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A Highly Specific Fluorescent Probe for Hypochlorous Acid and Its Application in Imaging Microbe-Induced HOCl Production

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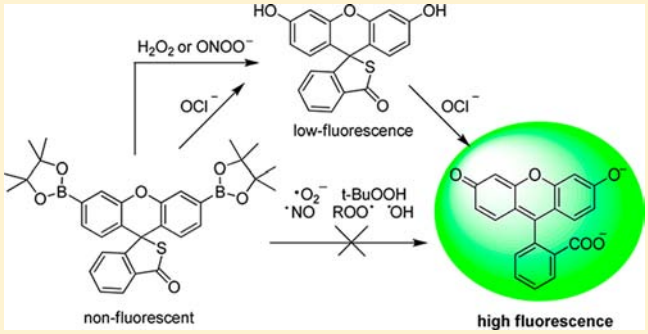
Supporting Information

ABSTRACT: Oxidative stress induced by reactive oxygen species (ROS) plays crucial roles in a wide range of physiological processes and is also implicated in various diseases, including cancer, chronic inflammatory diseases, and neurodegenerative disorders. Among the various ROS, hypochlorous acid (HOCl) plays as a powerful microbicidal agent in the innate immune system. The regulated production of microbicidal HOCl is required for the host to control the invading microbes. However, as a result of the highly reactive and diffusible nature of HOCl, its uncontrolled production may lead to an adverse effect on host physiology. Because of its biological importance, many efforts have been focused on developing selective fluorescent probes to image ROS. However, it is still challenging to design a fluorescent probe with exclusive selectivity toward a particular member of ROS. In the current work, we designed **FBS** as a new fluorescent HOCl probe which has high selectivity, sensitivity, and short response time in a broad range of pH. Compared with other sensors, the "dual-lock" structure of **FBS** has an advantage of eliminating interferences from other ROS/RNS. Importantly, we further showed that our HOCl probe could be applied for the *in vivo* imaging of physiological HOCl production in the mucosa of live animals. This probe provides a promising tool for the study of HOCl production.



INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an essential role in many biological processes such as aging and immunity. However, deregulation of ROS production and/or elimination may cause pathophysiological consequences such as aging and chronic inflammatory diseases of the human.[1] Many efforts have been focused on developing selective fluorescent probes[2] to image ROS including H_2O_2,[3] OCl^-,[4] NO,[5] $ONOO^-$,[6] $^{\bullet}O^{2-}$,[7] $^1O_2$.[8] Among the various ROS, hypochlorous acid (HOCl) plays as a powerful microbicidal agent in the innate immune system. It is generated from H_2O_2 and Cl^- by secreted myeloperoxidase (MPO) *in vivo* in response to inflammatory stimuli.[9] Regulated generation of hypochlorous acid is required for the host to control the invading microbes, while produced HOCl can also react with amino acids, proteins, cholesterol, and nucleosides[10] Uncontrolled production of HOCl derived from phagocytes is involved in some diseases such as cardiovascular disease and inflammatory disease.[11] Synthetic fluorescent probes are among the most powerful tools for the detection of HOCl due to their high sensitivities, simple manipulation, and lack of a requirement for sophisticated instrumentation. Fluorescent probes have advantages of facile visualization of intracellular dynamics and high-resolution localization of biomolecules of interest.[12] The design strategies are based on specific reactions between recognition groups and HOCl that give highly fluorescent products. The reactions include the oxidation reactions of *p*-methoxyphenol to benzoquinone,[4a] dibenzoyl hydrazide to dibenzoyl diimide,[4b] rhodamine-hydroxamic acid to rhodamine 19,[4c] thiol/thio ether to sulfonate derivatives,[4d] the cleavage of 4-aminophenyl,[4e] the release of oxazine fluorophore[4f] by HOCl, and so on.

Among the various ROS, H_2O_2 and HOCl are closely related since MPO converts H_2O_2 to HOCl. Accordingly, the detection of HOCl in the presence of H_2O_2 and other ROS is critical. For H_2O_2 imaging, arylboronates were heavily employed in the design of H_2O_2 sensors for the detection of H_2O_2 generation *in vivo*.[13] On the other hand, it is recently reported that arylboronates can react with peroxynitrite ($ONOO^-$) and OCl^- to yield hydroxyl derivatives much faster than does H_2O_2 using a stopped-flow kinetic technique and HPLC analysis.[14] A few boronate-based fluorogenic probes were developed to monitor $ONOO^-$ formation from $^{\bullet}O^{2-}$ and $^{\bullet}$NO.[15] In this work, "dual-lock" **FBS** is reported as a selective fluorescent probe for HOCl. We synthesized **FBS** and **FS** (Scheme 1) and studied their fluorescence responses to various ROS/RNS. H_2O_2 and $ONOO^-$ can react with arylboronates of **FBS** to give

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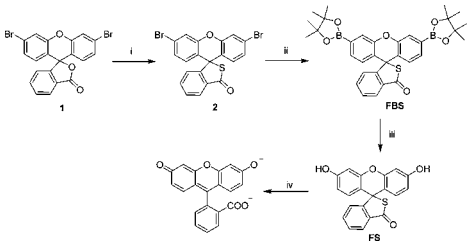
Scheme 1. Chemical Structure and Synthesis of FBS and FS[a]



[a]Reagents and conditions: (i) Lawesson's reagent, toluene, 90 °C. (ii) bis(pinacolato)diborane, potassium acetate, 1,4-dioxane, Pd(dppf)-Cl_2·CH_2Cl_2, 85 °C. (iii) NaOCl/CH_3CN/H_2O or H_2O_2/THF/CH_3OH. (iv) NaOCl/CH_3CN/H_2O.

only **FS**, which is still nonfluorescent. Only OCl^- can react with arylboronates and then hydrolyze thiolactone, which can induce large green fluorescence enhancement. Therefore, a selective "off–on" fluorescence enhancement was observed only in the presence of HOCl. Our results clearly demonstrate that not only H_2O_2 but also $ONOO^-$ and OCl^- can react with arylboronate. The high sensitivity and its *in vivo* compatibility permit imaging of microbe-induced HOCl production *in vivo* in the mucosa of *Drosophila*. At present, most of the reported ROS/RNS sensors rely on one kind of recognition group that can react with the target. The "dual-lock" structure of **FBS** has an advantage of eliminating interference from other ROS/RNS.

EXPERIMENTAL SECTION

Materials and Methods. Unless otherwise noted, materials were obtained from Aldrich and were used without further purification. ^1H NMR and ^{13}C NMR in $CDCl_3$ were measured on a Bruker AM-300 spectrometer with tetramethylsilane (TMS) as internal standard. Mass spectra were obtained using a JMS-HX 110A/110A tandem mass spectrometer (JEOL). UV–vis spectra were obtained using a Scinco 3000 spectrophotometer (1 cm quartz cell) at 25 °C. Fluorescence spectra were recorded on RF-5301/PC (Shimada) fluorescence spectrophotometer (1 cm quartz cell) at 25 °C. Deionized water was used to prepare all aqueous solutions.

Synthesis of Compound 2. 3′,6′-Dibromofluoran (1.0 g, 2.19 mmol), Lawesson's reagent (0.89 g, 2.19 mmol), and toluene (45 mL) were mixed in a 100-mL flask and heated to 90 °C. After stirring for 2 days, the mixture was cooled down, and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using CH_2Cl_2/hexane (1/4, v:v) as the eluent to get **2** as a white solid (0.11 g, 10.6%). ^1H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.91 (d, 1H), 7.58(m, 2H), 7.36 (d, 2H), 7.10–7.16 (m, 3H), 7.77 (d, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 195.48, 156.15, 150.75, 135.00, 130.52, 129.25, 127.70, 127.27, 123.47, 122.75, 121.12, 120.07, 59.46. IR spectrum: 1681 cm^{-1} (C═O). FAB-MS: m/z = 472.8849 $[M + H]^+$, calc for $C_{20}H_{12}Br_2O_2S$ = 472.8847.

Synthesis of FBS. **2** (0.15 g, 0.32 mmol), bis(pinacolato)diborane (0.185g, 0.73 mmol), potassium acetate (0.217g, 2.21 mmol), 1,4-dioxane (10 mL), and Pd(dppf)Cl_2·CH_2Cl_2 (10 mg) were mixed in 25-mL flask. After N_2 degassing, the mixture was stirred under 85 °C for 36 h. Then the solvent was removed under reduced pressure, CH_2Cl_2 was added, the mixture was washed with water three times and dried over anhydrous $MgSO_4$. After the solvent was removed, the residue was purified by silica gel column chromatography using CH_2Cl_2 as the eluent to obtain crude product. It was recrystallized from acetonitrile and dried under vacuum to give **FBS** as a white solid

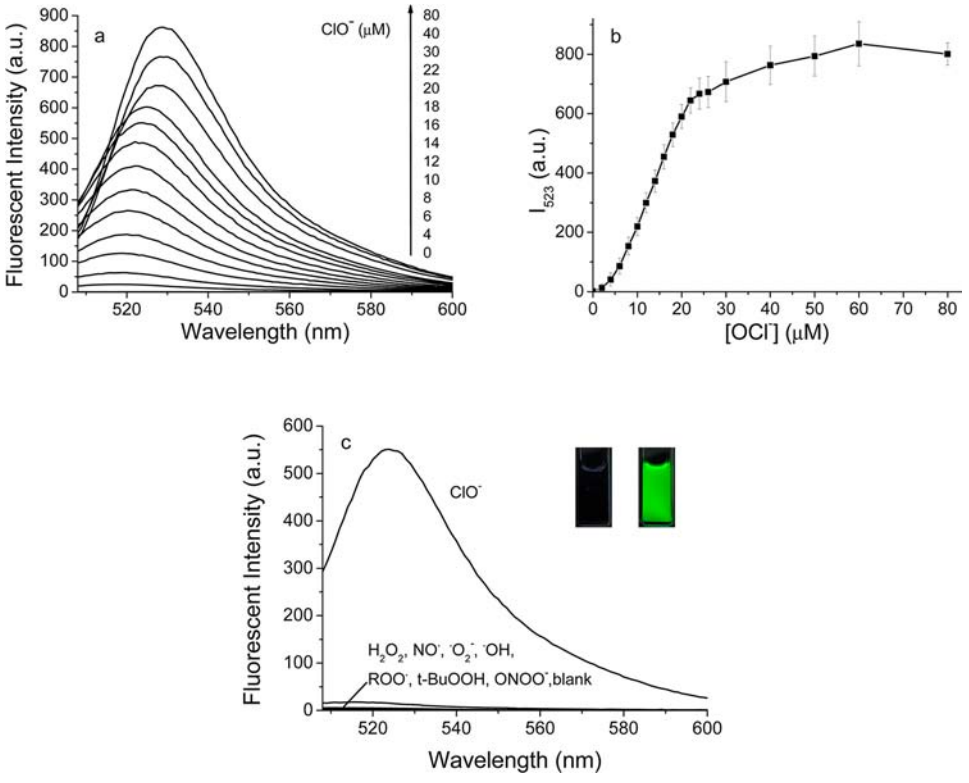


Figure 1. (a) Fluorescence spectra changes of **FBS** with titration of OCl^-. (b) Fluorescence intensity at 523 nm as a function of added OCl^-. (c) Fluorescence spectra of **FBS** before and after addition of various ROS: OCl^- (20 μM), ROO$^\bullet$ (1 mM), H_2O_2 (100 μM), $^\bullet O^{2-}$ (25 μM), $^\bullet$OH (100 μM). *tert*-butyl hyperoxide (100 μM), $ONOO^-$ (22 μM). Insets show the photos of **FBS** solution without (dark) and with (green) added OCl^- (20 μM). [**FBS**] = 2 μM, in KH_2PO_4 buffer (50 mM, pH 7.4), excitation wavelength 498 nm (slit widths: 3 nm/3 nm).

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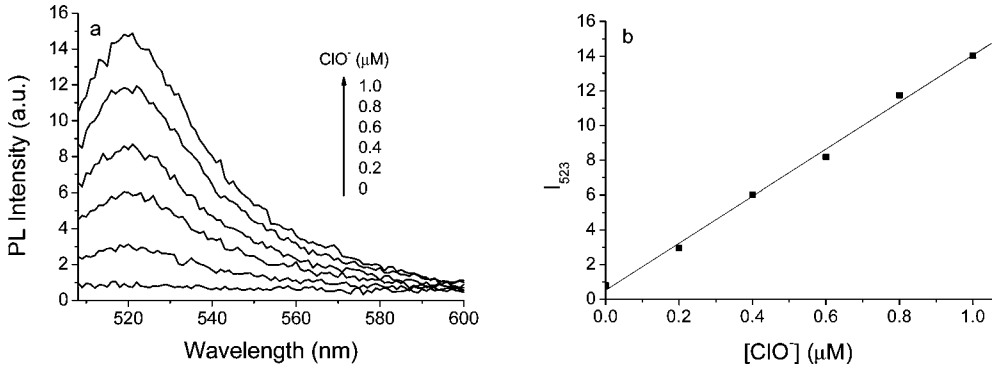

Figure 2. (a) Fluorescence spectra changes of **FBS** (2 μM) as the titration OCl^- from 0 to 1.0 μM in KH_2PO_4 buffer (50 mM, pH 7.4). (b) Fluorescence intensity at 523 nm as a function of added ClO^-. Excitation wavelength: 498 nm (slit widths: 3 nm/3 nm).

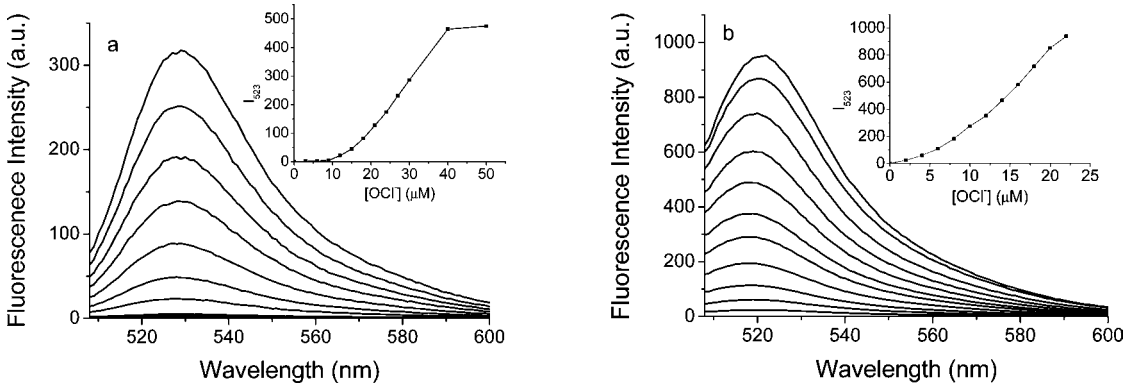

Figure 3. (a) Fluorescence spectra changes of **FBS** as the titration OCl^- (0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 μM) under pH 5.5 (KH_2PO_4 buffer, 50 mM). (b) Fluorescence spectra changes of **FBS** as the titration OCl^- (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 μM) under pH 9.3 (K_2HPO_4 buffer, 50 mM). Insets show fluorescent intensity at 523 nm as a function of added OCl^-. [FBS] = 2 μM. Excitation wavelength: 498 nm, slit width: 3 nm/3 nm.

(0.072 g, 40%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.91 (m, 1H), 7.61 (d, 2H), 7.54 (m, 2H), 7.37 (dd, 2H), 7.12 (m, 1H), 6.90 (d, 2H), 1.34 (s, 24H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 196.41, 156.95, 150.37, 135.13, 134.74, 129.64, 128.86, 128.44, 127.43, 124.58, 123.37, 123.23, 84.14, 60.49, 24.85. FAB-MS: m/z = 568.2260, $[M]^+$, calc for $C_{32}H_{34}B_2O_6S$ = 568.2262; m/z = 569.2343, $[M + H]^+$, calc for $C_{32}H_{35}B_2O_6S$ = 569.2340.

Synthesis of FS. H_2O_2 (0.5 mL, 28% in water) was added to **FBS** (0.128 g, 0.225 mmol) solution in THF (4 mL) and CH_3OH (2 mL), the mixture was stirred overnight. After reaction, solvents were removed under reduced pressure, and the residue was purified by silica gel column chromatography using CH_2Cl_2/CH_3OH (90/1, v:v) as the eluent to get crude product as a white solid (0.045g, 57.4%). ^{1}H NMR (CD_3OD, 300 MHz) δ (ppm): 7.85 (m, 1H), 7.62 (m, 2H), 7.17 (m, 1H), 6.67 (d, 2H), 6.55 (d, 2H), 6.46 (q, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 198.26, 159.97, 158.87, 153.18, 136.60, 135.95, 130.97, 129.90, 128.50, 123.63, 114.14, 113.53, 103.38, 62.86. FAB-MS: m/z = 348.0451 $[M]^+$, calc for $C_{20}H_{12}O_4S$ = 348.0456; m/z = 349.0536 $[M + H]^+$, calc for $C_{20}H_{13}O_4S$ = 349.0535.

Reaction of FBS and NaClO To Give FS. NaClO (3 mM in water, 30 mL) was added to **FBS** (10 mg, 0.0176 mmol) solution in CH_3CN (17 mL) slowly under stirring. After the solvents were removed under reduced pressure, the residue was purified on silica gel column chromatography using CH_2Cl_2/CH_3OH (90/1, v:v) as the eluent to get the product **FS** (3.0 mg, 48.9%).

The Reaction of FS and NaClO. NaClO solution (30 mL, 1 mM) was added to the solution of FS (10 mg, 0.029 mmol) in CH_3CN (28 mL) slowly and stirred for 30 min. Then the solvents were removed under reduced pressure, and the residue was purified on silica gel column chromatography using CH_2Cl_2/CH_3OH (5/1, v:v) as the eluent to get the product (0.32 mg). ^{1}H NMR (CD_3OD, with NaOH, 300 MHz) δ: 8.01 (m, 1H), 7.56 (m, 2H), 7.21 (m, 1H), 7.06 (d, 2H), 6.54–6.49 (m, 4H).

RESULTS AND DISCUSSION

Design and Preparation of FBS. In a cellular context, a variety of ROS (such as HOCl, H_2O_2 and $ONOO^-$) were simultaneously generated in response to physiological stimuli. Therefore, the *in vivo* applications of arylboronates-based sensors are limited due to lack of specificity toward a single species of ROS. To develop a specific HOCl sensor without noticeable activity toward other ROS including H_2O_2 and $ONOO^-$, we designed and synthesized a nonfluorescent xanthenone derivative which combined with boronic esters and thiolactone (**FBS**, Scheme 1). The reaction between 3′,6′-dibromofluoran[16] and Lawesson's reagent yielded thiolactone compound **2**. The following Miyaura borylation reaction with bis(pinacolato)diborane gave **FBS**. Although boronic esters can react with the three ROS, the product is expected to be the low-fluorescent **FS**. We expected that thiolactone of **FS** could be oxidized by HOCl and give the high fluorescent product fluorescein. The "dual-lock" structure of **FBS** is expected to exclude interferences from almost all other molecules. The detailed experimental procedures are explained in the Experimental Section and ^{1}H and ^{13}C NMR spectra are reported in the Supporting Information (SI).

Selectivity and Sensitivity of FBS for HOCl. To examine the sensitivity and selectivity of **FBS** to HOCl, we measured the fluorescence spectra of **FBS** with HOCl and other ROS/RNS.

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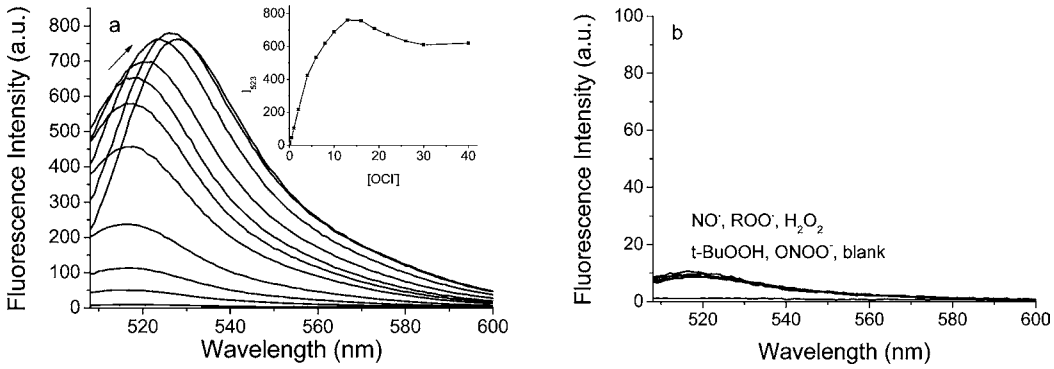

Figure 4. (a) Fluorescence spectra changes of **FS** as the titration of OCl^-: 0, 0.5, 1, 2, 4, 5, 6, 8, 10, 16, and 19 μM. (b) Fluorescence spectra of **FBS** before and after addition of OCl^- (19 μM) and other ROS for 30 min, $NO^\bullet$ (1 mM), $ROO^\bullet$ (1 mM), H_2O_2 (200 μM), *tert*-butyl hyperoxide (200 μM) and $ONOO^-$ (22 μM). [**FS**] = 2 μM, in KH_2PO_4 buffer (50 mM, pH 7.4), excitation wavelength: 498 nm (slits width: 3 nm/3 nm).

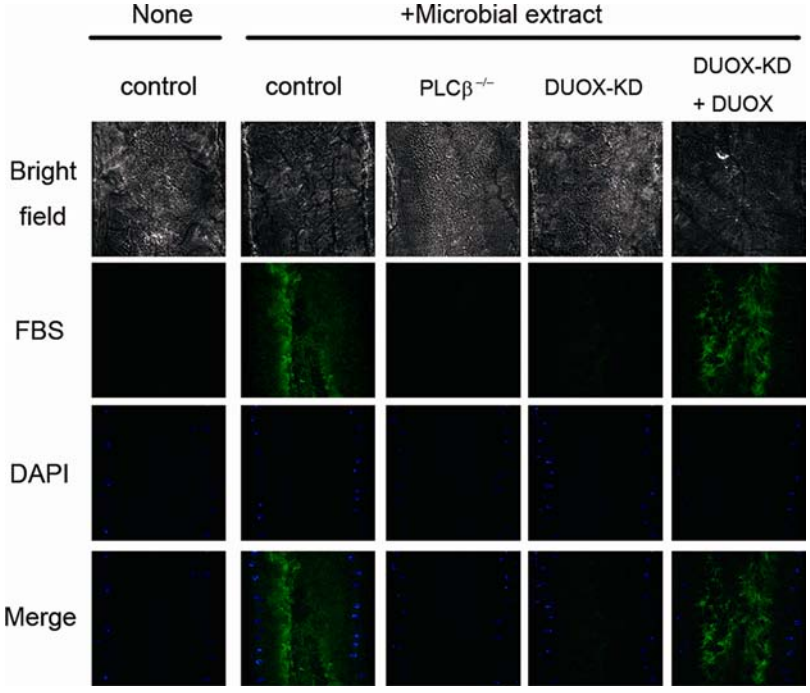

Figure 5. Detection of DUOX-dependent HOCl induction in the intestinal epithelia of *Drosophila*. Nuclear staining of midgut cells was performed with DAPI (blue). Representative confocal microscopic images of dissected guts from different genotypes in the presence or absence of oral ingestion of bacterial extract. The genotypes of the flies used in this study were as follows: Cont (*Da-GAL4/+*); PLC$\beta^{-/-}$ (*norpA*[7]); DUOX-knockdown (KD) (*UAS-DUOX-RNAi/+; Da-GAL4/+*); DUOX-KD + DUOX (*UAS-DUOX-RNAi/UAS-DUOX; Da-GAL4/+*).

As shown in Figure 1a, **FBS** was not fluorescent at all. When HOCl was titrated from 0 to 80 μM, the fluorescence emission increased remarkably and quickly. This can be attributed to the production of fluorescein by the reaction between **FBS** and HOCl (Scheme 1), which was confirmed by NMR and FAB-Mass. The fluorescence emissions as well as UV−vis absorptions (SI Figure S1) are red-shifted during the titration. This may be attributed to the chlorination of fluorescein.[17] As the concentration of HOCl increased to 100 μM, a small fluorescence decrease was observed. (SI Figure S2). The fluorescence intensity at 523 nm as a function of HOCl concentration was recorded, and a nearly linear relationship in the range of 0−20 μM was obtained (Figure 1b). Strong green fluorescence can be observed by the naked eye (Figure 1c). In contrast, other ROS (H_2O_2, $NO^\bullet$, $^\bullet O_2^-$, $^\bullet OH$, $ROO^\bullet$, t-BuOOH) of higher concentration could not induce measurable fluorescence changes even after incubation of 30 min (Figure 1c). $ONOO^-$ of less than 22 μM could induce a very limited increase in fluorescence, but then the fluorescence decreased at higher concentrations (SI, Figure S3). To find out the minimum concentration of HOCl that can induce fluorescence, titration of HOCl from 0 to 1.0 μM was carried out (Figure 2). HOCl concentration as low as 0.2 μM can be detected. In short, **FBS** is a good HOCl sensor with high sensitivity, selectivity, and short response time.

The Effect of pH. We also studied the performances of **FBS** in acidic or basic solutions with added HOCl. As shown in Figure 3, strong fluorescence enhancement was observed during titration of OCl^- to **FBS** solution of pH 5.5 and pH 9.3. These results clearly explain that this probe can be used in a broad range of pH.

Fluorescent Response of FS to ROS/RNS. The reactions of **FBS** with H_2O_2 and HOCl were carried out respectively, and the main product was found to be **FS**. The fluorescent

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performance of **FS** and its response to ROS/RNS are critical for the success of our strategy. As shown in Figure 4, **FS** has very weak fluorescence itself. After the titration of OCl^- from 0 to 19 μM, strong fluorescence enhancement was observed. We then incubated **FS** with other ROS/RNS: $NO^\bullet$ (1 mM), $ROO^\bullet$ (1 mM), H_2O_2 (200 μM), *tert*-butyl hyperoxide (200 μM), and $ONOO^-$ (22 μM) but failed to induce obvious fluorescence changes. Although **FS** was also found to have high selectivity for HOCl, **FS** showed background fluorescence, relatively stronger than that of **FBS**, and low stability after long-time storage in solution which is a drawback. Interestingly, we found that added OCl^- failed to react with **FBS** in HEPES buffer (20 mM, pH 7.4), as no fluorescence enhancement can be observed. It is probably due to the fact that OCl^- reacted with HEPES. Therefore, the use of HEPES buffer is suggested to be avoided for ROS detection.

***In Vivo* Imaging of Physiological HOCl Production Using FBS.** To test whether **FBS** can be used as a specific fluorescent sensor for the detection of physiological HOCl production *in vivo*, we applied **FBS** in the *Drosophila* gut system, a well-known HOCl producing organ.[17] In *Drosophila*, gut epithelia produce HOCl as a microbicidal agent via DUOX, a member of the NADPH oxidase family, in response to bacterial challenge.[18] To initiate physiological HOCl production, the flies were subjected to oral ingestion of bacterial extracts. **FBS** was subsequently introduced to the gut by oral ingestion to image bacterial-induced HOCl production *in situ*. As shown in Figure 5, the gut of the wide-type *Drosophila* shows green fluorescence following treatment of bacterial extracts. In contrast, there is no detectable fluorescence without the treatment. Recently it was found that phospholipase C-β (PLCβ) signaling is required for DUOX activity to produce microbicidal ROS.[19] As expected, no bacterial-induced fluorescence can be observed in the absence of PLCβ signaling pathway (in the gut of PLCβ mutant flies) as well as in the knockdown (KD) of DUOX expression (in the gut of DUOX-KD flies). Furthermore, normal level of bacteria-induced HOCl production was restored when DUOX-KD flies were rescued by overexpressing *Drosophila* DUOX. Taken together, these data indicated that **FBS** can detect PLCβ-DUOX-dependent HOCl production *in vivo* in response to a physiological signaling such as bacterial challenge.

■ CONCLUSIONS

In summary, we have developed a novel "dual-lock" fluorescent HOCl probe, **FBS**, bearing boronic esters and thiolactone. Its reaction with HOCl produces fluorescein as a product, which shows strong green fluorescence. **FBS** shows high selectivity for HOCl over H_2O_2, $ONOO^-$, and other ROS/RNS. H_2O_2 and $ONOO^-$ can convert **FBS** only to **FS**, which is still nonfluorescent, on the other hand, only HOCl can convert **FBS** to fluorescein. **FBS** can be used in neutral, acidic, and basic solutions. As a proof-of-principle, bacteria-induced HOCl generation was successfully visualized by **FBS** in the mucosa of live animals. We believe this "dual-lock" probe provides a promising tool for *in vivo* HOCl imaging.

■ ASSOCIATED CONTENT

Supporting Information

Generation of ROS/RNS, intestinal HOCl detection in live animals, absorbance spectra changes of **FBS** with added HOCl, fluorescence spectra changes of **FBS** as time with added OCl^- of 100 μM, fluorescence changes of **FBS** with added $ONOO^-$, infrared spectrum of compound **2**, ^{1}H NMR and ^{13}C NMR spectra of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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