Supporting Information

A Near-Infrared Fluorescent Probe for Monitoring Ozone and Imaging in Living Cells

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1. Materials and Apparatus

Materials

2-[4-Chloro-7-(1-ethyl-3,3-dimethyl(indolin-2-ylidene)]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-1-ethyl-3,3-dimethyl-3H-indolium (Cy.7.Cl) was synthesized in our laboratory. Ozone was obtained by an ozone generator (BEYOK ozone, Zhejiang, China) and ozone solutions were prepared in PBS buffer (pH = 6.0) and the concentrations were determined by UV absorption ($\lambda_{max} = 258$ nm; $\varepsilon = 2900$ L mol$^{-1}$ cm$^{-1}$). Glutathione (GSH), Xanthine/xanthine oxidase (X/XO), Mito Tracker Green FM and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (methyl thiazolyl tetrazolium, MTT) were purchased from Sigma-Aldrich (Beijing, China). Sodium nitroferricyanide (III) dihydrate (SNP) was purchased from Alfa Aesar (Tianjin, China). Ascorbic acid was purchased from Sinopharm (Shanghai, China). 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Shanghai DoBio Biotech. Co., LTD. Phorbol-12-myristate-13-acetate (PMA) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Ethyl 4-vinylbenzoate was purchased from Sigma-Aldrich (Shanghai, China). All other chemicals were obtained from standard reagent suppliers and of analytical reagent grade. Ultrapure water (18.2 MΩ cm) used in all experiments was from an ultrapurification system (Sartorius, Göttingen, Germany). A549 cells and RAW 264.7 cells were purchased from Shandong Academy of Medical Science (Shandong, Jinan, China). A stock solution (1.00 mM) of Trp-Cy was prepared by dissolving in DMSO.

In MS experiment, the sample injection flow rate was 10 µL/min. Data acquisition was performed using the Data Analyst software. HPLC-grade water, acetic acid and acetonitrile from Sigma-Aldrich were used. The deionized water used for sample preparation was obtained using a Nanopure Diamond Barnstead purification system.

Apparatus

Fluorescence spectra were obtained with a Fluorescence Spectrometer (FLS-920, Edinburgh, England) with a Xenon lamp and 1.0-cm quartz cells at the slits of 2.5/2.5 nm. Absorption spectrum was measured on a UV-visible spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Electrospray ionization (ESI) of samples was performed using a maXis UHR Time-of-Flight Mass Spectrometer System (Bruke Daltonics Inc.) and an UPLC-LTQ orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Ozone
was obtained by an ozone generator (BEYOK ozone, Zhejiang, China). All pH were measured with a pH-3c digital pH-meter (PB-21, Sartorius, Göttingen, Germany). The fluorescence images of cells were taken using a confocal laser scanning microscope (TCS SP5, Leica, Wetzlar, Germany) with an objective lens (×40) and the excitation wavelength was 633 nm (10 mW). Absorbance was measured in a microplate reader (RT-6000, Rayto, Guangdong, China) in the MTT assay. 1H NMR spectrum was taken on a nuclear magnetic resonance spectrometer (AVANCE 300, Bruker, Switzerland). Determination of organic elements was performed with a Model element analyzer (PE-2400(II), Perkin-Elmer, California, CA).

2. Synthesis Methods

Synthesis and Characterization of Trp-Cy
According to the synthesis of a series of near-infrared cyanine dyes proposed by Xiaojun Peng\(^2\), with some modification, we synthesized the Trp-Cy. Firstly, 0.2 g of Cy was dissolved in 10 mL DMF and 0.1 g of L-tryptophan was dissolved in 8 mL water. After mixing the two solutions thoroughly, the mixture was heated to 80 °C slowly under Ar atmosphere for 24 h in a 50-mL round bottom flask\(^3\). Scheme 1 showed the reaction between Cy and L-tryptophan. Then the solvent was evaporated on a rotary evaporator. The solid was purified on silica gel chromatography eluted with ethyl acetate/methanol (3:1 v/v) to give Trp-Cy as a blue solid (35% yield). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): 1.39 (t, 6H, J = 6 Hz), 1.66 (s, 12H), 1.85 (m, 2H), 2.53 (t, 4H, J = 6 Hz), 3.67 (d, 2H, J = 6 Hz), 3.92 (q, 4H, J = 6 Hz), 4.31 (t, 1H, J = 6 Hz), 5.64 (d, 2H, J = 12 Hz), 6.89 (m, 3H), 7.07 (m, 3H), 7.29 (m, 5H), 7.48 (m, 4H) ppm. \(^13\)C-NMR (300 MHz, CDCl\(_3\)): 175.37, 171.10, 167.02, 142.56, 140.58, 140.20, 130.87, 129.8, 128.99, 128.9, 128.25, 128.18, 125.98, 125.26, 122.85, 122.02, 108.44, 93.75, 65.51, 60.34, 47.82, 47.79, 38.29, 30.55, 29.31, 25.38, 21.61, 20.98, 19.65 ppm. MS: m/z Calcd 806.3, found 679.5 [M-I]\(^+\). Elemental Analysis: Calcd C, 66.99; H, 6.37; N, 6.94. Found C, 66.7; H, 6.25; N, 6.72.

Synthesis and Characterization of Trp-Cy Oxide
The oxidated Trp-Cy was obtained as follows: 500 μL of solution containing 20 μM Trp-Cy and 500 μL of solution containing 6 μM O\(_3\) were mixed together. The solvent was removed and the residue was determined by mass spectrometer. MS: m/z Calcd 838.3,
found 729.5 [M-I’ + H₂O]⁺.

3. The mechanism of detection of ozone by Trp-Cy

The fluorescence of Trp-Cy was quenched by twisted intramolecular charge-transfer (TICT)⁴, which can be validated by solvent effect in Figure S1. After the reaction of Trp-Cy with O₃ occurred, the molecular surface of L-tryptophan group of Trp-Cy was broken, resulting in TICT disappearance and the fluorescence recovery of tricarbocyanine (Cy).

![Figure S1](image.png)

**Figure S1.** Fluorescence intensities of 10 μM Trp-Cy dissolved in H₂O, CH₃CN and C₂H₅OH, respectively.
4. Optimization of pH value, buffer concentration and probe concentration

Effect of pH Value and Buffer Concentration

As shown in Figure S2, the probe was relatively stable in PBS medium with different pH from 6.8 to 8.0, whereas the fluorescence intensity of the probe after reacting with O₃ had somewhat changes and it was largest and stable when the pH ranging from 7.3 to 7.5. That was to say, the probe had good response at physiological condition (pH = 7.4). Furthermore, taking into the account of capacity of the buffer and ion strength, the experiment was performed in PBS buffer (pH = 7.4) with different concentrations ranging from 15 mM to 55 mM (Figure S3). At last, 30 mM was chosen.

![Figure S2](image.png)

**Figure S2.** The relationship between the fluorescence intensity and pH value (10 μM for Trp-Cy, 6 μM for ozone and 30 mM for PBS)
**Figure S3.** The relationship between the relative fluorescence intensity and concentration of the PBS buffer (10 μM for Trp-Cy and 6 μM for ozone, pH = 7.4)

**Effect of Probe Concentration**

Accuracy and sensitivity were also affected by the concentration of the probe. Figure S4 showed the relative fluorescence intensity of the probe after reacting with O₃ when different concentrations of probe were used. The results showed that, when the concentration was less than 9 μM, the relative fluorescence intensity increased with the increasing concentration of probe. When the concentration was more than 9 μM, the relative fluorescence intensity reached the greatest value and was stable. Therefore, a concentration of 10 μM was used as the optimal condition.
Figure S4. The relationship between of relative fluorescence intensity and the concentrations of the probe (6 μM for ozone and 30 mM PBS, pH=7.4)

5. Studies on interference from metal ions and ROS on determination of O$_3$ with Trp-Cy

An additional test was performed in order to determine whether metal ions such as K$^+$, Na$^+$, Ca$^{2+}$, Mg$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Ni$^{2+}$ and Cu$^{2+}$ are potential interferences. An error of <5.0% in the relative fluorescence intensity was considered tolerable in Figure S5a. In addition, considering some ROS molecules can slowly oxidize L-tryptophan, an experiment on extending the incubated time to 30 min was also performed in Figure S5b. As shown in Figure 5, no obvious interference was observed.

Figure S5 (a) The relative fluorescence response of 10 μm probe to some metal ion (6
μM for O₃; 0.5 mM for Ca²⁺, Cd²⁺, Ni²⁺, Zn²⁺, Co²⁺, Cu²⁺, Mg²⁺, K⁺ and Na⁺). (b) The relative fluorescence response of 10 μM probe to some small molecules (6 μM for O₃ and ONOO⁻; 120 μM for H₂O₂ and O₂⁻; 30 μM for t-BuOOH and ·OH; 24 μM for ¹O₂ and NaClO; 12 μM for NO; 0.3 mM for GSH and VC). Gray bars represent the addition of O₃ or the addition of O₃ plus one of these interferences to the probe solution in 30 mM PBS buffer, pH 7.4. Black bars represent one of these interferences to the probe solution in 30 mM PBS buffer, pH 7.4. All data in Figure S5b were obtained after incubation 30 min.

6. Studies on the molecular mechanism of Trp-Cy to recognize ozone based on MS technology

Figure S6 displays the ESI-MS spectrum showing the reaction of Trp-Cy (20 μM) with ozone generated by an ozone generator in acetonitrile/water (1:1 by volume) containing 1% acetic acid. According to product analysis, fluorescence properties and articles reported,⁵ we propose that the molecular mechanism of Trp-Cy to recognize ozone was as follows (Scheme S1).

![Figure S6. ESI-MS spectrum of the reaction solution of Trp-Cy and ozone](image_url)
Scheme S1. A proposed molecular mechanism of Trp-Cy to recognize ozone

7. Cell Culture and Confocal Imaging

Cell Culture

Human lung carcinoma A549 cells or RAW 264.7 cells were first grown in a circular petri dish (60 mm) using RPMI 1640 medium with 10% fetal bovine serum (FBS), NaHCO₃ (2 g/L), and 1% antibiotics (penicillin /streptomycin, 100 U/mL). Mouse macrophage RAW 264.7 cells were first grown in a circular petri dish (60 mm) using DMEM medium with 10% fetal bovine serum (FBS), NaHCO₃ (2 g/L), and 1% antibiotics (penicillin /streptomycin, 100 U/mL). When the cells reached 80% confluence, they were transferred from the petri dish and cultured directly on sterile coverslips, which were rinsed, and dried, in a circular petri dish (35 mm). The cells (0.1 mL, $1 \times 10^6$ cells/mL) were carefully added onto each coverslip to ensure uniform coverage and allowed to adhere onto the coverslips. The same cell medium was then added to cover the
coverslips in petri dishes, which were placed in a CO₂ incubator and maintained under the same conditions for 24 h.

Confocal Imaging
The A549 cells were incubated on the coverslips in petri dishes for 24 h before the confocal imaging performed. The RAW 264.7 cells were incubated on the coverslips in petri dishes for 24 h before the stimulated by 100 ng/mL PMA for 20 min. Subcellular locations of endogenous ozone in RAW264.7 cells were preformed by co-staining experiments with DAPI and Mito Tracker Green FM. Before the imaging was performed, the cells were washed with PBS (pH = 7.4, 0.1 mM) for three times after the original medium had been removed. Confocal fluorescence images were obtained on a confocal laser scanning microscope with an objective lens (×40). The excitation wavelength was 633 nm.

8. Tests of the cytotoxicity and photostability of Trp-Cy

MTT Experiment
To investigate Trp-Cy cytotoxicity, MTT assay were carried out when the probe existed in A549 Lung cancer cells. A549 cells (1×10⁶ cell/mL) were dispersed within replicate 96-well microplates to a total volume of 200 μL/well. Plates were maintained at 37 °C in a 5% CO₂/95% air incubator for 24 h. Then, A549 cells were incubated with the probe of different concentrations (10, 20, 50, 75, 100, 200, 350 and 500 μM) for 24 h. MTT solution (150 μL, 0.5 mg/mL, PBS) was then added to each well. After 4 h, the remaining MTT solution was removed, and 100 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a microplate reader.

Evaluation of Cytotoxicity of the Probe
We performed a MTT assay in A549 cells with concentrations of the probe from 10 μM to 500 μM, and calculated cell livability to investigate the cytotoxicity of the probe. The result in Figure S7 showed when the cell livability was 50%, the concentration of the probe was approximately 465 μM. The cell livability was 90% under our experimental conditions, which clearly demonstrated that the probe was low toxicity to cultured cell lines.
Photobleaching

Photobleaching is an irreversible photochemical inversion of fluorescent molecule into a non-fluorescent state. The photo-stability of oxidized Trp-Cy was investigated by time-sequential scanning of the living cells. After 300 s of continuous irradiation with a 633-nm laser, no obvious changes were observed in fluorescence brightness of oxidized Trp-Cy (Figure S8). In order to quantitatively determine the photobleaching rate, we choose three regions, calculated the average intensity and obtained a curve with scanning time. The results in Figure S9 showed that the intensities of Trp-Cy and oxidized Trp-Cy after 0 ~ 300 s of time-sequential scanning were about 90% of the initial value. These data indicated that the probe and its oxidized product are highly resistant to photobleaching.

**Figure S7.** MTT assay of A549 cells in the presence of different concentrations of Trp-Cy
Figure S8. Test of photostability of oxidized Trp-Cy. Confocal fluorescence images (T₀, T₀+100s, T₀+200s, T₀+300s) were achieved by means of time-sequential scanning of the probe-loaded A549 cells incubated with 6 µM O₃ for 10 min.

Figure S9. Normalized fluorescence intensity with 10 µM probe (a) and its oxidized product (b) in living cells from 0 to 300s of time-sequential scanning.
9. $^1$H NMR, $^{13}$C NMR and MS spectra of Trp-Cy and MS spectrum of Oxidized Trp-Cy

$^1$HNMR of Trp-Cy
$^{13}$C NMR of Trp-Cy
MS of Trp-Cy
MS of oxidized Trp-Cy
10. References


