Supporting Information for

A fast-responsive two-photon fluorescent probe for monitoring endogenous HClO with a large turn-on signal and its application in zebrafish imaging

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# Table of contents

Materials and instruments ................................................................. S3  
Determinination of the fluorescence quantum yield. .............................. S3  
Cell culture ......................................................................................... S4  
Cytotoxicity assays ............................................................................. S4  
Imaging of exogenous HClO in HeLa cells ......................................... S4  
Imaging of endogenous HOCl in RAW 264.7 cells ............................... S5  
Preparation of fresh mouse liver slices and two-photon fluorescence imaging... S5  
Imaging of HClO in zebrafish ............................................................. S5  
Synthesis of the probe NS-ClO ............................................................ S6  
Fig. S1 .................................................................................................. S7  
Fig. S2 .................................................................................................. S7  
Fig. S3 .................................................................................................. S7  
Fig. S4 .................................................................................................. S8  
Fig. S5 .................................................................................................. S8  
Fig. S6 .................................................................................................. S9  
Table S1 ................................................................................................. S9
Materials and instruments

Without other noted, all the solvents, reagents and materials were obtained from business company and used without other purification. Twice-distilled water was applied to all measurements and experiments. High-resolution electrospray mass spectra (HRMS) were gained from Bruker APEX IV-FTMS 7.0T mass spectrometer; NMR spectra were examined from AVANCE III 400 MHz Digital NMR Spectrometer with TMS as an internal standard; Electronic absorption spectra were recorded on a LabTech UV Power spectrometer; Fluorescence spectra were obtained with a HITACHI F4600 fluorescence spectrophotometer; The fluorescence images of cells and tissues were collected with Nikon A1MP confocal microscopy with a CCD camera; The pH measurements were implemented on a Mettler-Toledo Delta 320 pH meter; analysis was exhibited on silica gel plates and column chromatography was carried out over silica gel (mesh 200-300). Both TLC and silica gel were purchased from the Qingdao Ocean Chemicals.

Determination of the fluorescence quantum yield

Fluorescence quantum yields were determined by using fluorescein (0.1 M in NaOH) according to previous report.\(^1\) The fluorescence quantum yield of compound \(\text{NS-ClO}\) and \(\text{NS-ClO-adduct}\) was calculated according to the following equation:

\[
\eta_s = \frac{A_s I_s n_s^2}{A_r I_r n_r^2} \eta_r \quad (A \leq 0.05)
\]

In the equation, \(s\) and \(r\) represent the sample and the reference (fluorescein) molecule respectively, \(\eta\) represents the fluorescence quantum yield, \(A\) is the absorbance of molecules that were controlled below 0.05 at the excitation wavelength for both molecules in the experiment, \(I\) means the integrated emission area and \(n\) is the refractive index of the solvent.
Cell culture

The living HeLa cells, and RAW 264.7 cells were cultured in the Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (10% FBS) under the atmosphere containing 5% CO$_2$ and 95% air at 37 °C.

Cytotoxicity assays:

The living cells line were treated in DMEM (Dulbecco’s Modified Eagle Medium) supplied with fetal bovine serum (10%, FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) under the atmosphere of CO$_2$ (5%) and air (95%) at 37 °C. The HeLa cells were then seeded into 96-well plates, and 0, 1, 5, 10, 20, 30 μM (final concentration) of the probe NS-ClO (99.9% DMEM and 0.1% DMSO) were added respectively. Subsequently, the cells were cultured at 37 °C in an atmosphere of CO$_2$ (5%) and air (95%) for 24 hours. Then the HeLa cells were washed with PBS buffer, and DMEM medium (500 μL) was added. Next, MTT (50 μL, 5 mg/mL) was injected to every well and incubated for 4 h. Violet formazan was treated with sodium dodecyl sulfate solution (500 μL) in the H$_2$O-DMF mixture. Absorbance of the solution was measured at 570 nm by the way of a microplate reader. The cell viability was determined by assuming 100% cell viability for cells without NS-ClO.

Imaging of exogenous HClO in HeLa cells

Before using, the HeLa cells were washed with PBS three times and then incubated with NS-ClO (10 μM) for 30 min at an atmosphere of CO$_2$ (5%) and air (95%) in the condition of 37 °C. After incubating with NaClO (10 μM) for another 0.5 h under the same conditions. Subsequently, the HeLa cells were rinsed by PBS buffers three times. The ideal fluorescence images were obtained by means of Nikon A1MP confocal microscopy with the equipment of a cooled CCD camera.
Imaging of endogenous HOCl in RAW 264.7 cells

The RAW 264.7 cells were plated on 6-well plates and allowed to adhere for one day. And then the cells were incubated with probe **NS-CIO** (10 μM) for 0.5 h at 37 °C. After washing with PBS buffer for three times, 2 μg/ml PMA (phorbol 12-myristate13-acetate) and 2 μg/ml LPS (lipopolysaccharides) were added and incubated for another 2 h. As to the control experiments, the RAW 264.7 cells were not treated with PMA/LPS and only incubated with probe **NS-CIO** (10 μM) for 2 h at an atmosphere of CO₂ (5%) and air (95%) in the condition of 37 °C. Before the fluorescence imaging, the cells were washed with PBS buffer. The Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera were used for fluorescence images.

**Preparation of fresh mouse liver slices and two-photon fluorescence imaging**

The fresh mouse liver slices were obtained from the liver of 14-day-old mouse. The living liver slices were gained with 400 micron thickness using a vibrating-blade microtome in 25 mM PBS (pH 7.4). The fresh liver slices were pre-treated with **NS-CIO** (10 μM) for 0.5 h. Following another incubation with NaClO (100 μM) for 0.5 h in the condition of 37 °C, the slices were washed three times by PBS buffer and imaged. As to the control experiments, the fresh tissues were not treated with NaClO and only incubated with probe **NS-CIO** (10 μM) for 0.5 h at the same conditions. The two-photon fluorescent images were collected with excitation wavelength at 800 nm and emission wavelength at 450 nm by means of Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera and a femtosecond laser.

**Imaging of HClO in zebrafish**

The 5-day-old zebrafish was pre-treated with probe **NS-CIO** (10 μM) in embryo media for 0.5 h at 37 °C and then rinsed with PBS buffers for three times. After another incubation with NaClO (100 μM) for 0.5 h, the fresh zebrafish was washed with PBS buffers three times. As to the control group, the zebrafish incubated with
probe **NS-ClO** (10 μM) for 0.5 h at in the same condition. The zebrafish imaging was completed by Nikon A1MP confocal microscopy inverted fluorescence microscopy equipped with a cooled CCD camera.

**Synthesis of the probe NS-ClO**

Compound 1 (2.0 mmol, 510.0 mg, 1.0 equiv) and 2 (2.0 mmol, 340.0 mg, 1.0 equiv) were dissolved in DMF (15.0 mL). And the Na₂S₂O₅ (2.0 mmol, 380.0 mg, 1.0 equiv) was added under N₂. The reaction was refluxed for 5 h. Then, the distilled water was added (10.0 mL) for extracting with DCM. The product was purified by silica column chromatography to give the two-photon fluorescent probe **NS-ClO** with 60% yield. ¹H NMR (400 MHz, CDCl₃) δ 8.05 (d, J = 8.1 Hz, 1H), 7.92 - 7.84 (m, 1H), 7.82 (d, J = 2.1 Hz, 1H), 7.51 - 7.44 (m, 1H), 7.40 - 7.33 (m, 1H), 7.19 - 7.10 (m, 1H), 6.97 - 6.86 (m, 1H), 3.97 (q, J = 7.0 Hz, 1H), 1.45 (t, J = 7.0 Hz, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 171.4, 158.8, 152.1, 148.4, 139.4, 133.2, 132.4, 132.3, 132.2, 131.8, 130.4, 130.2, 128.7, 128.3, 127.7, 127.5, 127.0, 121.0, 120.8, 46.7, 17.7; HRMS (ESI) m/z calcd for C₁₉H₁₆NO₂⁺ (M+H)⁺: 361.0876; found 361.0859.

**Reference**

Fig. S1. The absorption spectra of **NS-ClO** (5 μM) in pH 7.4 PBS/DMF (v/v = 95/5) in the absence or presence of H₂O₂ (5 equiv) and NaClO (5 equiv).

Fig. S2. The linear fit of **NS-ClO** (10 μM) in pH 7.4 PBS buffer (5% DMF) in the absence or presence of NaClO (0-10 equiv).
Fig. S3. HRMS (positive ion mode) spectrum of NS-ClO.

Fig. S4. HRMS (positive ion mode) spectrum of NS-ClO (20 μM) after treatment with NaClO (200 μM) in pH 7.4 PBS/DMF (1: 1) for 20 min. The peak at m/z 377.0774 corresponds to NS-ClO-adduct.

Fig. S5. Cytotoxicity assays of NS-ClO at different concentrations (0 μM; 1μM; 5 μM; 10 μM; 20 μM; 30 μM) for HeLa cells
Fig. S5. $^1$H NMR (DMSO-$d_6$) spectrum of NS-ClO.

Fig. S6. $^{13}$C-NMR (DMSO-$d_6$) spectrum of NS-ClO.
Table S1 Comparison of the reported fluorescent probes based on phenothiazine for the detection of HClO

<table>
<thead>
<tr>
<th>Probes</th>
<th>Response time</th>
<th>Detection limit</th>
<th>Stokes shift</th>
<th>One or two photon</th>
<th>Imaging</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Compound 1" /></td>
<td>Few seconds</td>
<td>15.6 nM</td>
<td>128 nm</td>
<td>One photon</td>
<td>Living cells</td>
<td>Zebrafish, mice</td>
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<tr>
<td><img src="image2.png" alt="Compound 2" /></td>
<td>10 s</td>
<td>449.76 nM</td>
<td>168 nm</td>
<td>One photon</td>
<td>Living cells</td>
<td>Dyes Pigments, 2019, 162, 160-167.</td>
</tr>
<tr>
<td><img src="image3.png" alt="Compound 3" /></td>
<td>10 s</td>
<td>39 nM</td>
<td>170 nm</td>
<td>One photon</td>
<td>Endogenous Living cells</td>
<td>New J. Chem., 2018, 42, 5135-5141</td>
</tr>
<tr>
<td><img src="image4.png" alt="Compound 4" /></td>
<td>--</td>
<td>4.1 nM</td>
<td>none</td>
<td>One photon</td>
<td>Endogenous Living cells</td>
<td>Sensor. Actuat. B: Chem., 2018, 263, 137-142</td>
</tr>
<tr>
<td><img src="image5.png" alt="Compound 5" /></td>
<td>1 min</td>
<td>0.76 μM</td>
<td>90 nm</td>
<td>Two photon</td>
<td>Endogenous Living cells</td>
<td>Tissues zebrafish</td>
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</tbody>
</table>