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A Sensitive and Selective Fluorescent Probe for Real-Time Detection and Imaging of Hypochlorous Acid in Living Cells

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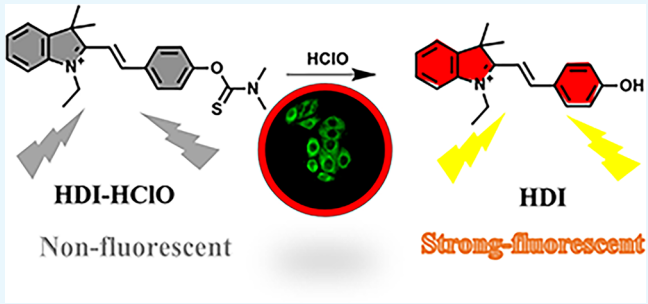
ABSTRACT: Hypochlorous acid (HClO), a reactive oxygen species, plays an essential role in the processes of physiology and pathology via reacting with most biological molecules. The abnormal level of HClO may cause inflammation, especially arthritis. To further understand its key role in inflammation, in situ detection of HClO is necessary. Herein, a water-soluble small molecule fluorescent probe (HDI-HClO) is employed to monitor and identify trace amounts of HClO in the biological system. In the presence of HClO, the probe releases a hydroxyl group emitting strong fluorescence because of the restoration of the intramolecular charge transfer process. Furthermore, this probe displays a 150-fold fluorescence enhancement accompanied by a large Stokes shift and a lower detection limit (8.3 nM). Moreover, the probe can make a rapid response to HClO within 8 s, which provides the possibility of real-time monitoring of intracellular HClO. Based on the advantages of rapid dynamics, good water solubility, and excellent biocompatibility, this probe could effectively monitor the fluctuations of exogenous and endogenous HClO in living cells. The fluorescence imaging of HDI-HClO indicated that it is an excellent potential approach for comprehending the relationship between inflammation and HClO.



1. INTRODUCTION

As excellent significant intracellular signaling molecules, reactive oxygen species (ROS) play a principal role in various physiological and pathological processes.[1−3] The overexpression of ROS can lead to abnormal cells and genetic structures, further causing various diseases, for example, DNA/protein mutations, atherosclerosis, inflammation, and cancer.[4−6] Hypochlorous acid (HClO), an extremely vital ROS, is mainly generated from the myeloperoxidase-catalyzed peroxidation reaction of chloride ions (Cl^-) and hydrogen peroxide (H_2O_2).[7−9] The presence of HClO can protect the human immune system from pathogens and bacteria.[9,10] For example, rheumatoid arthritis (RA) is an extremely general chronic inflammatory disease, which may cause chronic pain, damage, and even disability.[11−14] Accumulated evidence showed that RA is closely related to the level of HClO.[15,16] However, the excessive levels of HClO can also lead to the oxidation of nucleic acids, proteins, and lipids, which further leads to some diseases, for instance, arthritis and cancer.[17] Thus, it is of great importance to detect the fluctuations of HOCl in biological systems.

Compared with conventional analysis techniques, liquid chromatography−mass spectrometry (LC−MS), potentiometry, high-performance liquid chromatography (HPLC), and fluorescence imaging technology have become indispensable tools in the field of biological, chemical, and medical research because of their excellent sensitivity and specificity, noninvasiveness, real-time visual detection, and high spatiotemporal resolution.[18−21] Because of the strong reactivity and short existence of HClO, excellent fluorescent probes must possess high selectivity, sensitivity, fast response, and great biocompatibility for the monitoring of HClO.[22,23] In recent years, more and more fluorescent probes were proposed to monitor or image HClO. They are mainly the oxidation reaction of HClO with *p*-alkoxyaniline, *p*-methoxyphenol, oxime, electron-deficient C=C bonds, etc.[24−28] In spite of those probes displaying excellent characteristics, they also show shortcomings, for instance, low detection limit, slow reactivity, poor water solubility, and biocompatibility. Thus, exploiting small molecule fluorescent probes with great biocompatibility is necessary for the fast detection of HClO in living cells.

In this study, we design a small molecule fluorescent probe (HDI-HClO) based on intramolecular charge transfer (ICT). In this design, we introduce indole cations to enhance the water solubility of HDI-HClO and to obtain good biocompatibility. In the presence of HClO, the probe will rapidly transform into a fluorophore within 8 s, releasing a strong fluorescence signal. Furthermore, the probe exhibits specific

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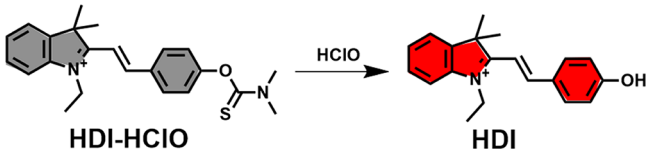
selectivity to HClO and is not interfered by other ROS, such as hydrogen peroxide (H_2O_2). Moreover, HDI-HClO displays a lower detection limit (LOD) and a satisfactory linear relationship for HClO. We also certified that HDI-HClO could be utilized to track exogenous and endogenous of HClO in cells, helping us to better understand inflammation, especially RA.

2. RESULTS AND DISCUSSION

2.1. Design and Synthesis of HDI-HClO. The structure of HDI-HClO and the proposed react mechanism of this probe toward HClO are displayed in Scheme 1. To specially detect the levels of HClO, the choice of fluorophores is critical for the excellent fluorescent probe. It can be seen that HDI-HClO composes a cyanine dye moiety and a response group of HClO. The introduction of indole cations greatly improves its water solubility and further increases the biocompatibility of the probe. Furthermore, some reports demonstrate that *N*,*N*-dimethylthiocarbamate can serve as a reaction site to achieve fast and specific detection of HClO.[29−32] Herein, a novel water-soluble fluorescent probe was synthesized by combining HDI with *N*,*N*-dimethylthiocarbamoyl chloride. The synthetic route and characterization of the probe are described in the Supporting Information. In the absence of HClO, HDI-HClO was non-fluorescent owing to *N*,*N*-dimethylthiocarbamate inhibiting the ICT process. Moreover, HPLC experiments were employed to further evaluate the response mechanism of HDI-HClO to HClO (Figure S1). As can been seen from Figure S1, the peak positions of HDI-HClO and HDI were at 5.43 and 6.78 min, respectively. Gratifyingly, after adding HClO, a small peak appeared, and its peak position was basically the same as that of HDI. In addition, this probe displayed an obvious fluorescence emission peak centered at 520 nm after the addition of HClO.

Scheme 1. Molecular Structure of HDI-HClO and Response Mechanism toward HClO



2.2. Spectroscopic Properties. The spectral characteristics of the probe were evaluated in 10 mM buffer solution (PBS/DMSO = 10:1, v/v, pH = 7.4). HDI-HClO displayed an obvious absorption at 400 nm. After adding HClO, there was an obvious 40 nm red shift to 440 nm (Figure 1a). Furthermore, the fluorescence response of HDI-HClO to variable concentrations of HClO was tested. In the initial state, the probe had almost no fluorescence. However, this probe HDI-HClO expressed a gradually increased fluorescence signal centered at 520 nm accompanied with a large Stokes shift of 80 nm. It should be ascribed to rapid reaction between HClO and *N*,*N*-dimethylthiocarbamate, thereby turning the fluorescence signal on (Figure 1b), suggesting that HDI-HClO could be utilized to the monitoring of HClO. Furthermore, an exciting linear relationship between the fluorescence intensity and HClO levels from 0 to 20 μM was obtained (Figure 1c). Furthermore, the regression equation was $F_{520\ nm}$ = 181.43-[HClO] + 133.25 with a linear functional relationship R^2 of 0.9863. According to the standard method of 3σ/k, the LOD was determined to be about 8.3 nM, which demonstrated that

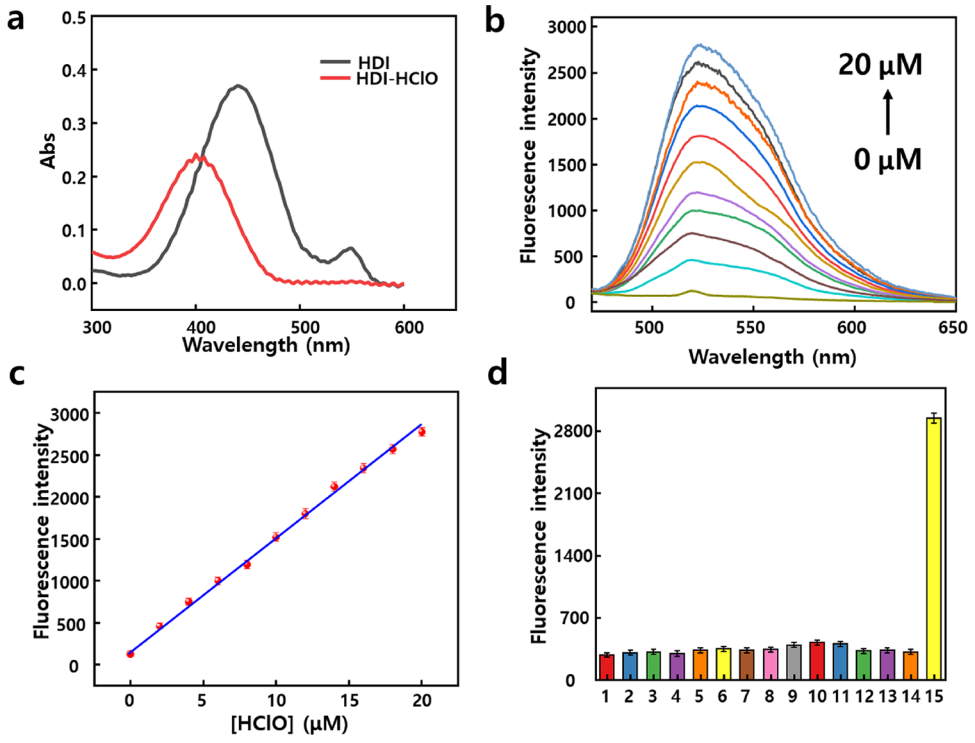


Figure 1. Spectral characteristics and selectivity of HDI-HClO. (a) UV−vis absorption and (b) the fluorescence emission spectrum with HClO (0−20 μM). (c) Linear relationship between the fluorescence intensity and HClO. (d) Response of HDI-HClO to various species: 1, blank; 2, 100 μM NO_2^-; 3, 100 μM NO; 4, 100 μM HNO; 5, 100 μM $ONOO^-$; 6, 100 μM ·OH; 7, 100 μM $O_2^{\cdot-}$; 8, 100 μM H_2O_2; 9, 100 μM ^{t}BuOO·; 10, 1 mM SO_3^{2-}; 11, 1 mM HSO_3^-; 12, 1 mM HSO_4^-; 13, 1 mM HCO_3^-; 14, 1 mM HS^-; and 15, 20 μM HClO.

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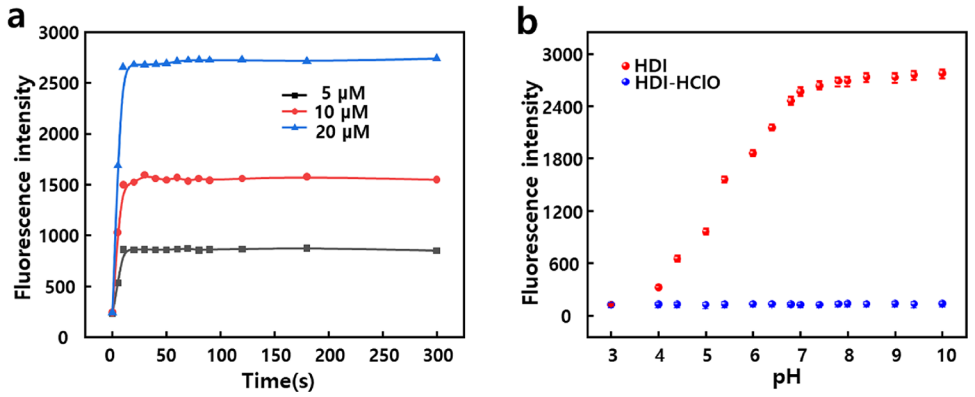

Figure 2. (a) Response speed and (b) pH effect of HDI-HClO to HClO.

this probe had a certain ability to identify trace HClO. Compared with other probes reported, our probe had a relatively LOD, which suggested that HDI-HClO possessed good potential to detect HClO (Table S1).

2.3. Selectivity. Great selectivity is very important to an ideal fluorescent probe. The response of the probe HDI-HClO was examined toward various biological interferents, including various reactive species (NO_2^-, ·OH, H_2O_2, NO, HNO, $ONOO^-$, OCl^-, $O_2^{\cdot-}$, and $^tBuOO\cdot$), common ions and anions (Cu^{2+}, Ba^{2+}, Na^+, K^+, Ca^{2+}, Hg^{2+}, Mg^{2+}, Fe^{3+}, SO_3^{2-}, HSO_3^-, HSO_4^-, HCO_3^-, HS^-, $S_2O_7^{2-}$, $S_2O_8^{2-}$, F^-, Cl^-, and Br^-), and amino acids (glutathione (GSH), tryptophane (Trp), methionine (Met), glutamic acid (Glu), cysteine (Cys), homocysteine (Hcy), alanine (Ala), arginine (Arg), threonine (Thr), serine (Ser), aspartic acid (Asp), leucine (Leu), and lysine (Lys)). It was gratifying to see that only HClO could lead to the increase of the fluorescence signal due to the fact that it could convert HDI-HClO into a HDI fluorophore, further revealing that the probe possessed excellent selectivity to HClO (Figure 1d and Figure S2). Subsequently, we studied the influence of some thiols on the probe for a long time (0–180 min). The result suggested that, after adding Cys, GSH, and HCy for 60 min, a weaker fluorescence signal appeared at 460 nm (Figure S3). The above results evidenced that HDI-HClO could specifically distinguish HClO instead of other species.

2.4. The Response Speed and pH Effect. The reaction kinetics of HDI-HClO on HClO was investigated. Notably, the fluorescence signal achieved a platform at 520 nm within 8 s and accompanied with a 150× signal enhancement, suggesting that HDI-HClO can quickly respond to HClO (Figure 2a). Next, the influence of pH on HDI-HClO was assessed. In the absence or presence of HClO, the changes of fluorescence intensities were analyzed with an emission wavelength at 520 nm ranging from pH 3.0 to 10.0. The fluorescence intensity of HDI-HClO remained steady under the range of the study, and HDI remained stable under physiological pH (Figure 2b), suggesting that the probe could be of great potential to qualitatively identify trace HClO in the living system.

2.5. Imaging of HClO in Living Cells. Inspired by the excellent performance of HDI-HClO, the potential application of the probe HDI-HClO to detect HClO was further evaluated in living cells. To test the biocompatibility and cytotoxicity of HDI-HClO, the cell counting kit-8 (CCK-8) assay was performed against RAW 264.7 cells and HeLa cells. As shown in Figure S4, HDI-HClO displayed lower cytotoxicity and great biocompatibility. Since HDI-HClO contained features of great sensitivity and specificity and low cytotoxicity, it could be suitable to detect the levels of HClO in living cells. Herein, HeLa cells and RAW 264.7 cells were selected as test cell models. Subsequently, we further evaluated the potential of the probe for monitoring intracellular HClO. HDI-HClO was employed to image HClO in living cells. Herein, the HeLa cell line was first incubated with HDI-HClO for around 20 min before imaging. As illustrated in Figure 3, we could observe a

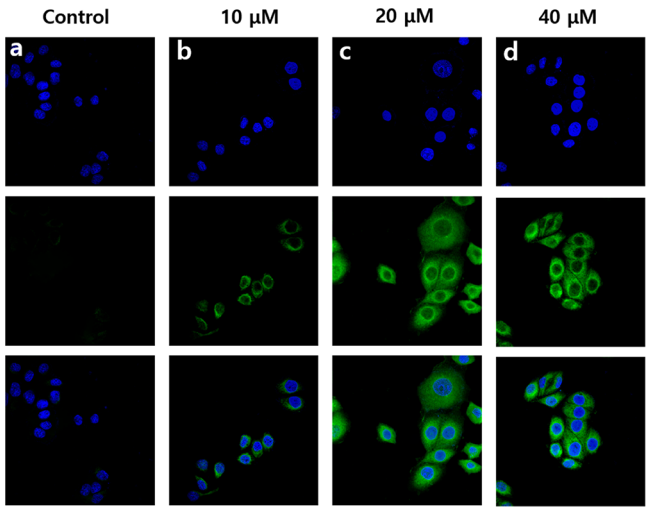

Figure 3. Imaging of exogenous HClO in HeLa cells incubated with the probe and (a) 0, (b) 10, (c) 20, and (d) 40 μM $HClO^-$. Blue channel: λ_{ex} = 405 nm, λ_{em} = 440–480 nm; green channel: λ_{ex} = 458 nm, λ_{em} = 500–580 nm.

weak fluorescence signal. However, after these cells were treated with 10 μM HClO, a significant fluorescence signal enhancement was obtained. The results indicated that HDI-HClO could make a specific response to HClO but not to other active species. Furthermore, to verify whether the signal changed with the change of the HClO level, we added different concentrations of HClO. As expected, the fluorescence signal was significantly enhanced when adding 20 and 40 μM HClO, which provided the possibility for the quantification of intracellular HClO. Therefore, the developed probe HDI-HClO could be used as an effective tool for the monitoring of HClO in living cells.

Since the probe could be capable of effectively monitoring exogenous HClO, it should be able to monitor endogenous HClO. The potential of the probe HDI-HClO for imaging

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endogenous HClO was further assessed. The assay was carried out in RAW 264.7 cells owing to the fact that the cells could produce high levels of HClO after being stimulated by lipopolysaccharide (LPS) as well as phorbol-12-myristate-13-acetate (PMA).[33−35] The RAW 264.7 cell lines were first treated with 10 μM HDI-HClO, and an inert fluorescence signal was found. However, after co-incubating RAW 264.7 cells with PMA/LPS, an apparent fluorescence enhancement signal was obtained, which suggested that the content of HClO was increased. The RAW 264.7 cell line was incubated with a particular inhibitor of myeloperoxidase and 4-aminobenzoic acid hydrazide (ABAH), which could suppress the production of HOCl. As expected, the weaker fluorescence signal was observed, which declared that ABAH inhibited the production of HClO and reduced its concentration. Moreover, the result also exhibited that the obtained fluorescence enhancement signal was caused by HClO and no other substance. To increase the reliability of the experiment, we used another HClO scavenger (NAC) (Figure 4). Similarly, we got a similar

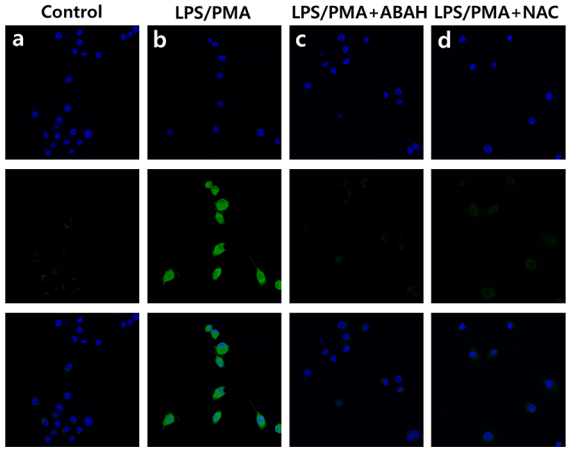

Figure 4. Imaging of endogenous HClO in RAW 264.7 cells. (a) Cells were preincubated with probes and washed with PBS buffer before imaging. The cells were preincubated with probes, washed with PBS buffer (b), and then stimulated with LPS (1 μg/mL)/PMA (1 μg/mL) and ABAH (200 μM) (c) and NAC (1 mM) (d) for 1 h. Blue channel: λ_{ex} = 405 nm, λ_{em} = 440−480 nm; green channel: λ_{ex} = 458 nm, λ_{em} = 500−580 nm.

experimental phenomenon that the intracellular fluorescence signal was significantly inhibited, revealing that the level of HClO was low. All above data evidenced that HDI-HClO could be able to detect fluctuations of endogenous and exogenous HClO.

3. CONCLUSIONS

In summary, we develop a new fluorescent probe HDI-HClO that can be used to track intracellular HClO levels. The probe features excellent specificity, sensitivity, and rapid response toward HClO. It is worth noting that the probe shows a low detection limit and good linearity for HClO, which provides a reliable basis for the quantitative analysis of HClO. Furthermore, the probe shows excellent water solubility and good biocompatibility. In addition, the proposed probe HDI-HClO has been employed to image and detect the content of HClO in living cells, which may be used as a tool to comprehend the relationship between HClO and inflammation, such as arthritis.

4. EXPERIMENTAL SECTION

4.1. Apparatus and Reagents. The absorption and fluorescence spectra were obtained on Hitachi UV-2910 and F-7000 fluorescence spectrophotometers, respectively. ^{1}H NMR and ^{13}C NMR measurements were carried out on a Bruker-500 MHz nuclear magnetic resonance spectrometer at room temperature. The fluorescence images of cells were acquired by an Olympus FV1000.

4.2. Synthesis of Compound 1. Toluene (20 mL), 5 mL of iodoethane, and 3.2 g of 2,3,3-trimethyl-3*H*-indole were added into a 100 mL round-bottom flask and heated to reflux for 18 h; the solvent was removed to obtain a deep blue solid. After heating with methanol in the flask to dissolve the solid, the mixture was transferred to a beaker and ether was added dropwise to precipitate the solid. It was then filtered with suction, and the precipitate was washed with ether to obtain a light blue powdery solid. After drying, the solid did not need to be purified and can be used directly in the next step.

4.3. Synthesis of HDI. Compound **1** (0.66 g) and *p*-hydroxybenzaldehyde (0.25 g) were put into a round-bottom flask. Then, 10 mL of absolute ethanol was added and heated to reflux overnight. When the mixture was cooled, a large amount of purple-red solid appeared. The mixture was filtered with suction and washed with absolute ethanol to obtain HDI.

4.4. Synthesis of HDI-HClO. Dimethylthiocarbamoyl chloride (123 mg, 1 mmol) and HDI (73 mg, 0.25 mmol) were dissolved in anhydrous CH_2Cl_2 (10 mL). Piperidine (43 μL, 0.50 mmol) was slowly dripped into the mixture and stirred vigorously overnight. After removing the solvent, the crude product was purified via silica column chromatography (CH_2Cl_2:CH_3OH = 10:1, v/v) to obtain HDI-HClO (34 mg, 37%). The specific synthetic route and the characterization of the compound are displayed in the Supporting Information. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 8.33−8.28 (m, 3H), 7.77−7.70 (dd, 2H), 7.60−7.58 (d, 3H), 7.24−7.7.22 (d, 2H), 5.00−4.99 (d, 2H), 3.45 (s, 3H), 3.36 (s, 3H), 1.87 (s, 6H), 4.62−1.59 (t, 3H).^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 186.45, 181.51, 158.29, 154.05, 143.58, 140.20, 132.86, 131.34, 130.14, 129.78, 124.17, 122.96, 115.05, 112.55, 69.97, 52.72, 44.55, 43.36, 39.16, 27.01, 25.61, 14.53. HR-MS: m/z $C_{23}H_{27}N_2OS^+$ calcd, 379.1839; found $[M]^+$, 379.1837.

4.5. Cell Culture and Cytotoxicity Experiment. The HeLa and RAW 264.7 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% antibiotics (streptomycin/penicillin, 100 U/mL). The cultures were maintained at 37 °C in a 95% humidified atmosphere with 5% CO_2. The cell cytotoxicity of HDI-HClO was evaluated by a CCK-8 assay. Both cells were put in a 96-well plate (5000 cells per well) for 24 h. Subsequently, phosphate-buffered saline (PBS) was used for cell washing and various concentrations of HDI-HClO were added in the cells and incubated for 24 h. The cells were washed with the DMEM medium; next, 10 μL of CCK-8 was added. After being incubated for about 4 h, the absorbance was measured at 450 nm using a microplate reader (Tecan, Austria).

4.6. Confocal Imaging. The fluorescence image was performed on an Olympus FV1000. Cells were plated in a culture dish and then adhered for 24 h before fluorescence imaging. Fluorescence collection windows were Ch 1: λ_{ex} = 405 nm, 440−480 nm and Ch 2: λ_{ex} = 458 nm, 500−580 nm.

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■ ASSOCIATED CONTENT

Supporting Information

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Structure characterizations of the probe, HPLC, and additional fluorescence spectra and images (PDF)

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Notes

The authors declare no competing financial interest.

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