Supporting Information

A nucleus targetable fluorescent probe for ratiometric imaging of endogenous NO in living cells and zebrafishes

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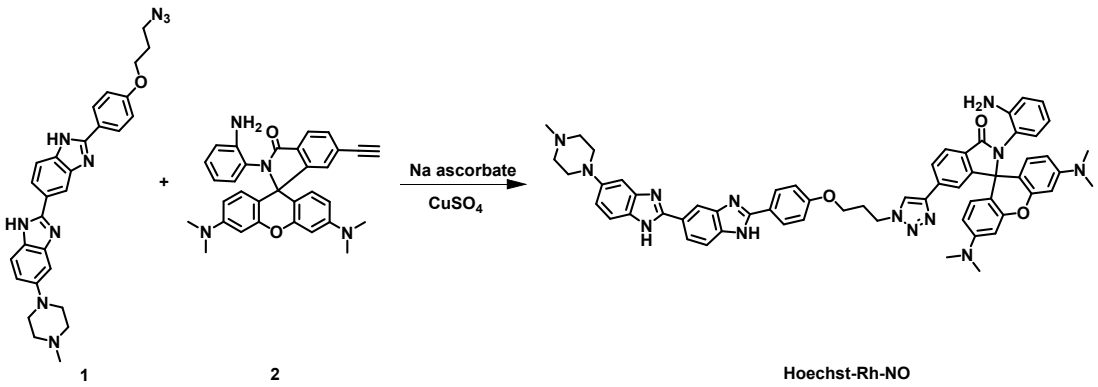
1. Experimental Procedures

1.1 Materials and apparatus.

Unless otherwise noted, reagents and solvents were purchased from TCI and Sigma-Aldrich and used without further purification. Hoechst 33342, LPS was obtained from Thermo Fisher and Sigma-Aldrich. All solvents were HPLC reagent grade, and ultrapure water from a Millipore Direct-Q system was used throughout the analytical experiments. Silica gel was used for column chromatography. 1HNMR spectra were collected in $CDCl_3$, DMSO-d6, and MeOD-d4 with TMS as internal reference (Cambridge Isotope Laboratories) on Bruker 400 MHz spectrometers. High-resolution electrospray-ionization (ESI) mass spectra were recorded on a Thermo Scientific LTQ Orbitrap XL mass spectrometer. Fluorescence measurements were recorded on an Agilent Cary Eclipse fluorescence spectrophotometer using quartz cuvettes with a path length of 1 cm. Fluorescence images were obtained with a confocal laser scanning microscope (Olympus Fluoview FV1000).

1.2 Synthesis of Hoechst-Rh-NO probe



Compound 1 (25 mg, 49.25 μmol) and compound 2 (21 mg, 41.95 μmol) were put into a 25 ml Schlenk bottle, vacuum and fill with argon. Add THF and DMF after bubbling. Take the centrifuge tube, dissolve Na ascorbate and $CuSO_4$(12 mg, 75.19 μmol) with a few drops of water, and add them to the Schlenk bottle. The reaction solvent is 1.5 ml, and the volume ratio is DMF : THF : water = 3 : 3 : 2. After reacting for 4 hours, pass the column with 100 mesh silica gel. Hoechst-Rh-NO 13 mg was obtained, and the yield was 26.18%. ^{1}H NMR (400 MHz, DMSO) δ 8.79 (s, 1H), 8.34 (s, 1H), 8.11 (d, J = 7.6 Hz, 3H), 7.99 (t, J = 11.7 Hz, 2H), 7.72 (d, J = 8.3 Hz, 1H), 7.60 (d, J = 8.4 Hz, 1H), 7.49 (s, 2H), 7.36 (s, 1H), 7.17 (d, J = 22.4 Hz, 1H), 7.07 (d, J = 6.3 Hz, 2H), 6.93 (s, 1H), 6.85 (t, J = 7.3 Hz, 1H), 6.74 (s, 2H), 6.55 (d, J = 8.0 Hz, 1H), 6.43 (s, 2H), 6.29 (s, 2H), 6.20 (t, J = 7.4 Hz, 1H), 5.93 (d, J = 7.9 Hz, 1H), 5.32 (s, 1H), 4.56 (s, 2H), 4.43 (s, 2H), 4.08 (s, 2H), 3.09 (d, J = 35.6 Hz, 5H), 2.88 (s, 12H), 2.34 (s, 2H), 2.26 (s, 3H), 2.00 (dd, J = 15.3, 7.5 Hz, 2H). HRMS (ESI) calced for $H_{60}C_{57}N_{13}O_3$, $[M+H]^+$ 1008.4707, found 1008.4789.

1.3 General Procedures for Fluorescence Spectroscopy.

Unless otherwise noted, all the spectrophotometric measurements were made in a spectral-pure solvent containing 1% DMSO as the cosolvent according to the following procedure. Dissolve Hoe-Rh-NO in DMSO to obtain a 4mM stock solution. The probe (5 μM) in a solution of 1.4ml Tris-HCl (pH=7.4) and 0.6 ml CH_3CN containing calf thymus DNA (500 μM base pairs) were cultured at 35 °C for 1.5 hours to bind the probe to DNA.

Preparation of aqueous NO solution. The preparation and concentration of NO were determined by the Griess method reported previously.[1] Since O_2 can rapidly oxidize NO to form NO_2, all apparatus was carefully degassed with argon for 30 min to remove O_2. The NO gas was bubbled through a saturated NaOH solution to eliminate NO_2 generated from the reaction of NO with O_2. To produce a NO solution as a stock solution, this gas was bubbled through 10 mL of deoxygenated deionized water for 30 min and kept under an NO atmosphere. The concentration of this stock solution was determined by the Griess method.

The detection limit was calculated based on the method reported in the literature.[2] The fluorescence emission spectrum of Hoe-Rh-NO was measured by ten times, and the standard deviation of blank

measurement was obtained. The ratio of fluorescence intensity at 603 nm and 460nm was plotted as a concentration of NO. The detection limit was calculated by 3σ/k, where σ is the standard deviation of blank measurement, and k is the slope of the ratio of fluorescence intensity versus NO concentration.

1.4 Ion selectivity.

Calculate the ratio of the fluorescence intensity of Hoe-Rh-NO (5 μM) in the presence of various reactive oxygen and nitrogen (10 equiv). The probe (5 μM) in a solution of 1.4ml Tris-HCl (pH=7.4) and 0.6 ml CH_3CN containing calf thymus DNA (500 μM base pairs) were cultured at 35 °C for 1.5 hours to bind the probe to DNA. Calculate the ratio of the fluorescence intensity of Hoe-Rh-NO (5 μM) in the presence of various reactive oxygen and nitrogen (10 equiv).

Hydrogen peroxide (H_2O_2) was diluted immediately from a stabilized 30% solution, and its concentration was determined by using its molar absorption coefficient of 43.6 M^{-1} cm^{-1} at 240 nm.[3] Freshly prepared aqueous solutions of $NaNO_2$ and $NaNO_3$ were used as nitrate (NO_2^-) and nitrate (NO_3^-) sources, respectively. Singlet oxygen was chemically generated from the $^-OCl/H_2O_2$ system in the buffer.[4]Hydroxyl radicals (•OH) were generated in the Fenton system from ferrous ammonium sulfate and hydrogen peroxide.[5] Superoxide (O_2^-) solution was obtained from 10 min vigorous stirring after adding KO_2(1 mg) to dry dimethyl sulfoxide (1 mL). $ONOO^-$ was synthesized from sodium nitrite (0.6 M) and H_2O_2 (0.65 M) (excess H_2O_2 was used to minimize nitrate contamination). After the reaction, the solution was treated with MnO_2 to eliminate the excess H_2O_2.

1.5 MTT assay.

Macrophages and Hela cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2/95% air incubator. The cells were seeded on 96 well plates (3 × 10^4 cells/well, 100 μL). After maintained for 24 h, Hoe-Rh-NO (0, 10 μM dispersed in 100 μL medium) was added to the wells and incubated for more 24 h. Then MTT (5.0 mg/mL, 20 μL) was added to the wells. The medium was removed after 4 h, and 150 μL DMSO was added to dissolve the formazan crystals. The optical density (OD) was recorded at 490 nm by a Thermo Scientific Multiskan GO spectrophotometer.

1.6 Cell culture.

Hela Cells were obtained from the Institute of Basic Sciences (IBMS) of the Chinese Academy of Medical Sciences (CAMS). The cell clones were cultured in MEM supplemented with 10% fetal calf serum (FBS) and streptomycin (0.1 mg/mL) at 37 °C in 95% air with 5% CO_2.

Macrophages were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The cells were all maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone). The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C (CO_2 incubator, Thermo Scientific) and grown on 25 mm coverslips (Fisherbrand, 12-545-102) for 1-2 days to reach 70 - 90% confluency before use.

1.7 Intracellular fluorescence imaging.

Hela cells in the exponential phase of growth were grown on 35 mm glass-bottom culture dishes for 24-36 h to reach 80% confluency. The cells were washed three times with PBS (pH = 7.4) and then incubated with 1mL of MEM containing Hoe-Rh-NO (5 μM) in an atmosphere of 95% air and 5% CO_2 for 2 hours at 37 °C. The cells were washed three times with PBS. Several groups of fluorescence images were obtained by gradually adding NO solution at the same position.

The culture medium of macrophages was removed out, add 1mL DMEM containing Hoe-Rh-NO (5.0 μM), incubate at 37 °C, 95 % air and 5 % CO_2 for 2 hours. The cells were washed with PBS for 3 times at room temperature. The culture medium was changed and the images were obtained after 12 hours of LPS stimulation.

The image was recorded with 60 × objectives using confocal microscopy (Olympus FV1000). The excitation wavelength was set to 405 nm for Hoe-Rh-NO, with an emission-signal range of 430-530 nm and 565-665 nm. Figures were processed with FV10-ASW Viewer (4.2 b, Olympus) and Image J.

1.8 Culture of Zebrafish.

The AB line zebrafish embryo was obtained from Shanghai Fish Bio Co. Ltd. and incubated with embryo culture fluid under 28.5 °C. Zebrafish embryo culture medium was changed daily, and following the 1.4:1 contrast ratio was cultured. All animal experiments comply with the "National Laboratory Animal Management Regulations" of MOST.

The zebrafish embryo were transferred to six-well plates and incubated with E3 embryo media (15 mM NaCl, 0.5 mM KCl, 1mM $MgSO_4$, 1 mM $CaCl_2$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 0.7 mM $NaHCO_3$, 5% methylene blue; pH 7.5). zebrafish embryo were cultured to 36 hpf and transferred to six-well plates with 20 tails per well.

Zebrafish and 5 μM Hoe-Rh-NO was stained in the culture medium for 6 hours, then the culture medium was changed, stimulated under different conditions for 12 hours, and anesthetized with MS-222. The excitation wavelength was set to 405 nm for Hoe-Rh-NO, with an emission-signal range of 430 - 530 nm and 565 - 665 nm. Figures were processed with FV10-ASW Viewer (4.2 b, Olympus) and Image J.

1.9 Ethical statement.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Dalian University of Technology and approved by the Animal Ethics Committee of Dalian University of Technology.

2. Supplementary figures and tables

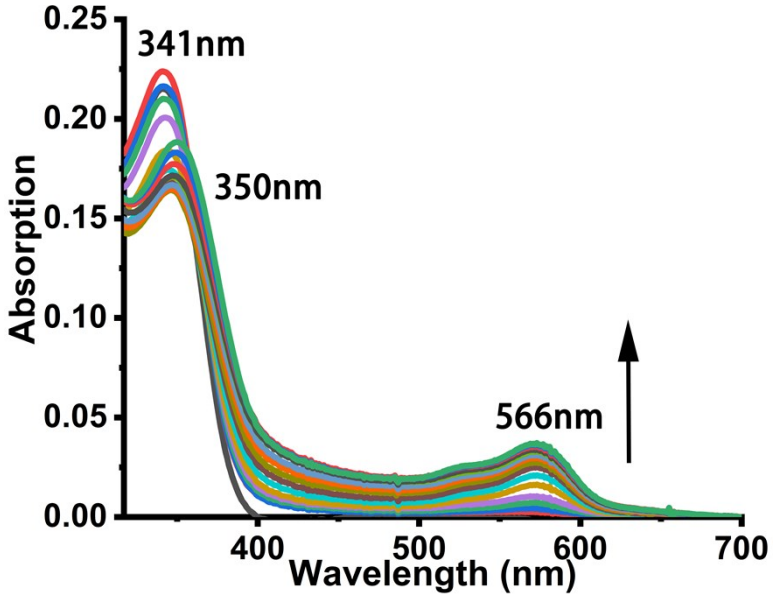


Fig. S1. In a solution of pH 7.4 (1.4 ml Tris-HCl) and 0.6 ml CH_3CN, containing Hoe-Rh-NO (5 μM), the absorption is measured by gradually adding NO solution from 0 to 170 μM Spectral changes.

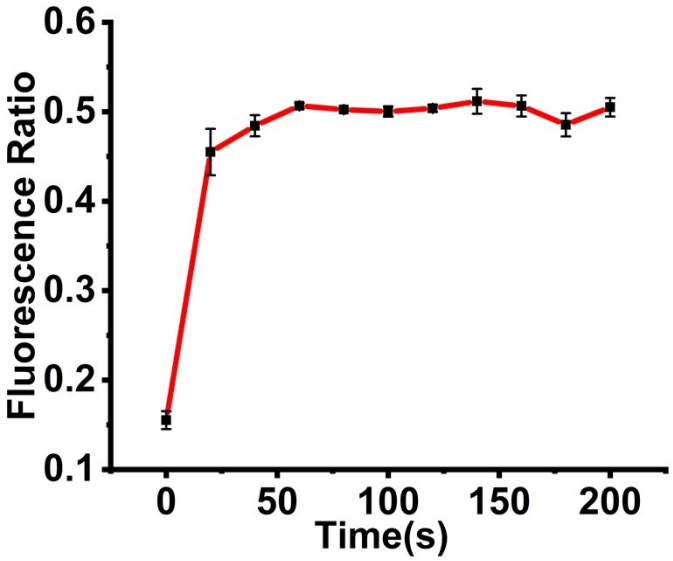


Fig. S2. In a solution of pH 7.4 (1.4 ml Tris-HCl) and 0.6 ml CH_3CN, containing Hoe-Rh-NO (5 μM). Add 200 μM NO and measure its fluorescence ratio (I_{603}/I_{463}).

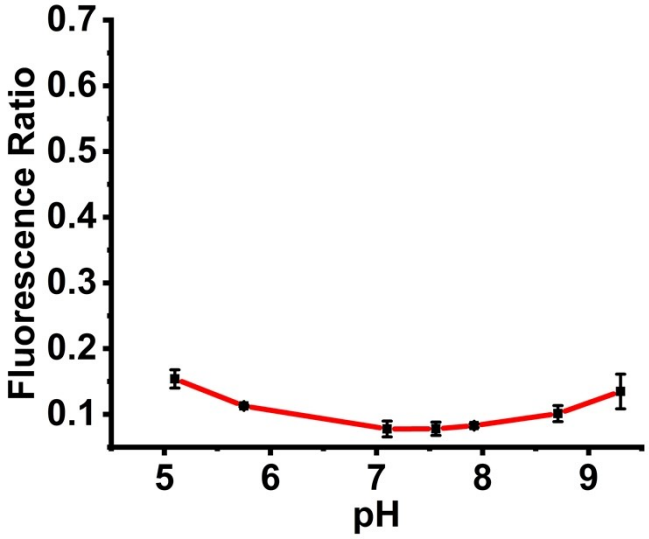


Fig. S3. pH response of Hoe-Rh-NO (5 μM) in solution (PBS/EtOH/CH_3CN, 1/1/1, v/v/v).

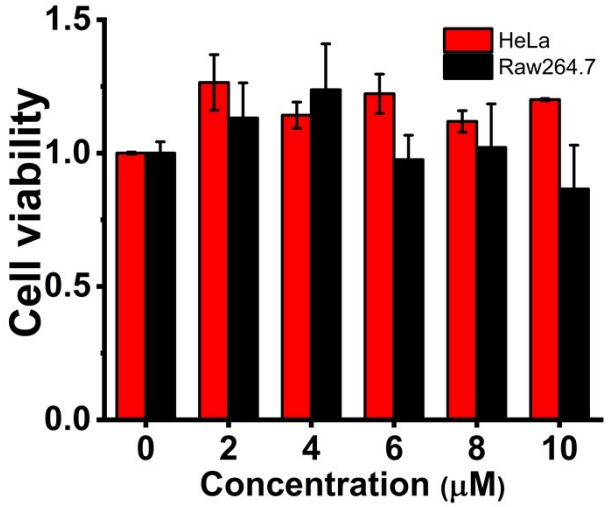


Fig. S4. Cell viability in 24 h of Hoe-Rh-NO at different concentrations.

3. References

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HRMS and ^{1}H NMR of Hoe-Rh-NO