Supporting information for

Development of a Ratiometric Fluorescent Sensor for Ratiometric Imaging of Endogenously Produced Nitric Oxide in Macrophage Cells

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Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, and all melting points were uncorrected; Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or Agilent 1100 HPLC/MSD spectrometer; NMR spectra were recorded on an INOVA-400 or Bruker AV-500 spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a LabTech UV Power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer; Cells imaging was performed with a Nikon Eclipse TE2000U inverted microscope; Confocal fluorescence imaging was performed with a Nikon 90i C1 Si laser scanning confocal microscopy; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

Cytotoxicity assay of ratiometric sensor Cou-Rho-NO: RAW264.7 Macrophages were grown in the modified Eagle's medium (MEM) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 ^{o}C. Immediately before the experiments, the cells were placed in a 96-well plate, followed by addition of increasing concentrations of ratiometric sensor **Cou-Rho-NO** (99% MEM and 1% DMSO). The final concentrations of the ratiometric sensor were kept from 5 to 200 μM (n = 3). The cells were then incubated at 37 $^{\circ}$C in an atmosphere of 5% CO_2 and 95% air at 37 ^{o}C for 24h, followed by the MTT assays. Untreated assay with MEM (n = 3) was also conducted under the same conditions.

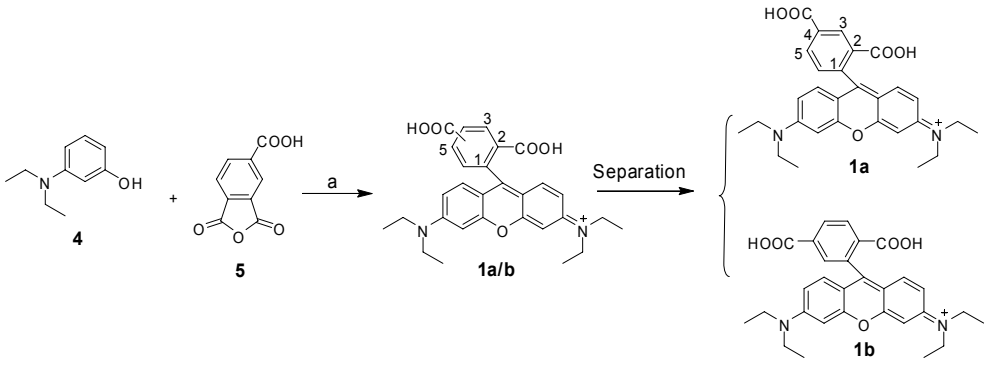
Spectroscopic studies of ratiometric sensor Cou-Rho-NO: DEA/NONOate (diethylamine NONOate, a commercially available NO donor) and dehydroascorbic acid (DHA) were purchased from Aldrich and used without further purification. Twice-distilled water and spectroscopic grade CH_3CN were used for spectroscopic studies. Superoxide ($O_2^{\bullet-}$) was added as solid KO_2. Hydroxyl radical was generated *in situ* by the Fenton reaction.[14] Singlet oxygen ($^1O_2$) was generated from ClO^- and H_2O_2. A stock solution of DEA/NONOate was prepared in 0.01M NaOH solution. Various analytes (100 equiv. for NO and 1000 equiv. for others analytes) represented by H_2O_2, NO_3^-, NO_2^-, HClO, $O_2^{\bullet-}$, OH•, $^1O_2$, ascorbic acid (AA), and dehydroascorbic acid (DHA) were added to the solution of compound **Cou-Rho-NO** (final concentration, 1 μM) in 0.1 M PBS, pH 7.4 (containing 20% CH_3CN as a cosolvent), respectively. The resulting solution was kept at ambient temperature for 30 min, and then the fluorescence spectra were recorded with excitation at 410 nm.

HeLa cell imaging using ratiometric sensor Cou-Rho-NO: HeLa cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 ^{o}C. The cells were plated on 6-well plates and allowed to adhere for 24 h. Immediately before the experiments, the cells were washed with phosphate-buffered saline (PBS) buffer. Subsequently, the cells were incubated with ratiometric sensor **Cou-Rho-NO** (5 μM) for 30 min at 37 ºC, and then washed with PBS three times. After incubating with 50 μM

DEA/NONOate for another 30 min at 37 ºC, the HeLa cells were rinsed with PBS three times, and the fluorescence images were acquired through a Nikon eclipase TE2000 inverted fluorescence microscopy equipped with a cooled CCD camera (Figure S9).

Raw 264.7 murine macrophages culture and imaging using ratiometric sensor Cou-Rho-NO. Raw 264.7 murine macrophages were obtained from Xiangya hospital and cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 ^oC. For nitric oxide detection studies, iNOS was induced in Raw 264.7 macrophages with 1.25 μg/mL of lipopolysaccharide (LPS) and 1000 U/mL of interferon-gamma (IFN-γ) and the cells were then incubated with 5 μM **Cou-Rho-NO**. Prior to imaging, the cells were washed three times with 1 mL of PBS and the fluorescence images were acquired through an Olympus fluorescence microscopy equipped with a cooled CCD camera (Figure 3).

Synthesis of Cou-Rho-NO: To a solution of compound **3** (53.0 mg, 0.066 mmol) in dry 1, 2-dichloroethane (6.0 ml) at room temperature, phosphorus oxychloride (0.3 ml) was added, and the mixture was heated to 80 °C. After the solution was further stirred at 80 °C for 4 h, the reaction mixture was cooled and concentrated under vacuum to give a crude acid chloride. The acid chloride was then dissolved in CH_2Cl_2 (8 mL), and then triethylamine (0.2 mL) and o-diaminobenzene (14.0 mg, 0.13 mmol) were added to the solution. After stirring for 0.5 hour at room temperature, the mixture was concentrated under vacuum and the crude product was purified by silica column chromatography (dichloromethane/methanol, 50 : 1) to give compound **Cou-Rho-NO** as a yellow powder (18.5 mg, 31.6% yield). ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.13-1.17 (t, J = 7.2 Hz, 12H), 1.21-1.25 (t, J = 7.2 Hz, 6H), 3.32-3.91 (22 H), 6.08-6.10 (d, J = 7.2 Hz, 1H), 6.27-6.34 (4 H), 6.39-6.43 (t, J = 7.6 Hz, 1H), 6.48 (s, 1H), 6.54-6.56 (t, J = 8.0 Hz, 1H), 6.60-6.69 (m, 3H), 6.93-6.97 (t, J = 7.6, 1.2 Hz, 1H), 7.32-7.34 (d, J = 8.8 Hz, 2H), 7.64-7.67 (dd, J = 8.0, 1.2 Hz, 1H), 7.93 (s, 1H), 8.04 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 11.40, 43.37, 43.98, 95.92, 97.03, 105.12, 106.77, 107.15, 108.49, 114.46, 116.12, 117.30, 120.96, 124.03, 127.86, 129.03, 130.83, 131.05, 143.35, 145.07, 148.12, 150.91, 152.87, 156.41, 158.25, 164.37, 168.74; ESI-MS m/z 888.4. $[M + H]^+$; HRMS (ESI) m/z calcd for $C_{53}H_{58}N_7O_6$ ($[M + H]^+$): 888.4443. Found 888.4454.

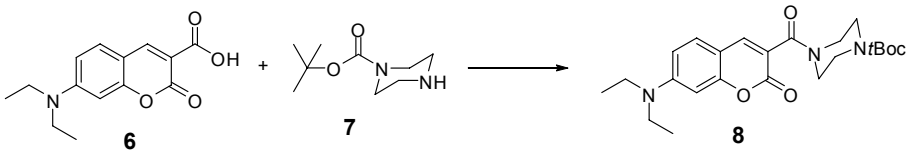


Synthesis of the mixture of 1a/b and pure regioisomers 1a and 1b. A mixture of powdered 1, 2, 4-benzenetricarboxylic anhydride **5** (2.46 g, 12.8 mmol) and 3-(diethylamino)phenol **4** (1.86 g,

11.30 mmol) was heated with stirring under nitrogen atmosphere up to 160-180°C for 3h. Then another portion of 3-(diethylamino)phenol **4** (1.48 g, 8.50 mmol) and 85% H_3PO_4 (7.0 ml) were added, and the mixture was heated at 160-180°C for 3 h. After cooling to room temperature, methanol (120 mL) and water (80 ml) were added, and the solution was stirred at room temperature for 4 h. Then, the solution was extracted with CH_2Cl_2 (3 × 100 ml). The combined organic solutions were dried, and the solvent was removed *in vacuo*. The resulting solid was purified by flash chromatography with methanol as the eluting solvent to yield the mixture of 4'- and 5'-carboxyrhodamines (**1a** and **1b**), which were then subjected to the preparative thin layer chromatography (CH_2Cl_2: CH_3OH 6: 1) to provide pure regioisomers **1a** and **1b**, separately.

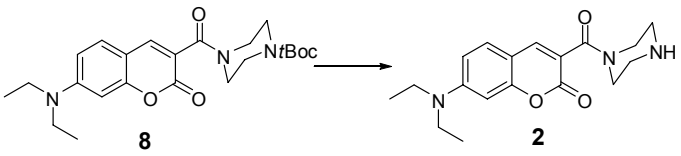
1a (4′-isomer): ^{1}H NMR (400 Hz, d_4-CD_3OD): δ = 1.30-1.64 (t, *J* = 6.8 Hz, 12H), 3.49-3.54 (q, 8H), 6.81 (d, *J* = 2.0 Hz, 2H), 6.85-6.88 (dd, *J* = 9.2, 2.0 Hz, 2H), 7.01-7.03 (d, *J* = 9.2 Hz, 2H), 7.28-7.30 (d, *J* = 8.0 Hz, 1H), 8.16-8.18 (d, *J* = 8.0, 1.2 Hz, 1H), 8.70 (d, *J* = 1.2 Hz,1H); ^{13}C NMR (100 Hz, CD_3OD): 13.81, 47.77, 98.15, 115.62, 116.26, 132.38, 133.57, 133.97, 134.05, 139.26, 158.00, 160.28, 162.19; ESI-MS m/z 487.3 $[M]^+$.

1b (5′-isomer): ^{1}H NMR (400 Hz, d_4-CD_3OD): δ = 1.12-1.15 (t, *J* = 7.0 Hz, 12H), 3.48-3.52 (q, 8H), 6.79 (d, *J* = 2.4 Hz, 2H), 6.81-6.85 (dd, *J* = 9.2, 2.4 Hz, 2H), 7.02-7.05 (d, *J* = 9.2 Hz, 2H), 7.71 (s, 1H), 8.05-8.11 (m, 2H); ^{13}C NMR (100 Hz, CD_3OD):13.81, 47.73, 98.05, 112.18, 115.94, 116.01, 132.61, 132.77, 133.96, 134.78, 157.89, 160.31, 163.69. ESI-MS m/z 487.3 $[M]^+$.



6 7 8

Synthesis of 8: To a CH_2Cl_2 solution (20 ml) of coumarin acid **6** (992.8 mg, 3.8 mmol) and mono-*t*Boc-piperazine **7** (1061.3 mg, 5.7 mmol), DCC (1030.0 mg, 5.0 mmol) and DMAP (6.1 mg, 0.05 mmol) were added at room temperature. The solution was stirred under reflux for 7 h, and then the solvent was removed under reduced pressure. The resulting residue was subjected to column chromatography on silica (CH_2Cl_2), yielding compound **8** as a yellow solid powder (1198.2 mg, 73.5%). mp. 176-179 °C; ^{1}H NMR (400 Hz, $CDCl_3$): δ = 1.23 (t, 6 H), 1.47 (s, 9 H), 3.38 (bs, 2H), 3.44 (q, 4H), 3.51 (bs, 4H), 3.72 (bs, 2H), 6.54 (s, 1H), 6.66-6.68 (d, *J* = 7.6, 1H), 7.31-7.34 (d, *J* = 9.2, 1H), 7.88 (s, 1H) ; ESI-MS m/z 430.4 $[M+H]^+$.

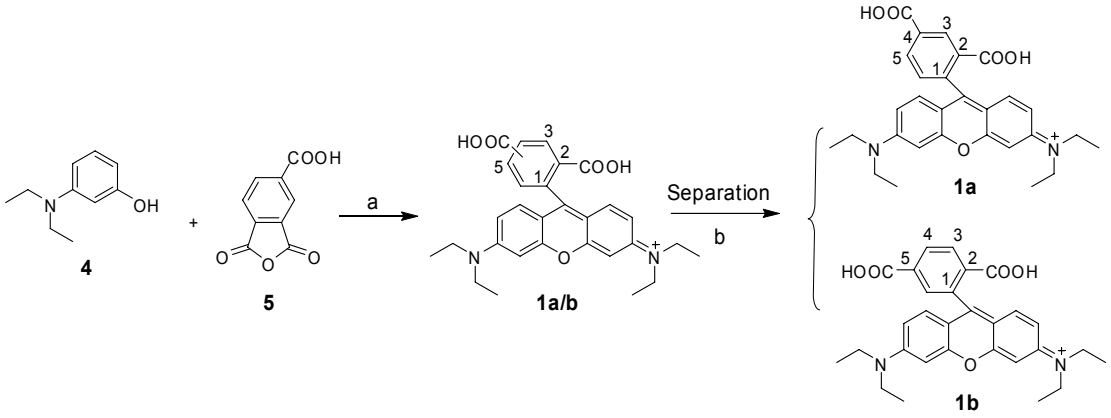


8 2

Synthesis of 2: Compound **8** (9.1 g, 21.3 mmol) was dissolved in CH_2Cl_2/CF_3COOH solution (10 ml, V/V = 1: 1), and the solution was stirred at room temperature for 2 h. The solution was concentrated under reduced pressure and then subjected to flash chromatography on silica (CH_2Cl_2:

C_2H_5OH = 10 : 3), yielding compound **2** as a yellow solid (6.5 g, 92.8%): mp 201-203 ^oC; ^1H NMR (400 Hz, $CDCl_3$): δ = 1.24 (t, 6 H), 3.38 (bs, 4H), 3.45 (q, 4H), 3.72 (bs,2H), 4.01 (bs, 2H), 6.48 (d, *J* = 2.4, 1H), 6.61-6.64 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.32-7.35 (d, *J* = 8.8 Hz, 1H), 7.97 (s, 1H) ; ESI-MS m/z 330.2 $[M+H]^+$.

Synthesis of compound 3: Compound **1a** (177.8 mg, 0.34 mmol) was suspended in CH_2Cl_2 (20 ml) at room temperature, and then DCC (72.2 mg, 0.34 mmol) and DMAP (3.0 mg, 0.02 mmol) were added. Upon the suspended compounds were completely dissolved, coumarin amine **2** (111.9 mg, 0.34 mmol) was slowly introduced to the solution with vigorous stirring, and the solution was further stirred at room temperature for 30 min. The solvent was removed under reduced pressure, and the resulting crude products were purified by preparative thin layer chromatography (CH_2Cl_2: CH_3OH 9: 1) to yield pure compound **3** as a dark purple solid (24.2 mg, 60.5%): mp 218-221 ^oC; ^1H NMR (400 Hz, CD_3OD): δ = 1.20-1.23 (t, *J* = 6.8 Hz, 6H), 1.26-1.30 (t, *J* = 6.4 Hz, 12H), 3.49-3.51 (q, 4H), 3.53-3.89 (16 H), 6.56 (s, 1H), 6.77-6.79 (d, *J* = 8.4 Hz, 1H), 6.92 (s, 2H), 6.98-6.99 (d, *J* = 7.6 Hz, 2H), 7.25-7.27 (d, *J* = 7.2 Hz, 2H), 7.37-7.39 (d, *J* = 7.2 Hz, 1H), 7.46-7.48 (d, *J* = 8.8 Hz, 1H), 7.69-7.71 (d, *J* = 7.6 Hz, 1H),7.98 (s, 1H), 8.14 (bs, 1H); ^{13}C NMR (125 Hz, CD_3OD): 12.71, 12.98, 45.85, 46.74, 97.23, 97.68, 108.98, 111.16, 115.04, 115.18, 115.21, 115.94, 131.53, 146.61, 153.69, 156.90, 158.69, 159.35, 167.64, 181.90; ESI-MS m/z 798.4 $[M]^+$; HRMS (ESI) m/z calcd for $C_{47}H_{52}N_5O_7$ ($[M]^+$): 798.3861. Found 798.3857.



Scheme S1. Synthesis of the intermediate compound **1a**. Reagents and Conditions: a) 85% H_3PO_4, 170-180 ^oC; b) Separation by preparative thin layer chromatography (CH_2Cl_2: CH_3OH = 6: 1).

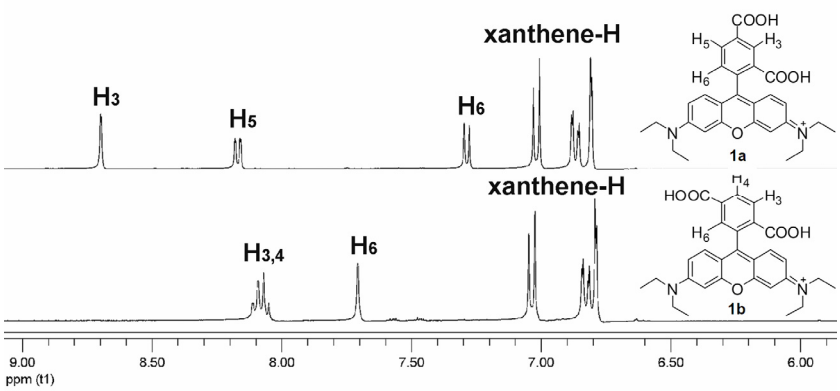
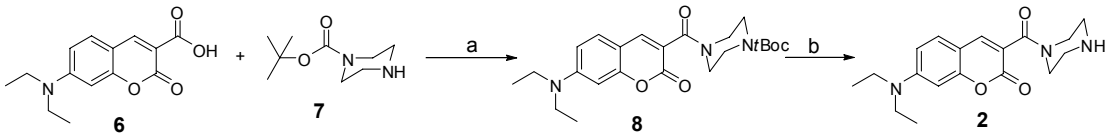
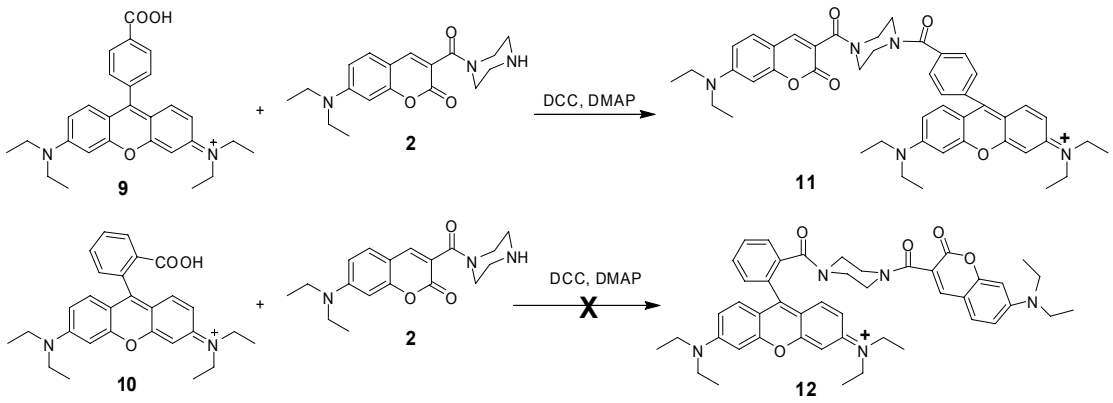


Figure S1. ^{1}H NMR spectra of 4- and 5-position regioisomers **1a** and **1b** in CD_3OD at 400 MHz.

The 4- and 5-position regioisomers **1a** and **1b** have distinct ^{1}H NMR profiles especially for the three phenyl protons, which provide a basis for their differentiation (Figure S1). For compound **1a**, signals for the two neighboring aryl protons at position-5 and position-6 (H_{arom}-5 and H_{arom}-6) were assigned as the two separate peaks at 8.16-8.18 (dd, J = 8.0 Hz, 1.2 Hz) and 7.28-7.30 (d, J = 8.0 Hz) ppm, respectively. The proton located at position-3 (H_{arom}-3) was assigned to the peak at 8.70 ppm (d, J = 1.2 Hz), which has a marked downshift when compared to those of H_{arom}-5 and H_{arom}-6 supporting that H_{arom}-3 is positioned between the two carboxyl groups. In the case of compound **1b**, the resonances for the two neighboring aryl protons (H_{arom}-3 and H_{arom}-4) appeared at 8.05-8.11 ppm. The sharp singlet peak at 7.71 ppm was assigned as H_{arom}-6. Thus, two regioisomers **1a** and **1b** can be readily distinguished by ^{1}H NMR.



Scheme S2. Synthesis of the intermediated compound **2**. Reagents and Conditions: a) DCC, DMAP, CH_2Cl_2; b) CF_3COOH, CH_2Cl_2.



Scheme S3. Synthesis of the compounds **11-12**.

Notably, reaction of rhodamine acid **9** with coumarin amine **2** by the standard coupling chemistry (DCC/DMAP) smoothly afforded amide compound **11**. By contrast, no reaction occurred when treatment of rhodamine acid **10** with coumarin amine **2** under the same conditions. The distinction in reactivity can be ascribed to the fact that the 4-position carboxylic acid is less sterically hindered than the 2-position carboxylic acid.

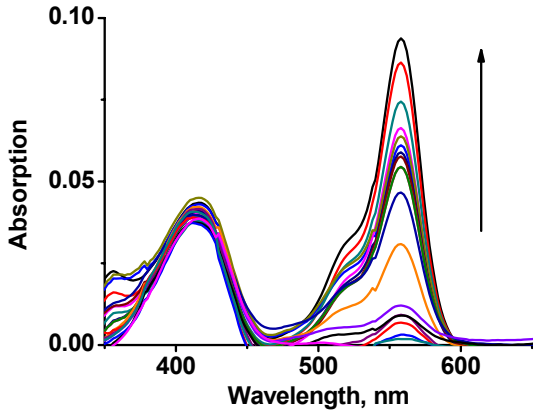
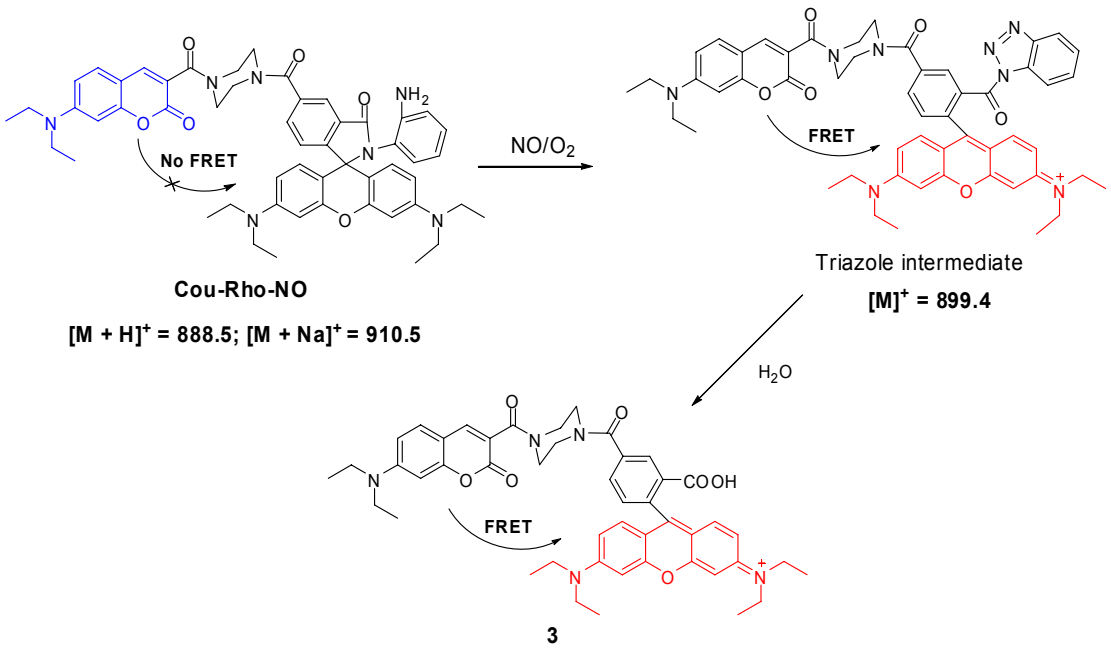


Figure S2. Absorption spectra of **Cou-Rho-NO** (1 μM) in the presence of various amounts of NO (0 - 100 equiv.) at 37 °C in PBS (pH 7.4, containing 20% CH_3CN as a cosolvent). The spectra were obtained after incubation of **Cou-Rho-NO** with DEA/NONOate (a commercially available NO donor) for 30 min.



Scheme S4. Proposed NO-induced FRET sensing mechanism for ratiometric **Cou-Rho-NO**.

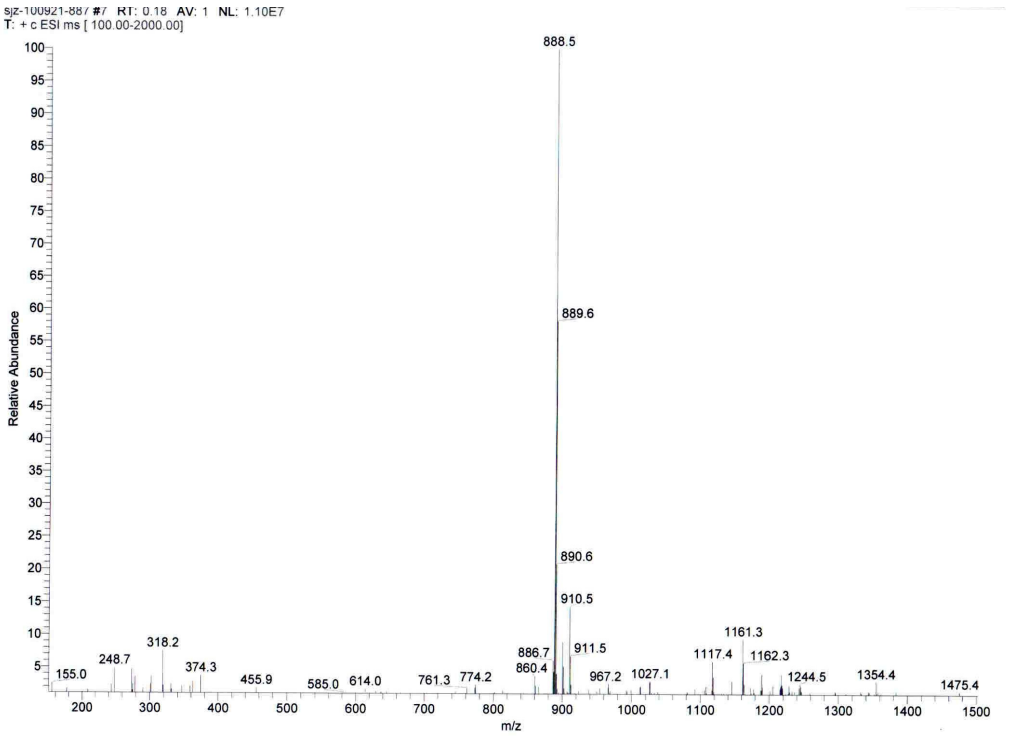


Figure S3. ESI-MS spectrum of **Cou-Rho-NO** in pH 7.4 PBS/CH_3CN (8: 2) under aerobic conditions. The peaks at m/z 888.5 and 910.5 correspond to (**Cou-Rho-NO** + H)$^+$ and (**Cou-Rho-NO** + Na)$^+$, respectively.

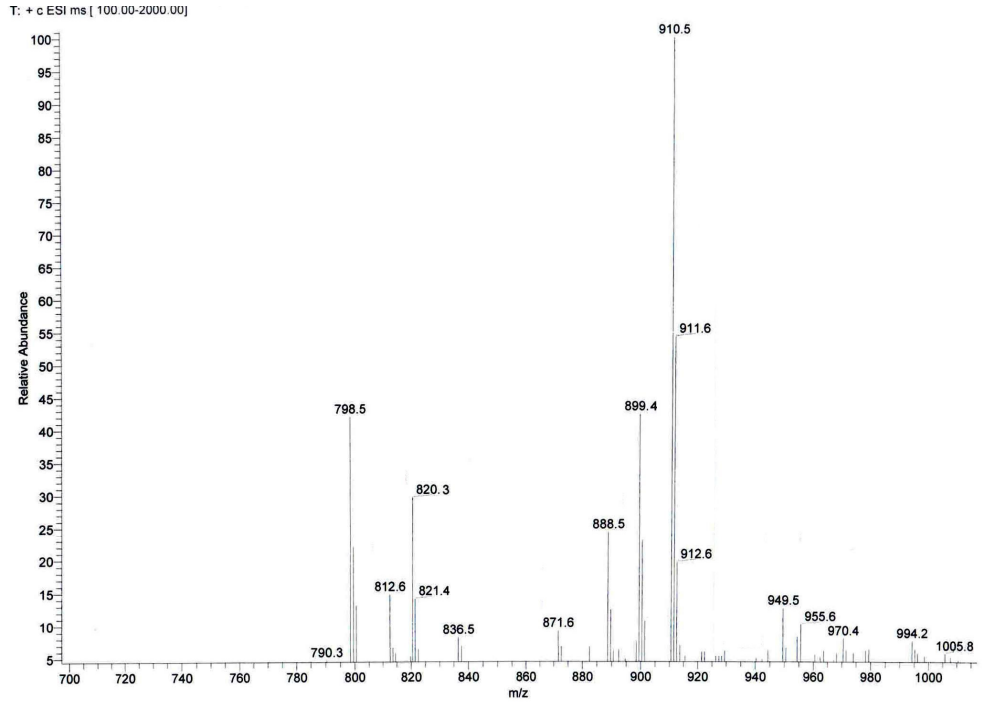


Figure S4. ESI-MS spectrum of **Cou-Rho-NO** after treatment with NO donor (DEA/NONOate) in pH 7.4 PBS/CH_3CN (8: 2) for 0.5 min under aerobic conditions. The peaks at m/z 888.5 and 910.5 are ascribed to (**Cou-Rho-NO** + H)$^+$ and (**Cou-Rho-NO** + Na)$^+$, respectively; the peak at m/z 899.4 corresponds to the intermediate triazole; the peaks at m/z 798.5 and 820.3 are ascribed to the (**3**)$^+$ and (**3** + Na - H)$^+$, respectively.

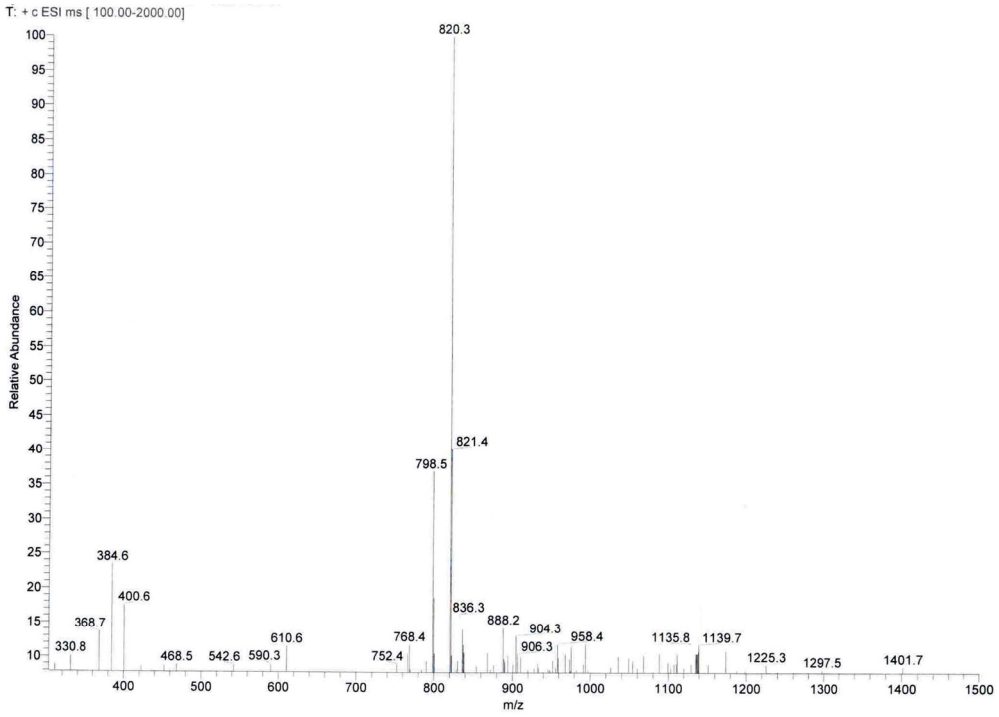


Figure S5. ESI-MS spectrum of **Cou-Rho-NO** after treatment with NO donor (DEA/NONOate) in pH 7.4 PBS/CH_3CN (8: 2) for 30 min under aerobic conditions. The peaks at m/z 798.5 and 820.3 are ascribed to (**3** + H)$^+$ and (**3** + Na)$^+$, respectively.

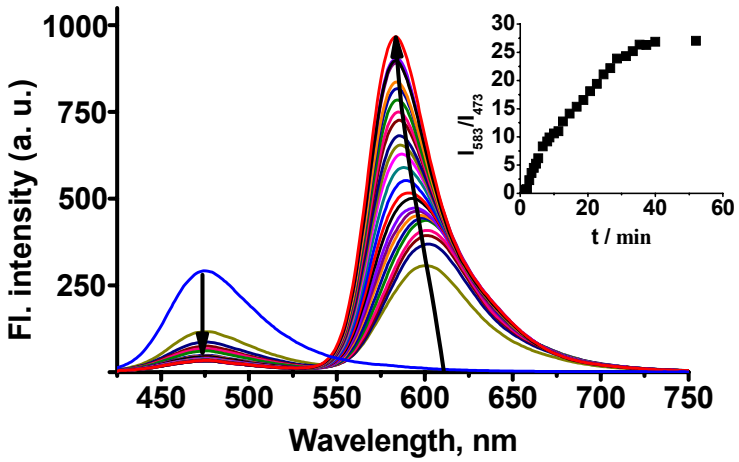


Figure S6. Fluorescence response of **Cou-Rho-NO** (1 μM) to NO (100 equiv.). The spectra were recorded after incubation of **Cou-Rho-NO** with NO donor (DEA/NONOate) for 0 to 37 min in PBS (pH 7.4, containing 20% CH_3CN as a cosolvent), with excitation λ = 410 nm. The arrow (in the right) indicates the changes of the emission intensities and *wavelengths (from around 600 to 583 nm)* with the incubation time variation from 0 to 37 min. The inset displays the emission ratio changes with the incubation time.

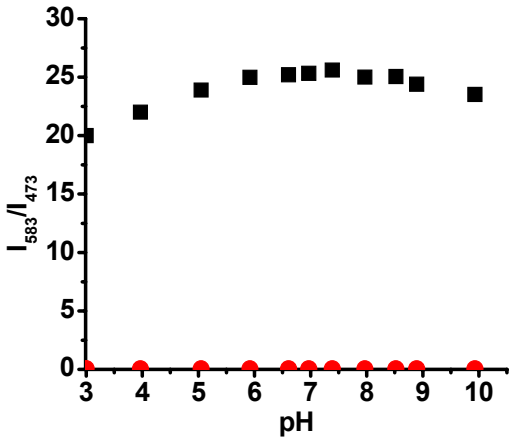


Figure S7. Effects of pH on the fluorescence ratio (I_{583}/I_{473}) of **Cou-RHO-NO** (1 μM) in the absence (●) or presence (■) of NO gas (100 equiv.).

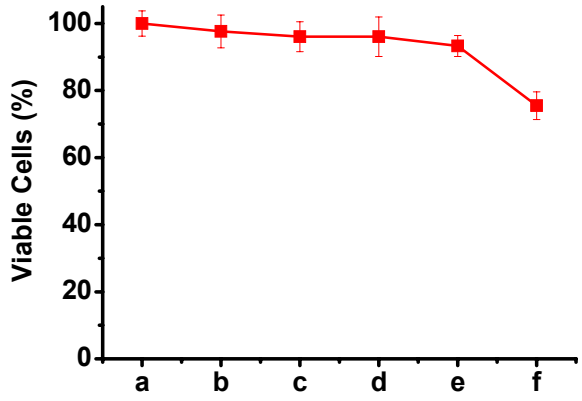


Figure S8. Cytotoxicity assay of ratiometric sensor **Cou-Rho-NO** at different concentration (a: 0 μM; b: 5 μM; c: 10 μM; d: 20 μM; e: 100 μM; f: 200 μM;) for RAW264.7 Macrophage.

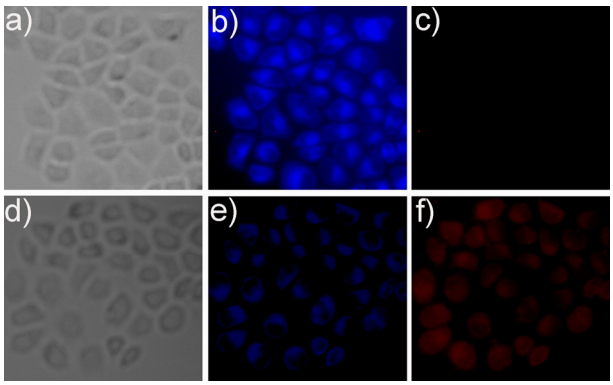


Figure S9. NO-induced ratiometric fluorescence response in live HeLa cells: (a) Bright field image of HeLa cells incubated with **Cou-Rho-NO** (5 μM); (b) Fluorescence image of (a) from blue channel; (c) Fluorescence image of (a) from red channel; (d) Brightfield image of HeLa cells incubated with **Cou-Rho-NO** (5 μM) for 30 min, and then further incubated with DEA/NONOate (50 μM) for 30 min at 37 °C; (e) Fluorescence image of (d) from blue channel; (f) Fluorescence image of (d) from red channel.

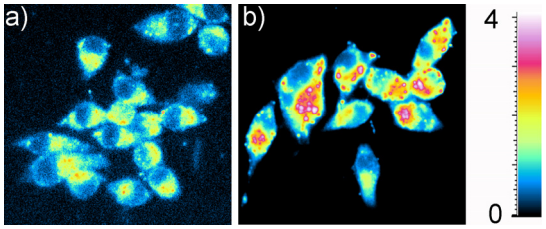


Figure S10. (a) Ratio fluorescence (I_{610} / I_{450}) image of Raw 264.7 macrophages incubated with only **Cou-Rho-NO** (5 μM). (b) Ratio fluorescence (I_{610} / I_{450}) image of Raw 264.7 cells co-incubated with 5 μM **Cou-Rho-NO**, 1.25 μg/mL LPS, and 1,000 U/mL IFN-γ.