubation with L ( $10\ \mu\text{M}$ ) for 2.5 hours in 0.01 M PBS buffer solution (Figure 6(a)). However, when the HepG2 cells were further treated with 6 equivalents of  $\text{Cu}^{2+}$  for 30 minutes, the green fluorescence intensity of L was nearly quenched (Figure 6(b)). During the whole experiment process (about 3 hours), the cells were visualized without obvious side effects. These results manifested that fluorescent L could be used for the recognition of copper ions within biological samples.

### 3. Conclusion

In conclusion, a new fluorescence probe L based on coumarin hydrazide and 2-acetylpyrazine moiety was synthesized to detect trace  $\text{Cu}^{2+}$  ions. A 1:1 stoichiometry of L and  $\text{Cu}^{2+}$  ions was established based on Job's plot analysis. Density functional theory (DFT) calculations confirm the four-coordination configuration of  $\text{Cu}^{2+}$  ions. The probe exhibits excellent stability over a wide range of pH, high selectivity, and sensitivity (detection limit of about  $3\ \mu\text{M}$ ) for  $\text{Cu}^{2+}$  over other relevant transition metal ions. Further, cell imaging test demonstrated that probe L was also used for the recognition of  $\text{Cu}^{2+}$  ions in living cells.

### Data Availability

The data used to support the findings of this study are included within the article and supplementary information file(s).

### Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

### Acknowledgments

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### Supplementary Materials

The synthesis process of probe L and specific test conditions for spectra and cell imaging reported in this article have been deposited in supplementary material. Figure S1: Job's plot fitting of probe L with  $\text{Cu}^{2+}$ . Figure S2: UV-vis absorption spectra of probe L upon addition of various metal ions. Scheme S1: the Cartesian coordinate

data of complex  $\text{L-Cu}^{2+}$  and  $\text{L}^{\prime}\text{-Cu}^{2+}$ . Figure S3, S4, and S5: the NMR and MS spectra of probe L. (*Supplementary Materials*)

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