Supporting Information

A Nitroreductase-Sensitive Near-IR Fluorescent Biosensor for Detecting Tumor Hypoxia in Vivo

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Synthesis of Cy7-Cl

Cy7-Cl was synthesized following a reported procedure.[1] Briefly, sodium acetate (164 mg, 2.0 mmol) was added to a mixture of 2-chloro-1-formyl-3-(hydroxymethylene)cyclohex-1-ene (173 mg, 1.0 mmol) and 1-(5-ethyl)-2,3,3-trimethylindoleninium bromide (670 mg, 2.5 mmol) in 5 mL of ethanol under nitrogen. The reaction mixture was stirred at 80 ℃ for 2 h. After removing the solvent under vacuum, 10 mL of water was added, and the mixture was extracted with DCM. The crude product was purified by silica gel column chromatography using a mixed eluent of DCM and methanol to provide the product as a green solid (480 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.34 (d, J = 14.1 Hz, 2H), 7.43-7.37 (m, 4H), 7.27-7.18 (m, 4H), 6.29 (d, J = 14.1 Hz, 2H), 4.30 (q, J = 7.2 Hz, 4H), 2.77 (t, J = 5.9 Hz, 4H), 1.99 (t, J = 5.9 Hz, 2H), 1.72 (s, 12H), 1.47 (t, J = 7.2 Hz, 6H).

Synthesis of Compound 1

A mixture of 4-nitrobenzyl bromide (216 mg, 1.0 mmol) and KSAc (228 mg, 2.0 mmol) in 3 mL of methanol was stirred at room temperature for 3 h under N_2. After removing the solvent by rotary evaporation, the crude was dissolved into DCM and washed with brine. The organic phase was dried over Na_2SO_4 and condensed under vacuum, yielding the final product a yellowish solid (205 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.15 (d, J = 7.5 Hz, 2H), 7.46 (d, J = 7.5 Hz, 2H), 4.17 (s, 2H), 2.37 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 194.35, 147.12, 145.55, 129.71, 123.86, 32.71, 30.30.

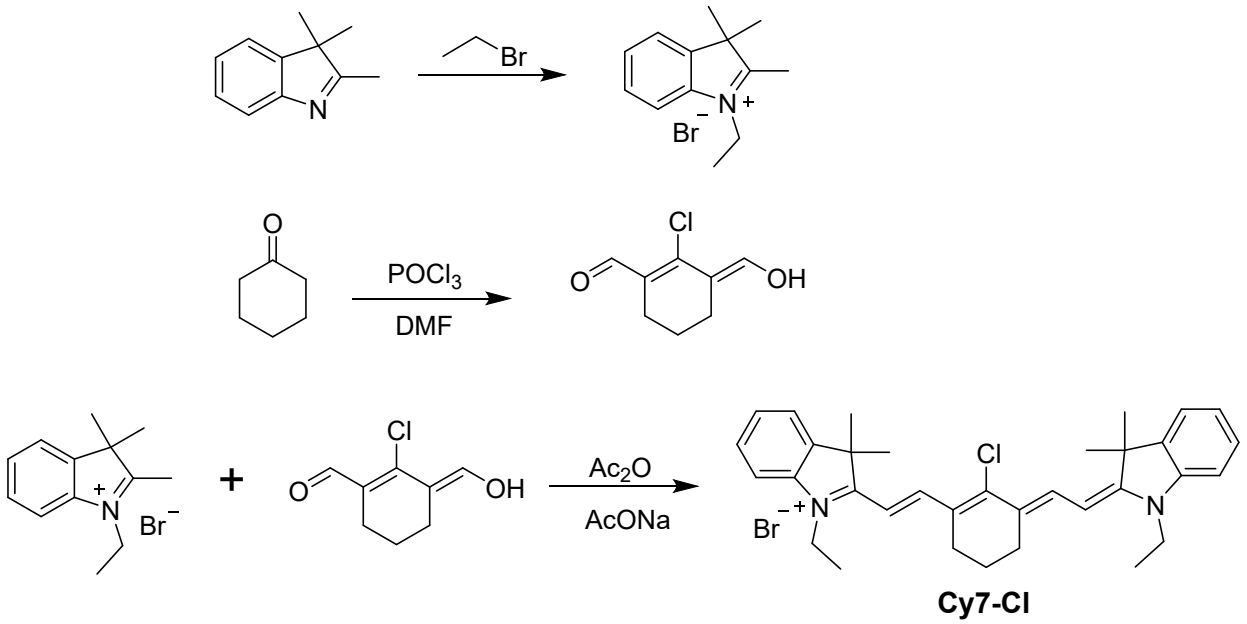
Synthesis of Compound 2

To a solution of compound **1** (211 mg, 1.0 mmol) in 2 mL of methanol, 1 mL of hydrogen chloride in ethanol (2.5 M) was added under N_2. The reaction was heated to 60 °C and stirred for 16 h under N_2. After condensation, the residue was dried under vacuum, and the final product was obtained as a light yellow solid (161 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.15 (d, J = 7.6 Hz, 2H), 7.46 (d, J = 7.6 Hz, 2H), 3.81 (d, J = 7.4 Hz, 2H), 1.84 (t, J = 7.4 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 130.10, 128.98, 123.10, 123.83, 28.42.

Synthesis of Cy7-NO_2

A solution of Cy7-Cl (118 mg, 0.2 mmol), compound **2** (42 mg, 0.25 mmol), and N,N-diisopropylethylamine (52 µL, 0.3 mmol) in 1 mL of methanol was stirred at room temperature for 1 h. After condensation, the residue was purified by flash column chromatography using silica gel as the stationary phase and a mixture of DCM and methanol as the mobile phase. The final product was obtained as a green solid (114.5 mg, 79%). ^{1}H NMR (400

MHz, $CDCl_3$): δ (ppm) 8.57 (d, *J* = 14.1 Hz, 2H), 8.10 (d, *J* = 8.4 Hz, 2H), 7.41-7.34 (m, 6H), 7.26-7.22 (m, 2H), 7.16 (d, *J* = 7.8 Hz, 2H), 6.20 (d, *J* = 14.1 Hz, 2H), 4.22 (q, *J* = 7.1 Hz, 4H), 4.06 (s, 2H), 2.67 (brs, 4H), 1.89 (brs, 2H), 1.62 (s, 12H), 1.45 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 171.67, 154.81, 147.09, 145.57, 145.45, 141.80, 140.89, 134.05, 129.64, 128.87, 125.28, 123.84, 122.34, 110.57, 101.16, 49.17, 40.97, 39.82, 27.81, 26.54, 20.83, 12.42.



Scheme S1. Synthesis of Cy7-Cl.

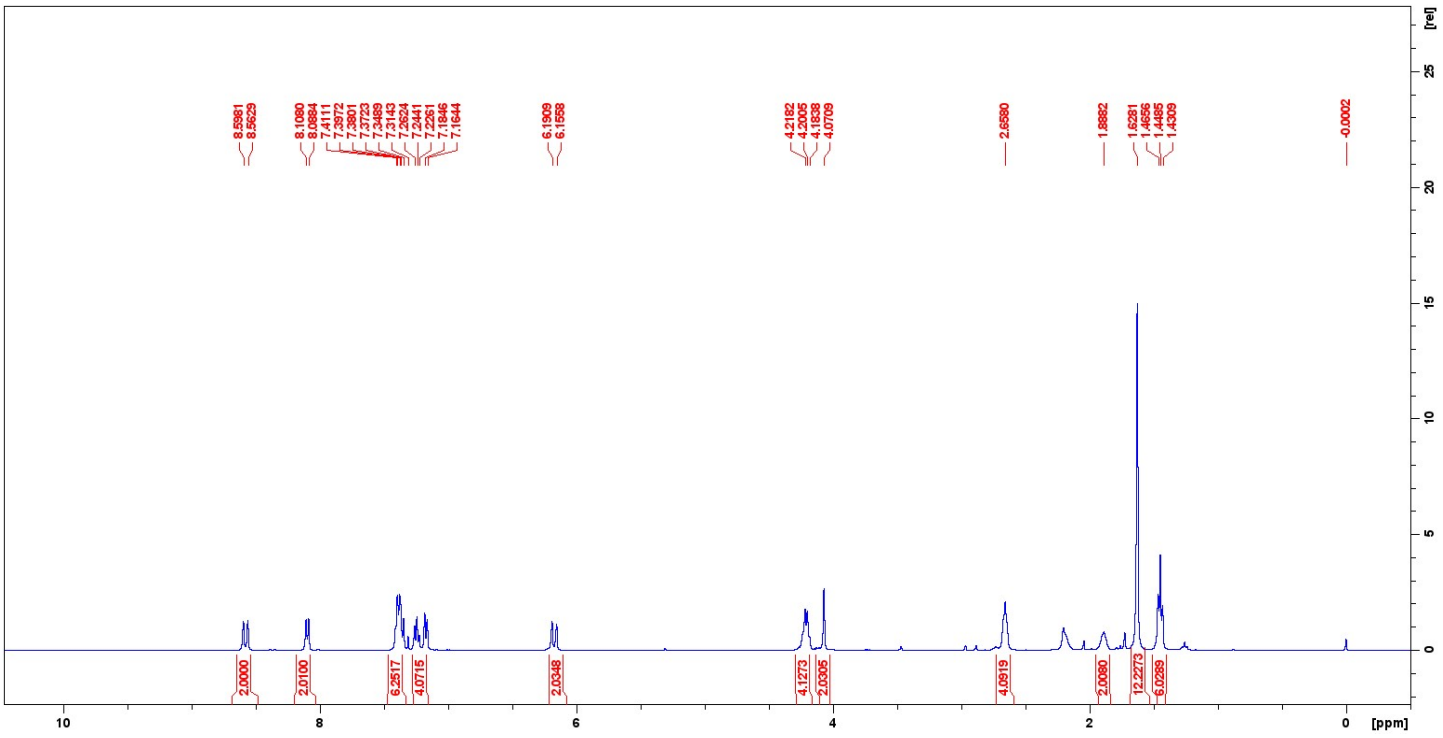


Figure S1. ^{1}H NMR spectrum of Cy7-NO_2.

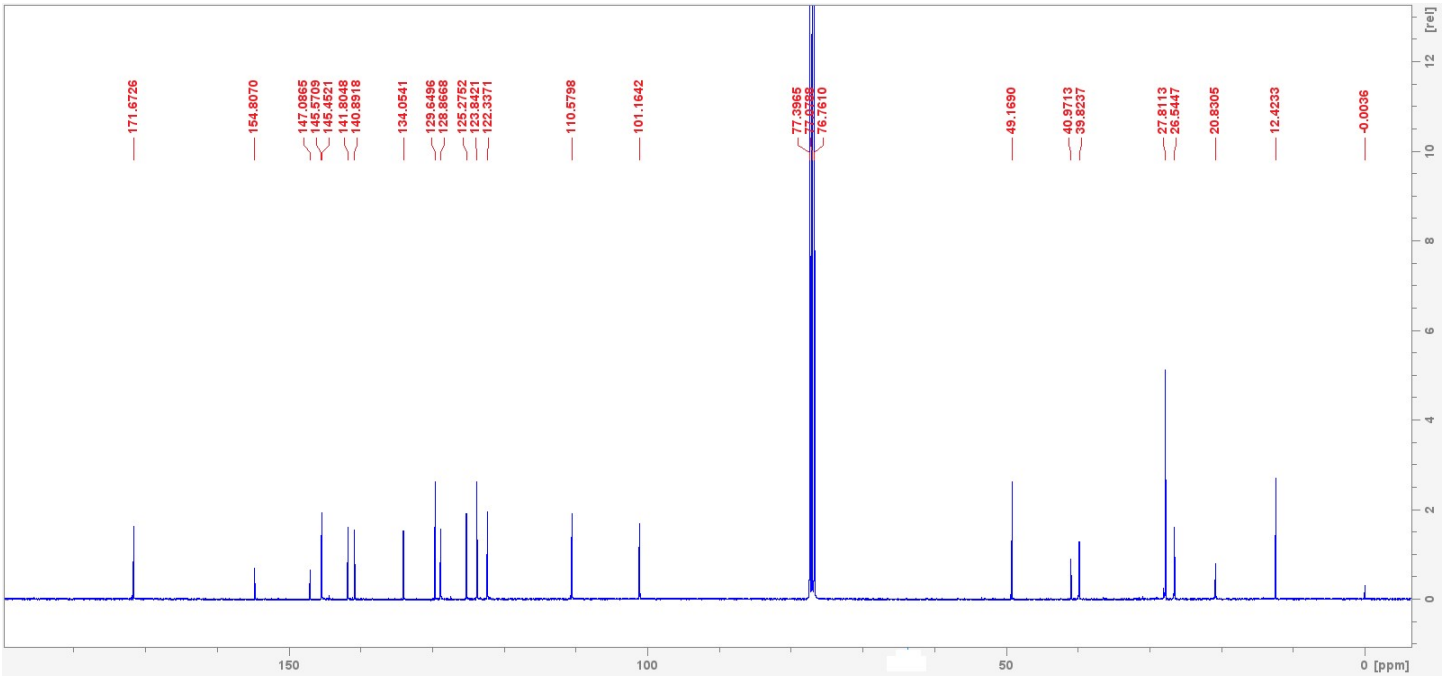


Figure S2. ^{13}C NMR spectrum of Cy7-NO_2.

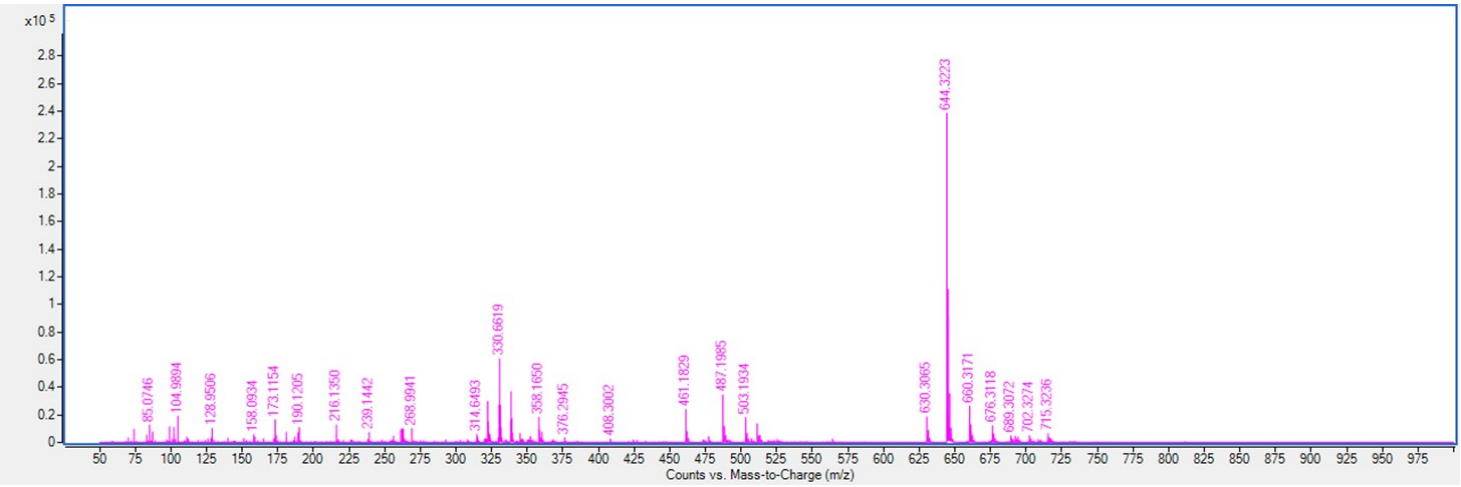


Figure S3. HRMS (ESI, m/z) spectrum of Cy7-NO_2. $C_{41}H_{46}N_3O_2S$ ($[M\text{-}Br]^+$): calcd 644.3305; found 644.3223.

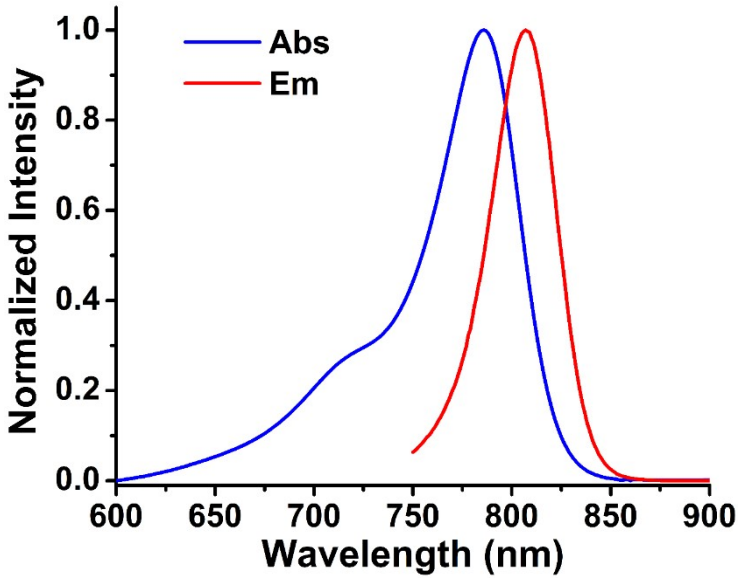


Figure S4. UV-vis absorption (blue) and fluorescence emission (red) spectra of Cy7-NO_2.

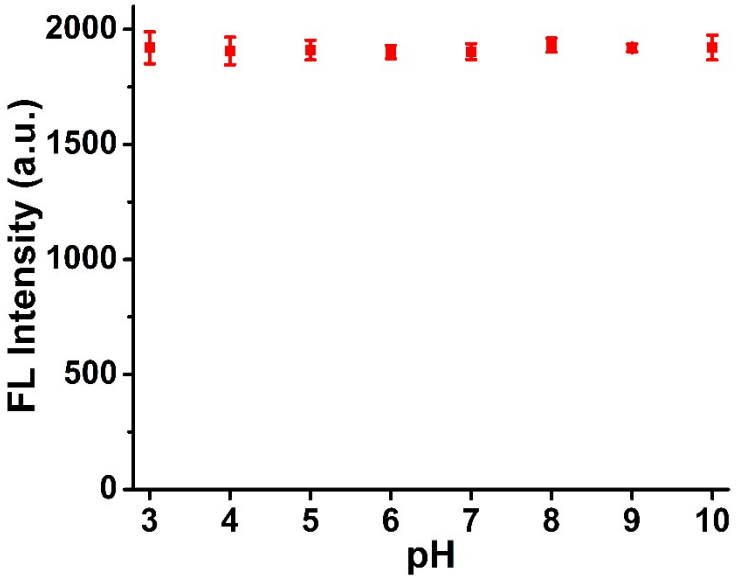


Figure S5. Fluorescence emission intensity of Cy7-NO_2 (10 μM) at varied pH.

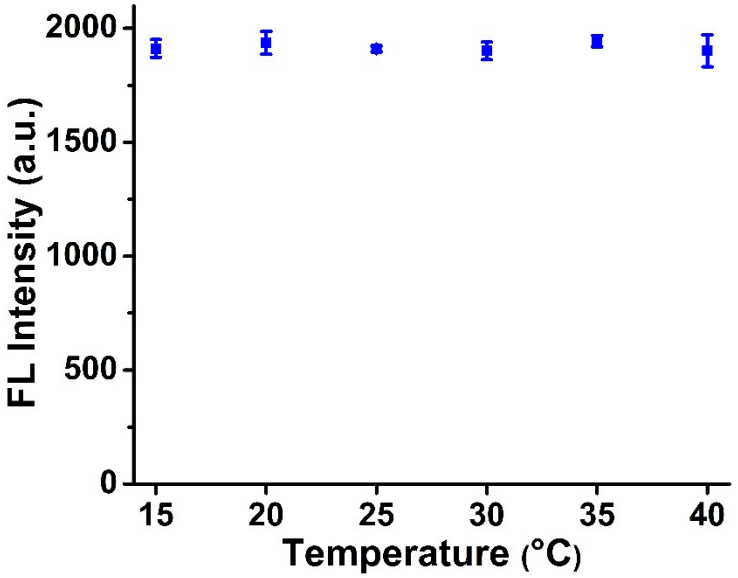


Figure S6. Fluorescence emission intensity of Cy7-NO_2 (10 μM) at varied temperatures.

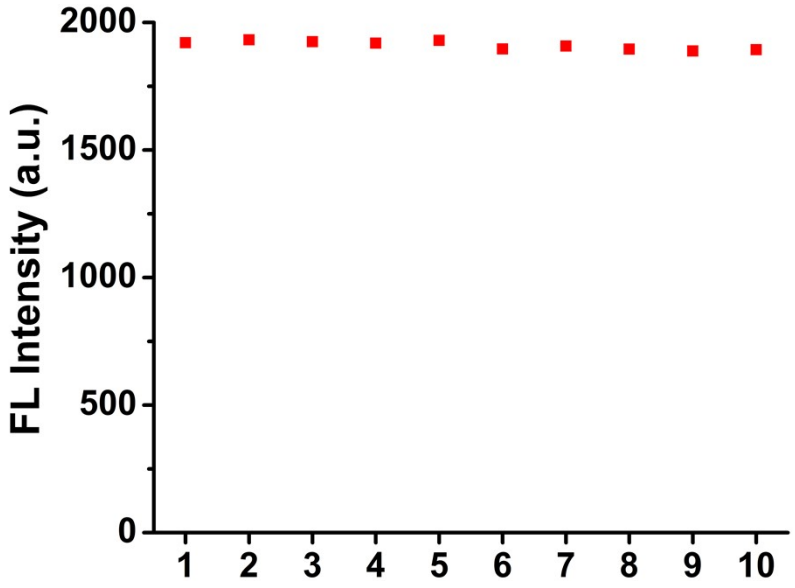


Figure S7. Fluorescence emission intensity of Cy7-NO_2 (10 μM) after 10 consecutive repeating measurements.

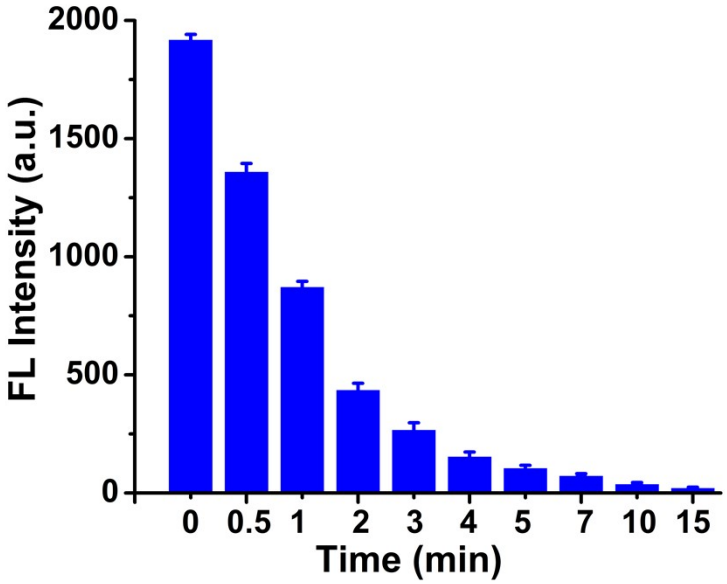


Figure S8. Intensity of time-dependent fluorescence emission of Cy7-NO_2 (10 μM) after the addition of NADH (1 mM) and NTR (2.5 μg/mL).

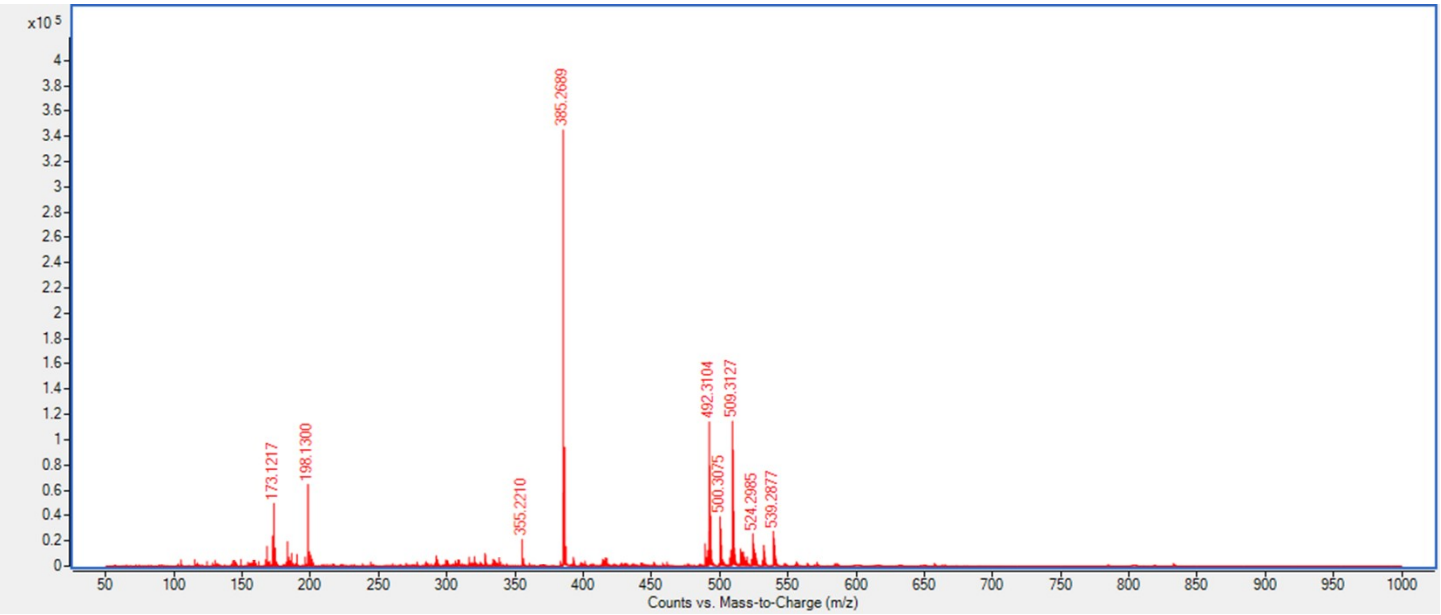


Figure S9. HRMS (ESI, m/z) spectrum of Cy7-SH. $C_{41}H_{46}N_3O_2S$ ([M-Br]$^+$): calcd 509.2985; found 509.3127.

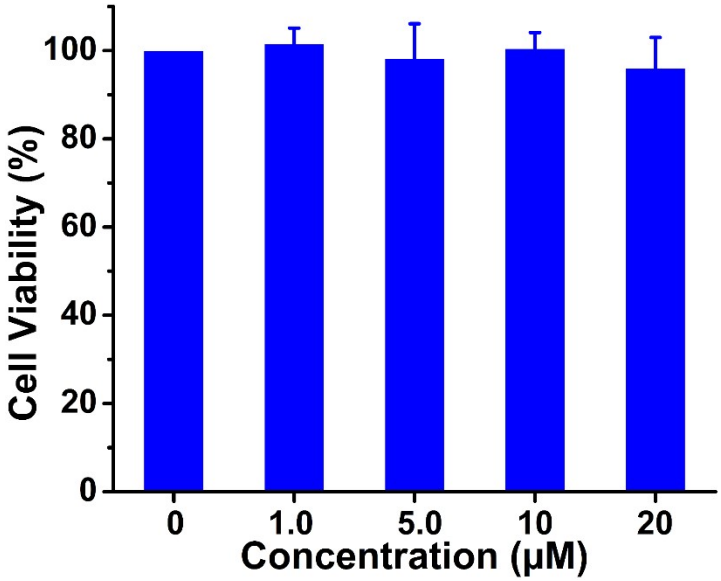


Figure S10. Cell viability of A549 cells after treatments with Cy7-NO_2 for 24 h. Data represent the means ± SD, n = 3.

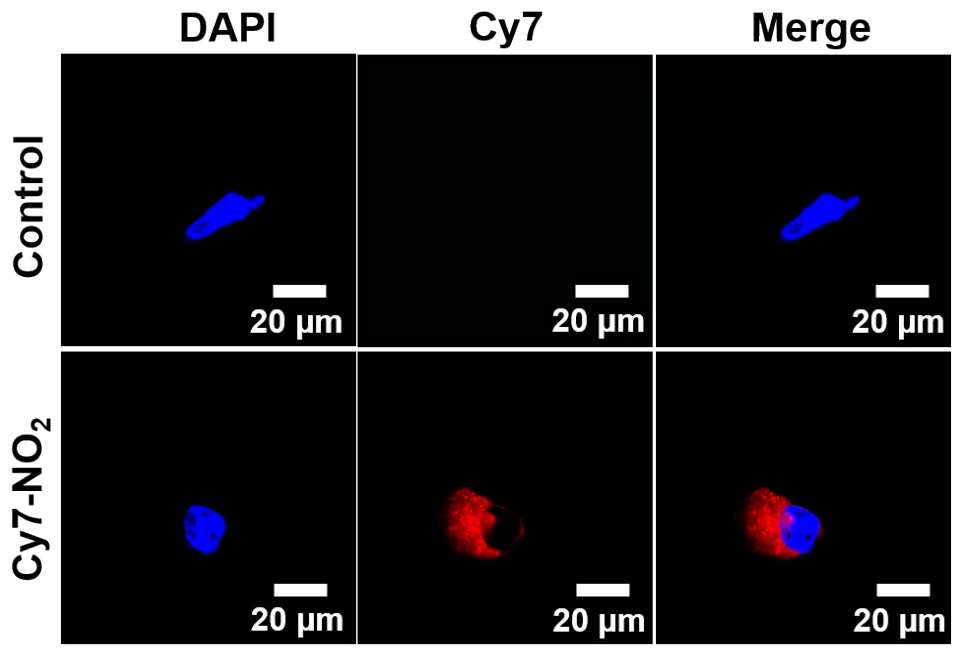


Figure S11. Confocal fluorescence images of A549 cells incubated with Cy7-NO_2. Cell nuclei were stained with DAPI. The blue and red fluorescence is from DAPI and Cy7, respectively.

References

1. Wang, W.; et al. A Self-Assembled "Albumin–Conjugate" Nanoprobe for Near Infrared Optical Imaging of Subcutaneous and Metastatic Tumors. *ACS Appl. Bio Mater.* **2020**, *3*, 327-334.