Electronic Supplementary Information (ESI):

A Cyanine-Derived "Turn-On" Fluorescent Probe for Imaging Nitroreductase in Hypoxic Tumor Cells

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Experimental section

I. Chemicals and apparatus

Unless otherwise stated, all chemicals for synthesis were purchased from commercial suppliers and were used without further purification. Nitroreductase (NTR) expressed in *Escherichia coli* was purchased from Sigma-Aldrich, NADH was obtained from J&K scientific Reagent Ltd (Beijing, China). Cell culture products were purchased from Corning Co., Ltd (NY, USA). All phosphate-buffered solutions (PB) were obtained from Beijing Dingguo Changsheng biotechnology Co., Ltd (Beijing, China). Deionized water from a Millipore water system was used throughout.

Fluorescence spectra measurements were performed on a Thermo Scientific

Varioskan Flash multimode reader (Waltham, MA, USA) and a HITACHI F-7000 spectrofluorimeter (Tokyo, Japan). Confocal fluorescence images were recorded with a FV-1000 confocal laser scanning microscopy (Olympus Co., Ltd, Germany). Nuclear magnetic resonance (NMR) spectra were measured on a JEOL JNM-ECA 300 spectrometer (Tokyo, Japan) using tetramethylsilane (TMS) in the solvent of $CDCl_3$ or MeOD as an internal standard. ESI Mass spectra measurements were performed on a Thermo Fisher LTQ linear ion-trap mass spectrometer (San Jose, CA, USA).

II. Synthesis of the fluorescent probe 1

The desired compound was readily synthesized in reasonable yield from the isophthalaldehyde and the indolinium by a condensation reaction.

4-(4-nitrobenzyloxy) isophthalaldehyde was prepared from 4-hydroxy-benzene- 1,3-dicarbaldehyde with p-Nitobenzylbromide in DMF described as our previous work. While 2,3,3-trimethyl-1-(3-sulfopropyl)-3H-benzo[f]indolinium was prepared from 2,3,3-trimethyl-3H-benzo[f]indolenine (0.21 g, 1 mmol) and 1,3-propane sultone (0.15 g, 1.2 mmol) in toluene (5 mL) at 90℃ for 4h under N_2, which was cooled to room temperature, filtered and purified by column chromatography with petroleum ether/ethyl acetate (v/v, 2:1) on silica gel as a pale grey solid (0.29 g, 88% yield, Rf = 0.45).

A mixture of 4-(4-nitrobenzyloxy) isophthalaldehyde (0.14 g, 0.5 mmol), NaOAc

(0.091 g, 1.1 mmol), and the indolinium (0.37 g, 1.1 mmol) in Ac_2O (8 mL) and stirred at 80℃ for 30 min. After completion, the reaction mixture was cooled to room temperature and concentrated under vacuum. The residue was purified by column chromatography with dichloromethane/methanol (v/v, 5:1) on silica gel to afford the probe **1** as an orange solid (0.37 g, 40%, Rf=0.55, melting point >300℃). ^{1}H NMR ((400MHz, DMSO-d_6): δ = 9.38 (s, 1H), 8.75-8.66 (m, 3H), 8.44 (d, 1H, *J*=8.9 Hz), 8.38-8.34 (m, 4H), 8.31-8.22 (m, 8H), 8.13-8.10 (m, 1H), 7.90-7.81 (m, 5H), 7.75-7.72 (m, 3H), 7.57-7.55 (m, 1H), 5.72 (s, 2H), 5.14-5.02 (m, 4H), 2.12-2.07 (m, 8H), 2.04-1.90 (m, 8H). ^{13}C NMR (100MHz, $CDCl_3$ + drop of MeOD): δ = 142.8, 132.3, 131.8, 128.2, 124.2, 114.2, 112.0, 111.9, 77.4, 77.2, 76.9, 70.2, 49.4, 49.2, 49.1, 49.0, 48.8, 29.6, 27.0, 26.6, 25.0, 24.9. MS (ESI): m/z calc. for $C_{51}H_{49}N_3O_9S_2$: 911.29, found: 934.18, $[M+Na]^+$

III. Kinetic Parameters Measurements

All kinetic parameters determinations were measured in phosphate buffer (50 mM, pH 7.4) at 37℃ containing NTR (10 μg/mL) and NADH (0.5 mM) as an electron donor. The kinetic rate was measured by adding varied concentrations of Probe **1** (0-50 μM) into the reaction solutions. The parameters of the kinetic enzymatic reaction, Michaelis-Menten constant (K_m), maximum rate (V_{max}) and catalytic rate constant (k_{cat}) were investigated from Lineweaver-Burk plot. The excitation and emission wavelengths were 605 nm and 720 nm.

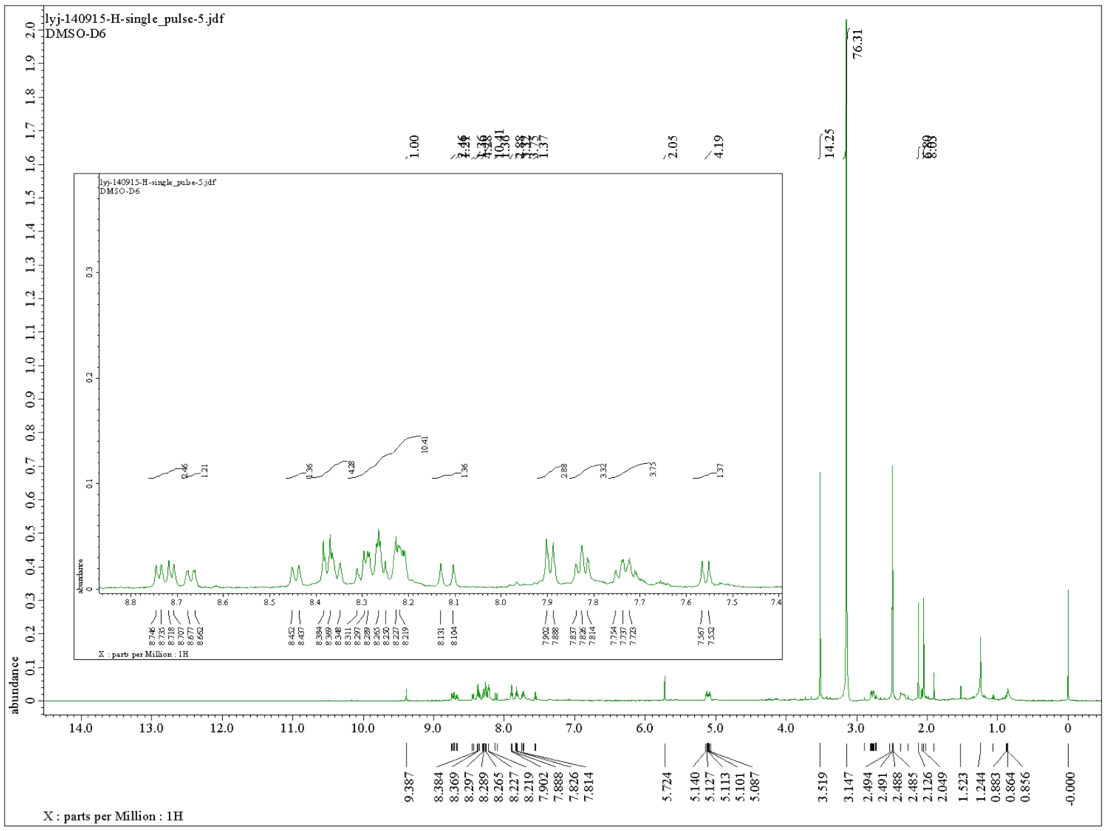
IV. Selectivity Measurements of Probe 1

Stock solutions (20 mM) of biological reductants in phosphate buffer (50 mM, pH 7.4) were prepared. The determinations were carried out by adding 50 μL stock solutions of biological reductants into the test solutions (final volume 1 mL) in the presence of Probe **1** (10 μM) with or without NTR (10 μg/mL). The test solution was kept for 20 min at 37℃, then measured with excitation at 605 nm and recorded the fluorescence intensity at 720 nm.

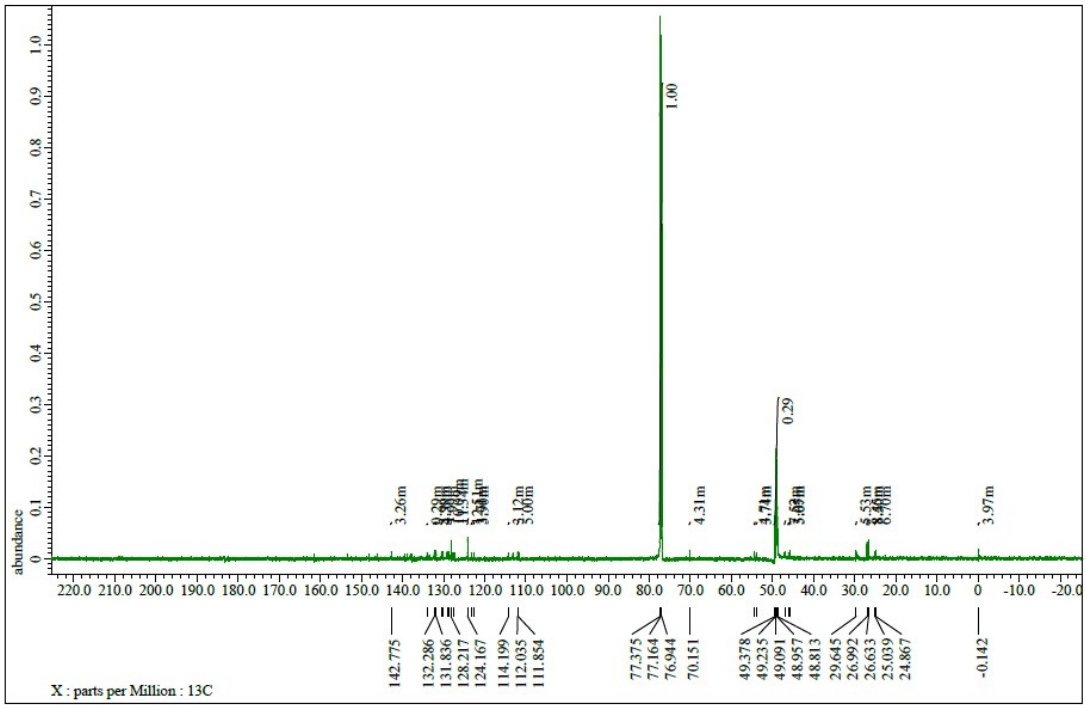
V. Confocal Fluorescence Imaging of HeLa Cells

HeLa cells were obtained from Cell Resource Center, IBMS, CAMS/PUMC and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ mL penicillin-streptomycin. Cells were incubated at 37℃ in a humidified atmosphere under normoxic (5% CO_2, 95% air) and hypoxic (5% CO_2, 94% N_2 and 1% O_2) conditions. Cells were passed by 0.25% trypsin (2.21 mM EDTA and 0.25% trypsin) in sodium bicarbonate buffer solution before use. For cell imaging experiments, HeLa cells were incubated with 10 μM probe **1** for 1 hour in FBS-free DMEM at 37℃. Before imaging, Cells were washed three times with prewarmed DMEM to remove the residue probe and then rinsed with DMEM.

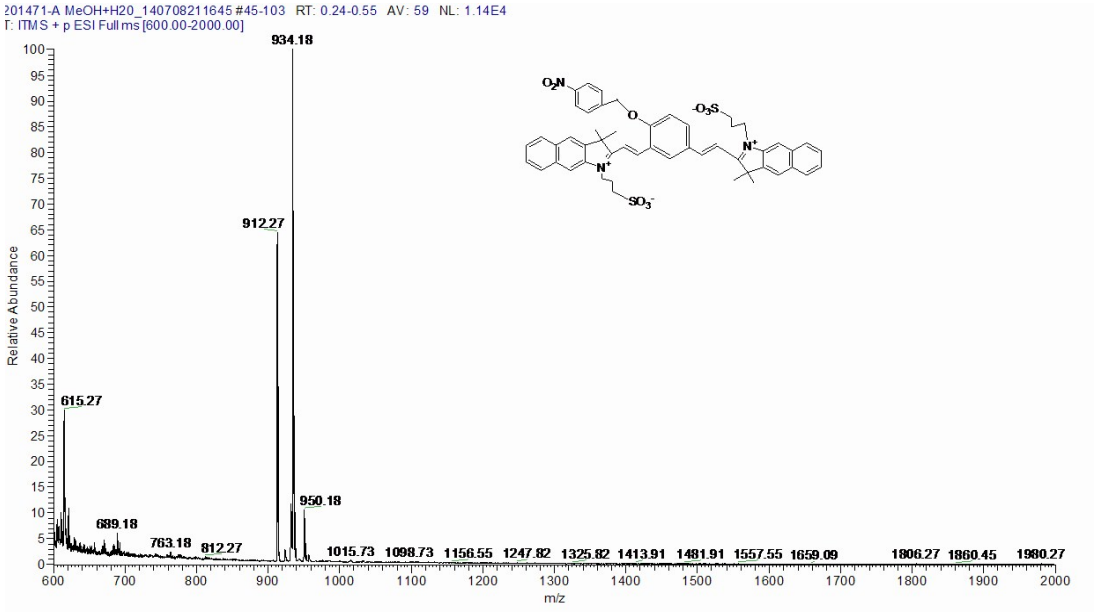
VI. NMR and ESI mass spectra of probe 1



^{1}H NMR spectrum of probe **1**



^{13}C NMR spectrum of probe **1**



ESI mass spectrum of probe **1**