Supporting information

Fluorescence turn-on detection of hypochlorous acid via HOCl-promoted dihydrofluorescein-ester oxidation and its application in vivo

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Reagents and Apparatus

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents were purified and dried using standard procedures. Electrospray ionization mass spectra (ESI-MS) were measured on a Micromass LCTTM system. $^1$H-NMR and $^{13}$C-NMR were measured on a BrukerAV-500 or BrukerAV-300 spectrometer with chemical shifts reported in ppm (in CDCl$_3$ or DMSO-d$_6$; TMS as internal standard). UV-visible spectrums were recorded on a Perkin-Elmer 35 spectrometer. Fluorescence measurements were performed at room temperature on a Perkin-Elmer LS 50B fluorescence spectrophotometer. TLC analysis was performed on silica gel plates. Column chromatography was conducted over silica gel (mesh 200–300), and both were obtained from the Qingdao Ocean Chemicals.

Preparation of ROS and RNS

FCN1 ~ FCN3

A stock solution of FCN1 ~ FCN3 (1 mM) was prepared in DMSO and was stored at -20 °C for spectrum and *vivo* investigation.

HOCl

HOCl was prepared from the source of NaOCl at room temperature in HEPES buffer (pH 7.2). The concentration of HOCl was determined by titration with Na$_2$S$_2$O$_3$.

NO$_2^-$

NO$_2^-$ was prepared from the source of NaNO$_2$ at room temperature in HEPES buffer (pH 7.2).

ONOO$^-$

The synthesis of peroxynitrite involved nitrosation of H$_2$O$_2$ at pH $\geq$12.0 by isoamyl nitrite. The peroxynitrite concentration was determined by using an extinction coefficient of 1670 ± 50 cm$^{-1}$(mol/L)$^{-1}$ at 302 nm.$^1$

OH$^•$

Hydroxyl radicals was generated by the addition of Fe$^{2+}$ (100 mM) and H$_2$O$_2$ (100 mM) at room temperature in HEPES buffer (pH 7.2) and the mixture was then stirred for 30 min.

O$_2^-$

Superoxide was prepared from the source of KO$_2$ at room temperature in HEPES buffer (pH 7.2).
NO
Nitric oxide was prepared from a saturated NO aqueous solution (2 mM) at room temperature.

HNO
Nitroxyl donor (HNO) was generated from sodium trioxodinitrate (Na$_2$N$_2$O$_3$, Angeli’s salt). Angeli’s salt was prepared as described by King and Nagasawa and was stored at -20 °C until needed.$^2$

ROO•
ROO• was generated from 2,2’-azobis(2-amidinopropane)dihydrochloride (CAS: 2997-92-4), which was dissolved in deionized water first and then added into probe testing solutions at room temperature in HEPES buffer (pH 7.2) for 30 min.

Synthetic details
Our initial attempt to convert fluorescein as the starting material into dihydrofluorescein by sodium borohydride failed, presumably its carboxyl group partially forms spirocyclic ring to resist the reduction (Fig. S1, ESI†). However, deprotonation of fluorescein afforded fluorescein-ester, which was further installed by the NaBH$_4$-mediated reduction to give FCN1 and FCN2 successfully.

2-(3’-Hydroxy-6’-ethoxy-9’H-xanthen-9’-yl)-benzoic acid ethyl ester (FCN2)

![Chemical structure](image)

2-(6’-ethoxy-3’-oxo-3’H-xantene-9’-yl)-benzoic acid ethyl ester (FCN-2Et): EtI (4.68 g, 30.0 mmol) was added to the mixture of fluorescein (3.32 g, 10.0 mmol) and K$_2$CO$_3$ (4.14 g, 30.0 mmol) in 50 ml of CH$_3$CN. After heating at 60 °C for 24 h, the reaction mixture was concentrated under reduced pressure and diluted with saturated NaHCO$_3$ (40 ml) solution. The resulting mixture was extracted three times with CH$_2$Cl$_2$ (40 ml). The combined organic layer was dried over anhydrous Na$_2$SO$_4$, and concentrated under vacuum. Compound FCN-2Et was isolated using a silica gel chromatographic column eluted with Dichloromethane/MeOH (v/v, 95:5), resulting a light yellow solid (R$_f$ = 0.38, 2.91 g, yield: 75%).$^1$H-NMR (500 MHz, DMSO-$d_6$): δ = 8.20 (dd, J= 7.9 Hz, J= 1.1 Hz, 1H, Ar-H), 7.86 (t,
J = 7.6 Hz, 1H, Ar-H), 7.79 (t, J = 7.8 Hz, 1H, Ar-H), 7.50 (dd, J = 7.6 Hz, J = 1.0 Hz, 1H, Ar-H), 7.21 (d, J = 2.4 Hz, 1H, Ar-H), 6.89 (dd, J = 8.9 Hz, J = 2.4 Hz, 1H, Ar-H), 6.85 (d, J = 8.9 Hz, 1H, Ar-H), 6.81 (d, J = 9.7 Hz, 1H, Ar-H), 6.39 (dd, J = 9.7 Hz, J = 2.0 Hz, 1H, Ar-H), 6.24 (d, J = 2.0 Hz, 1H, Ar-H), 4.18 (q, J = 7.0 Hz, 2H, -CH2-), 3.96 (m, 2H, -CH2-), 1.36 (t, J = 7.0 Hz, 3H, -CH3), 0.86 (t, J = 7.1 Hz, 3H, -CH3).

13C-NMR (125 MHz, CDCl3): 183.76, 164.90, 163.12, 158.27, 153.53, 149.83, 133.42, 132.93, 130.62, 130.50, 130.29, 129.99, 129.91, 129.30, 128.86, 116.67, 114.21, 113.76, 104.52, 100.81, 64.38, 60.81, 14.28, 13.27. Anal. Calcd for C24H20O5: C, 74.21; H, 5.19. Found: C, 74.01; H, 5.06. ESI-MS: m/z 389.1 [M+H]+.

**FCN2:** To a solution of **FCN-2Et** (776 mg, 2.0 mmol) in MeOH (30 mL), NaBH4 (152 mg, 4.0 mmol) was added at 0 °C. The resulting mixture was stirred at 0 °C for 10 min, and slowly brought to room temperature for another 2 hour. After removal of the solvent, the residue was diluted with water. The mixture was then extracted three times with CH2Cl2 (40 ml), dried over Na2SO4, and concentrated in vacuo to yield the crude product. Compound **FCN-2** was purified using a silica gel chromatographic column eluted with Dichloromethane/petroleum ether (v/v, 7:3), resulting a light yellow solid (Rf = 0.52, 710 mg, yield: 91%). 1H-NMR (500 MHz, DMSO-d6): δ = 7.69 (t, J = 7.6 Hz, 1H, Ar-H), 7.39 (t, J = 6.6 Hz, 1H, Ar-H), 7.24 (t, J = 7.6 Hz, 1H, Ar-H), 6.97 (t, J = 7.6 Hz, 1H, Ar-H), 6.89 (dd, J = 8.6 Hz, J = 2.4 Hz, 1H, Ar-H), 6.81 (dd, J = 8.5 Hz, J = 2.6 Hz, 1H, Ar-H), 6.68 (d, J = 2.4 Hz, 1H, Ar-H), 6.56 (dd, J = 8.6 Hz, J = 2.3 Hz, 1H, Ar-H), 6.53 (d, J = 2.3 Hz, 1H, Ar-H), 6.44 (dd, J = 8.4 Hz, J = 2.1 Hz, 1H, Ar-H), 6.00 (s, 1H, Ar3-CH), 4.38 (q, J = 7.1 Hz, 2H, -CH2-), 4.00 (q, J = 7.0 Hz, 2H, -CH2-), 1.34 (t, J = 7.1 Hz, 3H, -CH3), 1.30 (t, J = 7.0 Hz, 3H, -CH3). 13C-NMR (125 MHz, CDCl3): 168.23, 167.81, 158.18, 157.10, 150.88, 147.87, 132.32, 132.19, 130.91, 130.19, 129.43, 129.07, 126.13, 116.35, 114.77, 111.45, 110.69, 102.41, 101.44, 63.22, 61.16, 37.04, 14.55, 14.06. Anal. Calcd for C24H22O5: C, 73.83; H, 5.68. Found: C, 73.69; H, 5.61. ESI-MS: (positive ion mode) m/z 413.1 [M+Na]+; (negative ion mode) m/z 389.1 [M-H].
2-(3'-Hydroxy-6'-methoxy-9'H-xanthen-9'-yl)-benzoic acid methyl ester (FCN1)

\[
\text{HO} \quad \text{COOH} \quad \text{MeI, K}_2\text{CO}_3 \quad \text{MeO} \quad \text{COOMe} \quad \text{NaBH}_4 \quad \text{EtOH} \quad \text{MeO} \quad \text{COOMe}
\]

2-(6'-Methoxy-3'-oxo-3'H-xanthene-9'-yl)-benzoic acid methyl ester (FCN-2Me): This compound was synthesized from fluorescein 1 using a synthetic procedure for FCN-2Et (82%, yellow solid). 

\[\text{H-NMR (300 MHz, DMSO-d}_6\text{):} 8.18 (\text{dd, J= 7.8 Hz, J= 1.1 Hz, 1H, Ar-H}), 7.73-7.81 (\text{m, 2H, Ar-H}), 7.49 (\text{dd, J= 7.9 Hz, J= 1.2 Hz, 1H, Ar-H}), 7.20 (\text{d, J= 2.5 Hz, 1H, Ar-H}), 6.80-6.89 (\text{m, 3H, Ar-H}), 6.39 (\text{dd, J= 9.5 Hz, J= 2.2 Hz, 1H, Ar-H}), 6.21 (\text{d, J= 2.3 Hz, 1H, Ar-H}), 3.87 (\text{s, 3H, -CH}_3), 3.79 (\text{s, 3H, -CH}_3). \text{ESI-MS: m/z 361.1 [M+H]}^+.

FCN1: FCN1 was synthesized from FCN-2Me using a synthetic procedure for FCN2 (88%, yellow solid). 

\[\text{H-NMR (500 MHz, CDCl}_3\text{):} 7.76 (\text{d, J= 7.6 Hz, 1H, Ar-H}), 7.31 (\text{td, J= 7.5 Hz, J= 1.4 Hz, 1H, Ar-H}), 7.21 (\text{t, J= 7.6 Hz, 1H, Ar-H}), 7.06 (\text{dd, J= 7.5 Hz, J= 1.5 Hz, 1H, Ar-H}), 6.90 (\text{d, J= 8.3 Hz, 1H, Ar-H}), 6.85 (\text{d, J= 8.3 Hz, 1H, Ar-H}), 6.60 (\text{d, J= 2.5 Hz, 1H, Ar-H}), 6.57 (\text{d, J= 2.5 Hz, 1H, Ar-H}), 6.48 (\text{dd, J= 8.5 Hz, J= 2.4 Hz, 1H, Ar-H}), 6.40 (\text{dd, J= 8.5 Hz, J= 2.4 Hz, 1H, Ar-H}), 6.10 (\text{s, 1H, Ar}_3\text{-CH}), 3.91 (\text{s, 3H, -CH}_3), 3.74 (\text{s, 3H, -CH}_3). \text{C-NMR (125 MHz, CDCl}_3\text{):} 169.23, 159.21, 155.46, 151.57, 151.41, 148.36, 132.57, 131.90, 130.95, 130.18, 129.62, 129.41, 126.28, 116.97, 116.90, 111.15, 110.21, 103.16, 101.17, 55.51, 52.47, 37.86. \text{Anal. Caled for C}_{22}\text{H}_{18}\text{O}_5\text{: C, 72.92; H, 5.01. Found: C, 72.70; H, 4.96. ESI-MS: (positive ion mode) m/z 385.1 [M+Na]}^+; (negative ion mode) m/z 361.1 [M-H]^-.}

2-(3'-Hydroxy-6'-ethoxy-9'H-xanthen-9'-yl)-phenylethyl alcohol (FCN3)

\[
\text{EtO} \quad \text{COOEt} \quad \text{LiAlH}_4 \quad \text{THF} \quad \text{EtO} \quad \text{OH}
\]
FCN3: To a solution of FCN-2Et (388 mg, 1.0 mmol) in anhydrous THF (20 ml, was prepared by refluxing the commercial THF over LiAlH₄ under an argon atmosphere for 24 hours followed by distillation), LiAlH₄ was added slowly in small portions at 0 °C until the starting materials were consumed (over 30 min). The suspended mixture was stirred at 0 °C with another 2 h and neutralized with 5 M HCl (40 ml) solution. After evaporation of the solvent, the residue was extracted three times with CH₂Cl₂ (20 ml). The combined organic layer was dried over anhydrous Na₂SO₄, and concentrated under vacuum. Compound FCN-3 was purified using a silica gel chromatographic column eluted with Dichloromethane/petroleum ether (v/v, 7:3), resulting a yellow viscous solid (Rₐ = 0.31, 219 mg, yield: 63%). ¹H-NMR (300 MHz, CDCl₃): δ = 7.37 (t, J = 4.9 Hz, 1H, Ar-H), 7.14~7.24 (m, 3H, Ar-H), 6.73 (d, J = 8.6 Hz, 1H, Ar-H), 6.67 (d, J = 8.4 Hz, 1H, Ar-H), 6.59 (d, J = 2.6 Hz, 1H, Ar-H), 6.51 (d, J = 2.4 Hz, 1H, Ar-H), 6.45 (dd, J = 8.6 Hz, J = 2.6 Hz,1H, Ar-H), 6.32 (dd, J = 8.4 Hz, J = 2.6 Hz,1H, Ar-H), 5.41 (s, 1H, Ar₃-CH), 4.59 (s, 2H, -CH₂OH), 3.97 (q, J = 7.0 Hz, 2H, -CH₂-), 1.37 (t, J = 7.0 Hz, 3H, -CH₃). ¹H-NMR (75 MHz, CDCl₃): 158.56, 155.89, 151.13, 151.08, 144.37, 137.81, 131.26, 130.21, 130.01, 129.26, 128.34, 127.03, 116.23, 115.96, 111.01, 110.59, 103.05, 101.67, 67.93, 63.60, 39.69, 14.72. Anal. Calcd for C₂₂H₂₀O₄: C, 75.84; H, 5.79. Found: C, 75.59; H, 5.68. ESI-MS: (positive ion mode) m/z 471.1 [M+Na]⁺; (negative ion mode) m/z 347.1 [M-H]⁻.

Detection of HOCl-promoted FCN2 oxidation (HPLC-MS positive ion mode)
The probe FCN2 (39.1 mg, 0.1 mmol) was dissolved in 50% CH₃CN/H₂O (10 mL) and then diluted to 10 μM with HEPES buffer at pH 7.2. Then 20 equiv of HClO was injected into the probe. The reaction mixture was stirred at room temperature for 40 min and conversion was checked by analytical HPLC. (4.6 mm x 150 mm 5 μm C18 column; 5 μL injection; 50% CH₃CN/H₂O, linear gradient, with constant 0.1% v/v TFA additive; 20 min run; 1 mL/min flow; ESI; positive ion mode; UV detection at 254 nm); ESI-MS: m/z 361.0 [M+H]⁺.
Supplementary spectra data and tables

**Fluorescence Analysis:** Fluorescence emission spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. The probe FCN2 (DMSO, 1.0 mM) was diluted to 1.5 μM in 20 mM HEPES buffer, and was further added to a 10.0-mL color comparison tube for test. The mixture was equilibrated for 30 min before measurement. The fluorescence intensity was measured simultaneously at λ<sub>ex</sub>/λ<sub>em</sub> = 415/485 nm, respectively. Φ<sub>F</sub> was determined with rhodamine B as a standard (Φ<sub>F</sub> = 0.69 in ethanol).³

**Figure S1:** Time course of fluorescein (5.0 μM) and FCN-2Et (5.0 μM) was measured by a spectrofluorometer in EtOH. FCN-2Et was reduced by 1.5 equiv NaBH₄ for 70 min (blue line), then further treated with 5 equiv of HClO for another 50 min (red line).
**Figure S2:** The fluorescence intensity of FCN1~FCN2 showed 6.5-fold and 2.4-fold enhancement upon the exposure to 20 mM HEPES buffer (pH 7.2) for 24 h in air, indicating its auto-oxidation characteristic. However, FCN3 did not show any auto-oxidation for more than 48 h.

**Table S1.** Fluorescence intensity (F.I.) of FCN2 to biologically relevant ROS and RNS in 20 mM HEPES buffer.

<table>
<thead>
<tr>
<th></th>
<th>HOCl</th>
<th>H₂O₂</th>
<th>NO₂⁻</th>
<th>ONOO⁻</th>
<th>OH⁻</th>
<th>O₂•⁻</th>
<th>NO</th>
<th>HNO</th>
<th>ROO•</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.I.</td>
<td>944.95</td>
<td>0.65</td>
<td>0.47</td>
<td>26.53</td>
<td>6.45</td>
<td>0.56</td>
<td>0.59</td>
<td>2.78</td>
<td>0.94</td>
</tr>
</tbody>
</table>

**Detection limit:**

The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of probe FCN2 in the absence of HClO was measured. The value of [DL] was estimated on the basis of the signal-to-noise ratio: For FCN2 with HClO: [DL]= 6.68 nM (0.71 ppb).
Figure S3: (a) Fluorescence titration of FCN2 (0.5 μM) upon addition of HClO (by 25 nM ~ 150 nM) in 20 mM HEPES buffer (pH 7.2) with excitation at 415 nm. (b) The fluorescence intensities at 485 nm.

Kinetic Studies:

The kinetic Studies of probe FCN1~FCN3 (1 μM) with HOCl were determined in HEPES buffer (20 mM, 0.1M KNO3, pH7.2) at room temperature. The pseudo-first-order rate constant value was fitted from the emission intensity data at 485 nm following the modified pseudo-first-order equation:

\[
\ln \left( \frac{\Delta I_{\text{max}} - \Delta I_t}{\Delta I_{\text{max}}} \right) = -k't
\]

Here \( \Delta I_t = I_t - I_{\text{min}} \) and \( \Delta I_{\text{max}} = I_{\text{max}} - I_{\text{min}} \), where \( I_{\text{min}} \), \( I_t \), and \( I_{\text{max}} \) are the fluorescence intensities of FCN1~FCN3 considered in the absence of HOCl, at an intermediate time \( t \), and at a reaction was complete. \( k' \) is the pseudo-first-order rate constant. The pseudo-first-order plots for the reaction of HOCl with FCN1~FCN3 are shown in Figures S4-6, respectively. From the plot of \( \ln \left( \frac{\Delta I_{\text{max}} - \Delta I_t}{\Delta I_{\text{max}}} \right) \) against \( t \) for FCN1~FCN3, the value of \( k' \) was determined by fluorescence time course method for FCN1~FCN3 with HOCl: \( k' = 5.50 \times 10^{-3} \text{ s}^{-1}, 2.54 \times 10^{-3} \text{ s}^{-1}, \) and \( 5.94 \times 10^{-4} \text{ s}^{-1} \), respectively.
Figure S4: (a) Time course of reaction of FCN1 (1.5 μM) with HOCl (5 μM) in 20 mM HEPES buffer (pH 7.2) at room temperature for 0-25 min. (b) Pseudo-first-order kinetic plot of the reaction of FCN1 (1.5 μM) with addition of HOCl in 50 mM HEPES buffer (pH 7.2) at room temperature. [G1] = (ΔI_{max} − ΔI / ΔI_{max}), Slope = 5.50×10^{-3} s^{-1}, R= 0.99281.

Figure S5: (a) Time course of reaction of FCN2 (1.5 μM) with HOCl (5 μM) in 20 mM HEPES buffer (pH 7.2) at room temperature for 0-40 min. (b) Pseudo-first-order kinetic plot of the reaction of FCN2 (1.5 μM) with addition of HOCl in 50 mM HEPES buffer (pH 7.2) at room temperature. [G2] = (ΔI_{max} − ΔI / ΔI_{max}), Slope = 2.54×10^{-3} s^{-1}, R= 0.99234.
Figure S6: (a) Time course of reaction of FCN3 (1.5 μM) with HOCl (5 μM) in 20 mM HEPES buffer (pH 7.2) at room temperature for 0-90 min. (b) Pseudo-first-order kinetic plot of the reaction of FCN3 (1.5 μM) with addition of HOCl in 20 mM HEPES buffer (pH 7.2) at room temperature. $[G3] = (\Delta I_{\text{max}} - \Delta I_t / \Delta I_{\text{max}})$, Slope = 5.94 × 10^{-4} s^{-1}, R=0.98924.

The second-order rate constant value was related to pseudo-first-order rate constant following the equation:

$$k' = k \, [M]$$

Where $[M]$ is the concentration of HOCl, $k$ is the second-order rate constant, $k'$ is the pseudo-first-order rate constant. The second-order plots for the reaction of HOCl with FCN1~FCN3 are shown in Figures S7-9. The second-order rate constant for this reaction is the slope of the linear plot of $k'$ against the concentration of HOCl: $k' = 1.51 \times 10^3$ M^{-1}s^{-1}, and 2.14 × 10^{-4} s^{-1}, respectively.
Figures S7: Dependence of the rate constant of the observed processes on FCN1 concentration for HOCl. The solid line is the best fit to a straight line. Conditions: 20 mM HEPES buffer (pH 7.2) at room temperature. Slope = $1.51 \times 10^3$ M$^{-1}$ s$^{-1}$.

![Graph of Figures S7](image1)

Figures S8: Dependence of the rate constant of the observed processes on FCN2 concentration for HOCl. The solid line is the best fit to a straight line. Conditions: 20 mM HEPES buffer (pH 7.2) at room temperature. Slope = $2.14 \times 10^3$ M$^{-1}$ s$^{-1}$.

![Graph of Figures S8](image2)

Figures S9: FCN3 does not meet the strict definition of the second-order equation.
Table S2. The comparison of the photophysical properties and rate constants of FCN1~FCN3 with HOCl.

<table>
<thead>
<tr>
<th>Probe</th>
<th>FCN1</th>
<th>FCN2</th>
<th>FCN3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{\text{HOCl}}/F_{\text{ap}}$</td>
<td>1232.8-fold</td>
<td>1643.4-fold</td>
<td>31.6-fold</td>
</tr>
<tr>
<td>$\Phi_{\text{HOCl}}$</td>
<td>0.63</td>
<td>0.71</td>
<td>~0.02</td>
</tr>
<tr>
<td>$K'(s^{-1})/(K'_{\text{FCN2}}/K')$</td>
<td>5.50×10^{-3}(0.46)</td>
<td>2.54×10^{-3}(1.0)</td>
<td>5.94×10^{-4}(4.28)</td>
</tr>
<tr>
<td>$K(M^{-1}s^{-1})$</td>
<td>1.51×10^{3}</td>
<td>2.14×10^{3}</td>
<td>—</td>
</tr>
<tr>
<td>auto-oxidation</td>
<td>6.5-fold</td>
<td>2.4-fold</td>
<td>stable</td>
</tr>
</tbody>
</table>

*a* The relative emission intensity of FCN1~FCN3 with HOCl compared to the blank probe.  
*b* The pseudo-first-order rate constant of FCN1~FCN3 with HOCl.  
*c* The relative pseudo-first-order rate constant.  
*d* The second-order rate constant of FCN1 and FCN2 with HOCl.  
*e* FCN3 does not meet the strict definition of the second-order equation.  
*f* The exposure of probe to 20 mM HEPES buffer for 24 h in air.

Figure S10: Fluorescence responses of FCN2 (1.5 μM) with HOCl (5 μM) at different pH.
Table S3 Comparison of the recently reported HOCl fluorescent probes

<table>
<thead>
<tr>
<th>probe</th>
<th>detection limit</th>
<th>selectivity</th>
<th>Comments</th>
</tr>
</thead>
</table>
| ![Chem. Eur. J. 2009, 15, 2305](image1) | no data available | good | PBS/DMF (v/v, 1:4)  
$I_{505}/I_{439} = 9.79$-fold  
Ratiometric Fluorescent Probe |
| ![Chem. Commun. 2011, 47, 11978](image2) | no data available | good | HEPES/DMSO (v/v, 9:1)  
$I_{580} = 61$-fold  
Fluorescence enhancement  
Living cell imaging |
| ![Org. Lett. 2008, 10, 2171](image3) | no data available | medium | 100% aqueous media  
$I_{541} = 1079$-fold  
Fluorescence enhancement  
Living cell imaging |
| ![Org. Lett. 2009, 11, 859](image4) | $\sim 25$ nM | good | PBS/DMF (0.1%)  
Fluorescence enhancement  
Living cell imaging |
| ![J. Am. Chem. Soc. 2011, 133, 5680](image5) | no data available | good | 100% aqueous media  
NIR Fluorescence enhancement  
mouse imaging  
Living cell imaging |
| ![Chem. Commun. 2011, 47, 12691](image6) | 0.2 $\mu$M | good | PBS/DMF (v/v, 2:8)  
$I_{505}/I_{585} = 235$-fold  
Ratiometric Fluorescent Probe  
Living cell imaging |
**Cell Culture and Fluorescence Imaging**

The **FCN2** working solution for cell staining was prepared from a 1 mM stock solution (DMSO, 1.0 mM) of probe by diluting with PBS to a final concentration of 5 μM. Myeloperoxidase (human neutrophils) was purchased from J&K (CAS: 9003-99-0). NIH3T3 cells (mouse embryonic fibroblast cells) were dropped on the poly-D-lysine-coated 35 mm glass bottom dishes (Mat Tek Corp) at a density of 2×10^3 cells per well in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin (100 μg mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air for 24 h prior to staining. All cellular fluorescent images were collected on an FV1000-IX81 confocal microscope.

Experiments to detection of HOCl were performed in the PBS media supplemented with MPO (1.5 U/100 mL), NaCl (250 mM) and 5 μM **FCN2** for 30 min. After washing twice with PBS (phosphate...
buffered saline, pH=7.2, Gibco) to remove the remaining sensor, then the treated cells were stimulated with 10 μM H₂O₂. Images were collected using IPP software (Olympus) by confocal microscope.

Figures S11: Similar fluorescence enhancements were observed by using **FCN1** under the H₂O₂ stimulated production of HOCl. DIC (a) and confocal fluorescence (b) images of NIH3T3 cells preincubated with NaCl (250 mM) and 5 μM **FCN1** in MPO (1.5 U/100 mL) enzymatic system. (c) overlay image of (a) and (b).

Figures S12: Negligible fluorescent enhancements were observed by using **FCN3** under the H₂O₂ stimulated production of HOCl. DIC (a) and confocal fluorescence (b) images of NIH3T3 cells preincubated with NaCl (250 mM) and 5 μM **FCN3** in MPO (1.5 U/100 mL) enzymatic system.

**MTT Assay**

To ascertain the cytotoxic effect of **FCN2** treatment over a 24 h, the MTT assay was performed. NIH3T3 cells (5×10⁴) were passed and plated to 70% confluence in 96-well plates 24 h before treatment. 5, 25, 50, 75, and 100 μM **FCN2** was added to the cells and incubated at 37°C for 12 h. Cytotoxicity was then determined by the method of Thiazolyl Blue Tetrazolium Bromide (MTT) assay (Cell Proliferation Kit; keygen biological products, Nanjing, China), following the instructions of the kit. Subsequently, the cells were incubated with 5 mg/mL MTT reagent at 37°C for 4 h and the
absorbance of each well was measured by a microplate reader (SPECTRA SLT; Labinstruments, Salzburg, Austria). The excitation wavelength was 492 nm, and the emission was read at 690 nm. Each treatment was done in six wells, and the experiments were repeated three times. Cytotoxicity was calculated relative to the absorbance of the control for each treatment. Data were expressed as means ± SD. The reported percent cell survival values are relative to untreated control cells.

![Graph showing cell viability](image)

**Figure S13:** Cell viability was quantified by the MTT assay (NIH3T3 cells, 12h).

### Experimental for the fluorescence imaging of zebrafish

Zebrafish were kept at 28.5°C and maintained at optimal breeding conditions. For mating, male and female zebrafish were maintained in one tank at 28.5°C on a 12 h light/12 h dark cycle and then the spawning of eggs were triggered by giving light stimulation in the morning. Almost all the eggs were fertilized immediately. The 19 dpf and 54 dpf old zebrafish was maintained in E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 0.15 mM KH$_2$PO$_4$, 0.05 mM Na$_2$HPO$_4$, 0.7 mM NaHCO$_3$, 10-5% methylene blue; pH 7.5). Experiments to detection of HOCl were performed in E3 embryo media with 5 μM FCN2 for 30 min. All zebrafish fluorescent images were collected on a fluorescent dissecting microscope (Leica) equipped with a DP70 digital imaging system (Olympus, Tokyo, Japan) with a GFP filter set. Images were collected using IPP software (Olympus).
Ethical statement:
In this paper, all experiments with live animals were performed in compliance with guidelines issued by Ethical Committee of Model Animal Research Center of Nanjing University, and the institutional committee(s) have approved the experiments.

Figure S14: Images of adult zebrafish organs treated with 5 μM FCN2 in (a) the absence and (b) presence of external HOCl.
Figure S15: Fluorescence microscopy images of AB/Tubingen larvae zebrafish incubated with 10 μM HOCl (E3 embryo media, 28.5°C) during the development. 1-Phenyl-2-thiourea (PTU, 0.003%) was added to depress the development of pigment after 8 h of incubation. Fluorescence and DIC images of (a, b) 18 h-old, (c, d, e) 28 h-old, (f, g) 54h-old, and (h, i) 78h-old zebrafish were further incubated with 5 μM FCN2 for 1 h.
References


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$^1$H NMR, $^{13}$C NMR and ESI-MS spectra Supplementary spectra data