Supporting Information

Triple-locked fluorescent probes sequentially activated by hNQO1, LAP and FA: application in bioimaging of cancer cells

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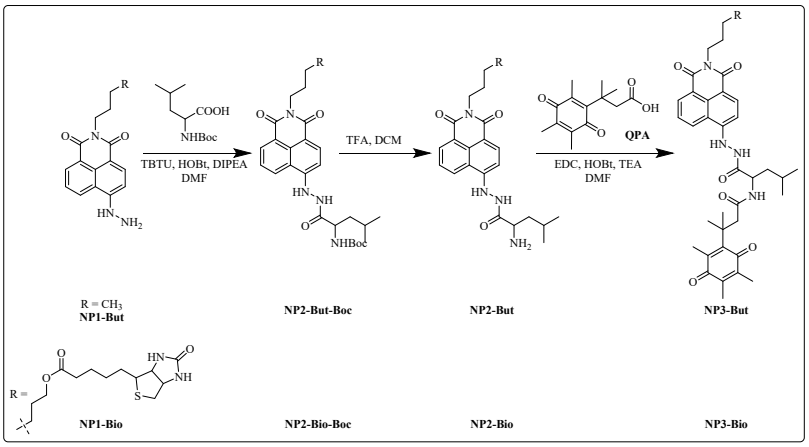
Contents

Experimental section

Reagents and instruments

All chemical reagents and solvents were purchased from commercial companies without further purification. Ultrapure water was from a Sartorius Arium 611DI system. ^{1}H-NMR and ^{13}C-NMR were measured with an AV-400 nuclear magnetic resonance spectrometer (Bruker, Switzerland) with tetramethylsilane (TMS) as the internal standard. High resolution mass spectra ware performed with a Xevo G2-XS Tof high resolution mass spectrometer (Waters, USA) under standard condition (ESI, 70 eV). The absorption and emission spectra were recorded on an Evolution 220 UV-Vis spectrophotometer and a Lumina fluorescence spectrometer (Thermo Fisher Scientific, USA), respectively.

Synthesis of probes



Scheme S1 Synthesis of probes.

The synthesis routes are shown in Scheme S1, and the starting material **NP1-But**/**NP1-Bio** were synthesized according to the reference.[1]

NP2-But and **NP2-Bio**: A solution of DMF containing Boc-Leu-OH (231 mg, 1 mmol), TBTU (321 mg, mmol), HOBt (135 mg, 1 mmol) and DIPEA (515 μL, 3 mmol) were stirred at room temperature for 5 min, then **NP1-But** (283 mg, 1 mmol) was added and stirred for 24 h. After evaporating the solvent, the residue was dissolved in 5 mL of dichloromethane. Then a solution of dichloromethane (5 mL) containing TFA (5 mL) was added dropwise to above solution at 0 ℃. After stirred for 1 h at room temperature, the solvent was evaporated and the crude product was purified with column chromatography (DCM:MeOH = 50:3) to afford orange solids **NP2-But** (301 mg, 76%). ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 8.70 (d, J = 8.4 Hz, 1H), 8.48 (d, J = 7.2 Hz, 1H), 8.30 (d, J = 8.4 Hz, 1H), 7.76 (dd, J = 8.5, 7.3 Hz, 1H), 6.97 (d, J = 8.4 Hz, 1H), 4.02 (t, J = 7.4 Hz, 2H), 3.60 (dd, J = 8.3, 6.1 Hz, 1H), 1.85-1.74 (m, 1H), 1.66-1.54 (m, 3H), 1.48 (ddd, J = 13.8, 8.3, 6.4 Hz, 1H), 1.37-1.29 (m, 2H), 1.00-0.90 (m, 9H). **NP2-Bio** was obtained by the same procedure with **NP1-Bio** instead of **NP1-But**. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 8.70 (d, J = 8.5 Hz, 1H), 8.50 (d, J = 7.2 Hz, 1H), 8.31 (d, J = 8.4 Hz, 1H), 7.79 (t, J = 7.9 Hz, 1H), 7.07 (d, J = 8.4 Hz, 1H), 6.42 (s, 1H), 6.36 (s, 1H), 4.31-4.25 (m, 1H), 4.14-4.09 (m, 1H), 4.00 (q, J = 7.3, 6.5 Hz, 4H), 3.94 (t, J = 7.2 Hz, 1H), 3.08 (dt, J = 10.4, 5.6 Hz, 1H), 2.79 (dd, J = 12.4, 5.0 Hz, 1H), 2.55 (d, J = 12.5 Hz, 1H), 2.27 (t, J = 7.4 Hz, 2H), 1.82-1.74 (m, 1H), 1.73-1.64 (m, 2H), 1.64-1.50 (m, 8H), 1.38-1.31 (m, 6H), 0.99 (dd, J = 12.5, 6.3 Hz, 6H).

NP3-But and **NP3-Bio**: A solution of DMF (5 mL) containing **QPA** (30 mg, 0.12 mmol), HOBt (20 mg, 0.14 mmol), EDC (28 mg, 0.14 mmol) and TEA (30 μL, 0.22 mmol) was stirred at room temperature for 1 h. Then 80 mg of **NP2-But** (0.12 mmol) was added into the above solution. After stirred for another 12 h, the solvent was evaporated under reduced pressure and the crude product was purified with column chromatography (DCM:MeOH = 50:3, v:v) to afford a yellow solid **NP3-But** (60 mg, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 8.53 (d, *J* = 2.2 Hz, 1H), 8.39 (d, *J* = 7.2 Hz, 1H), 8.35 (d, *J* = 8.2 Hz, 1H), 8.10 (d, *J* = 8.4 Hz, 1H), 7.71 (d, *J* = 2.2 Hz, 1H), 7.40 (t, *J* = 7.9 Hz, 1H), 6.84 (d, *J* = 8.2 Hz, 1H), 6.08 (d, *J* = 8.1 Hz, 1H), 4.59-4.49 (m, 1H), 4.17-4.12 (m, 2H), 3.10 (d, *J* = 14.6 Hz, 1H), 2.80 (d, *J* = 14.6 Hz, 1H), 2.19 (s, 3H), 1.94 (d, *J* = 1.3 Hz, 3H), 1.92 (d, *J* = 1.3 Hz, 3H), 1.73-1.62 (m, 7H), 1.45 (s, 6H), 1.00-0.91 (m, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ(ppm): 191.70, 187.87, 172.97, 172.20, 164.16, 163.90, 151.70, 148.54, 143.00, 139.43, 139.06, 132.99, 130.86, 128.95, 126.10, 125.55, 122.78, 119.69, 114.54, 106.00, 49.81, 48.98, 40.13, 39.64, 39.06, 30.23, 29.78, 29.66, 24.84, 22.74, 22.02, 20.45, 14.12, 13.88, 12.69, 12.24. ESI-MS: *m/z* $(M+H)^+$ calcd 629.3339, found 629.3338. **NP3-Bio** was obtained by the same procedure with **NP2-Bio** instead of **NP2-But**. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.38 (s, 1H), 9.58 (s, 1H), 8.66 (d, *J* = 8.5 Hz, 1H), 8.47 (d, *J* = 7.3 Hz, 1H), 8.30 (d, *J* = 7.7 Hz, 1H), 8.17 (d, *J* = 8.4 Hz, 1H), 7.74 (t, *J* = 7.9 Hz, 1H), 6.81 (d, *J* = 8.4 Hz, 1H), 6.42 (s, 1H), 6.34 (s, 1H), 4.38 (q, *J* = 7.8 Hz, 1H), 4.28 (t, *J* = 6.5 Hz, 1H), 4.12 (d, *J* = 5.6 Hz, 1H), 4.00 (q, *J* = 6.4 Hz, 4H), 3.10-3.05 (m, 1H), 2.85-2.75 (m, 3H), 2.55 (d, *J* = 12.4 Hz, 1H), 2.27 (t, *J* = 7.4 Hz, 2H), 2.06 (s, 3H), 1.95 (s, 3H), 1.91 (s, 3H), 1.69-1.43 (m, 13H), 1.38-1.36 (m, 4H), 1.35-1.32 (m, 6H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.86 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ(ppm): 190.70, 187.34, 173.37, 172.48, 172.11, 164.14, 163.39, 163.18, 155.05, 151.05, 144.27, 136.88, 136.25, 133.70, 131.36, 129.43, 128.92, 125.56, 122.50, 119.33, 111.13, 105.39, 64.12, 61.49, 59.64, 55.82, 49.96, 47.76, 40.73, 40.50, 38.22, 33.80, 28.62, 28.49, 28.44, 27.98, 26.64, 25.67, 24.99, 24.71, 23.30, 22.53, 22.00, 14.43, 14.23, 13.25, 12.23. ESI-MS: *m/z* $(M+H)^+$ calcd 899.4377, found 899.4376.

Spectral measurements

All stock solutions, including 3 mmol/L of **NP3-But** and **NP3-Bio** in DMSO, 1 mg/mL of hNQO1 in PBS (100 mmol/L, pH 7.40, containing 100 mmol/L KCl and 0.007% BSA), 30 mmol/L of NADH in PBS, 10 U/ml of LAP in PBS, 30 mmol/L of FA in PBS, 30 mmol/L of dicumarol and bestatin in DMSO, 30 mmol/L of amino acids/ions ($NaHSO_3$, Na_2S, DTT, Lys, Gly, L-Arg, L-Glu, GSSG, GSH, Hcy, Cys, glucose, NH_4^+, Mg^{2+}, Cu^{2+}, NO_3^-, SO_4^{2-}, Cl^-) in Tris-HCl or (NAC, His, Met, Trp) in DMSO and 30 mg/mL of proteins (HSA, BSA, OVA, α-chy, β-lac, mucin, pepsin, tripsin) in PBS were prepared in advance. The excitation/emission wavelengths were 425 nm/535 nm.

Dynamic experiments. Stock solution of the probes (10 μL), hNQO1 (30 μL), NADH (10 μL), LAP (150 μL) and FA (13.3 μL) were added to 3 mL of buffer solution. The absorption and emission spectra of the above solution were detected at different times.

pH effect. The pH of the probes (10 μM) and reaction products (10 μM) in PBS were adjusted with NaOH or HCl aqueous solution, and then recorded the emission spectra of the above solutions with different pH values.

Selectivity. Stock solutions of amino acids/ions (50 μL) or proteins (100 μL) were added to 3 mL of PBS containing probes (10 μmol/L). The fluorescence spectra were measured after the mixture equilibrium for 90 min.

High-performance liquid chromatography (HPLC) traces

HPLC spectra were obtained on an iChrom 5100 LC system and a Sinopak C18 reversed-phase colum (4.6 mm × 25 cm). The mobile phase was degassed with an ultrasonic device for 10 minutes. Mobile phase: A-water, B-acetonitrile; injection volume: 20 μL; flow rate: 1.0 mL/min; detection wavelength: 440 nm; elution condition: gradient elution, 0-30 min 5-95% B.

Computational methods

The density functional theory (DFT) was employed to optimize the structures of the probes through Gaussian 16. All structure optimizations were performed using DFT-M062X functionals and Def2SVP basis set for the ground states. Solvation effects were taken into account using the SMD mode and the solvent was water. Frequency calculations were performed to confirm that we obtained stable structures without imaginary vibrational frequencies.

MTT Assay

L929, MDA-MB-231, HeLa and HepG-2 cell lines (National Collection of Authenticated Cell Cultures, Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium (High glucose) containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2. The cells were transferred to 96-well plate (5000 cells/well) and incubated for 24 h. After removal of the medium, the cells were cultured with different concentrations (0-20 μM) of probes in serum-free DEME medium for 24 h followed by incubated with 5 mg/mL of MTT for another 4 h. After removal of the medium, 100 μL of DMSO were added to the plate. The absorbance at 570 nm of each solution was measured with a microplate reader. The cell viability was calculated according to the following equation.

$$\text{Cell viability (\%)} = (OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$$

Where $OD_{control}$ and OD_{sample} are the optical densities of the wells in which the cells incubated without and with certain concentration of the probe, respectively; OD_{blank} represents optical density of the well only containing culture medium and MTT.

Confocal fluorescent imaging

The cells were transferred to confocal dishes and incubated for 24 h, and followed by washed with PBS (20 mmol/L, pH 7.4) for three times. Then the cells were cultured with serum-free DEME medium containing probes (10 μmol/L) for 30 min and washed with PBS for three times again. For the control experiments, before incubation with 10 μmol/L of probes, the cells were pre-incubated with 500 μmol/L dicumarol for 6 h, 100 μmol/L bestatin for 1 h, 200 μmol/L $NaHSO_3$ for 30 min, 1 μg/mL LPS for 12 h or 1 μg/mL hNQO1 (containing 100 μmol/L NADH) for 30 min and washed with PBS. The fluorescence was collected at 425-475 nm for the blue channel and 500-550 nm for the green channel as excited at 405 nm and 488 nm, respectively.

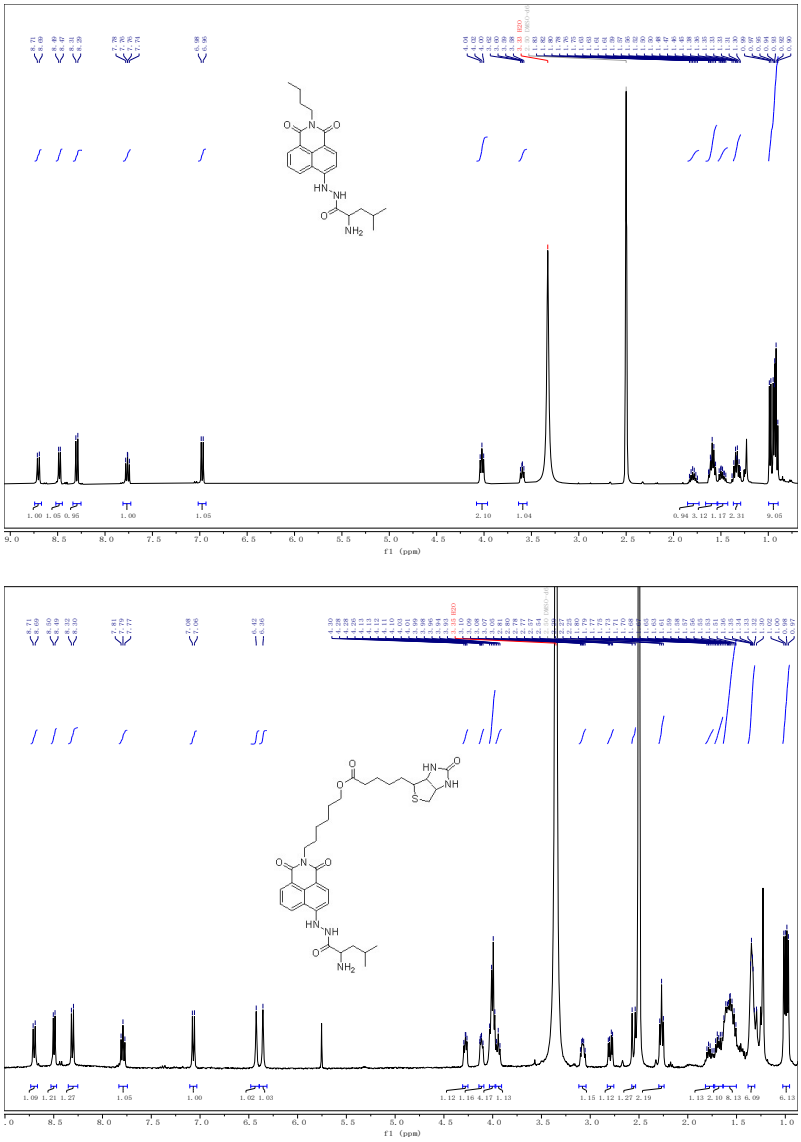


Fig. S1 ^{1}H-NMR spectra of **NP2-But** and **NP2-Bio**.

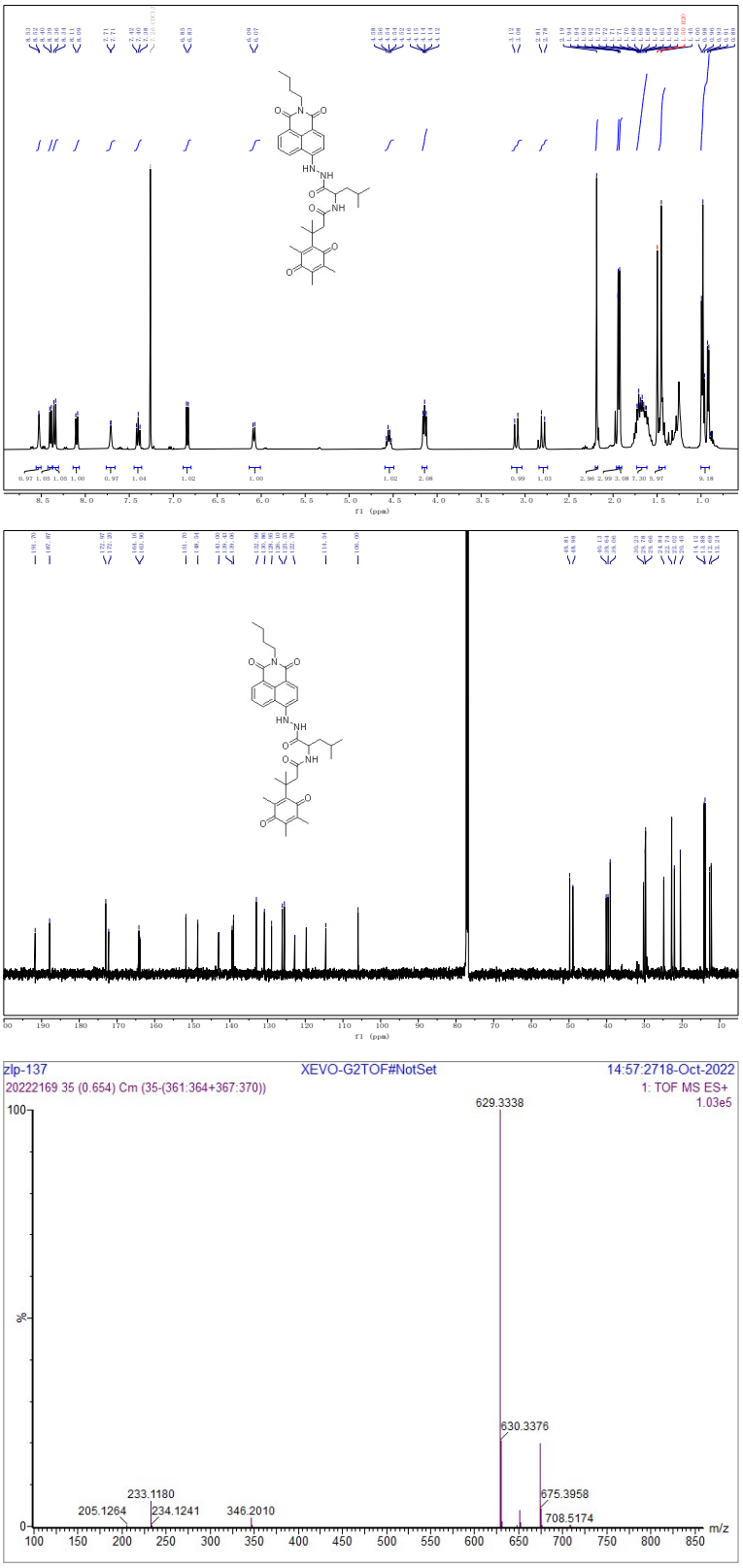


Fig. S2 ^{1}H-NMR, ^{13}C-NMR and ESI-Mass spectra of **NP3-But**.

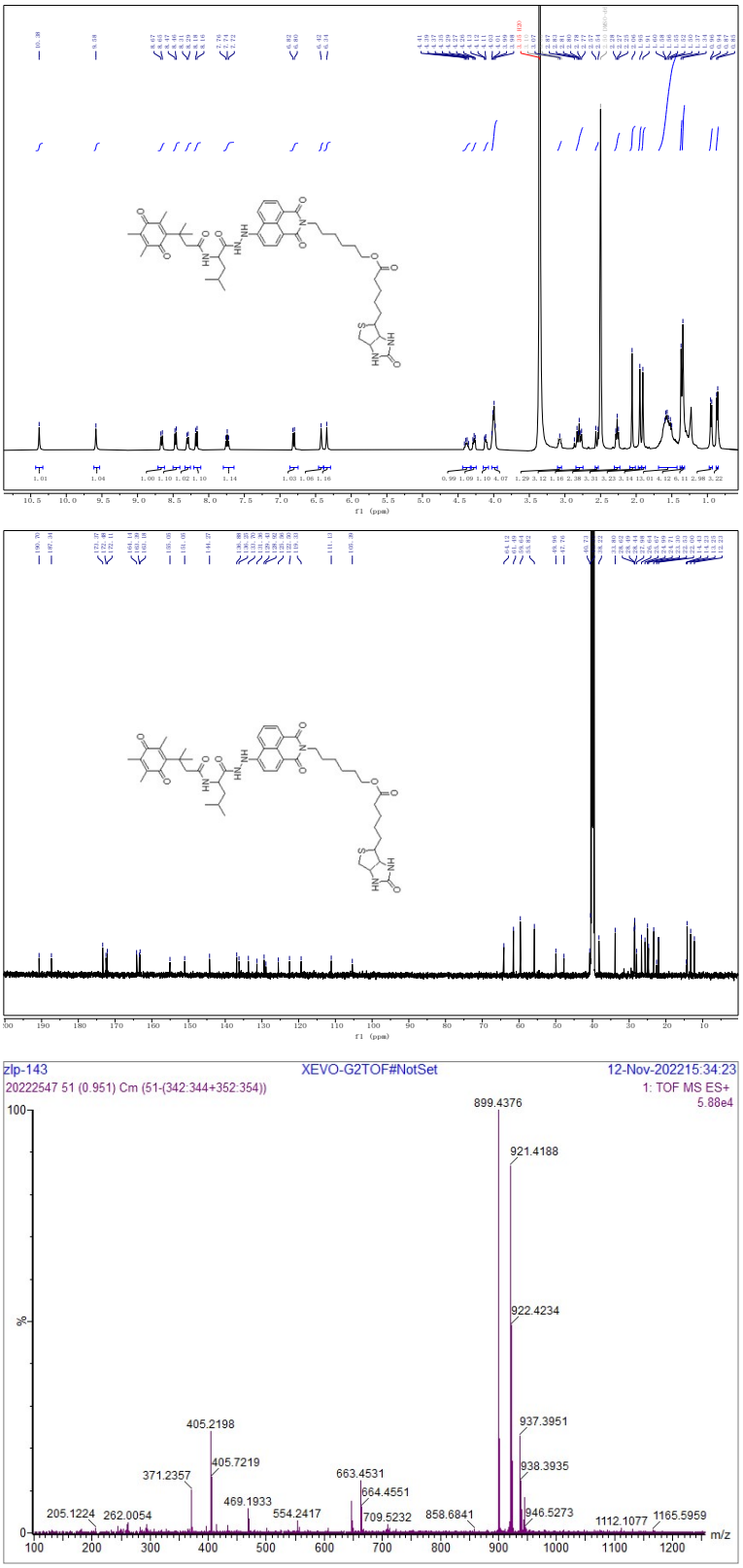


Fig. S3 ^{1}H-NMR, ^{13}C-NMR and ESI-Mass spectra of **NP3-Bio**.

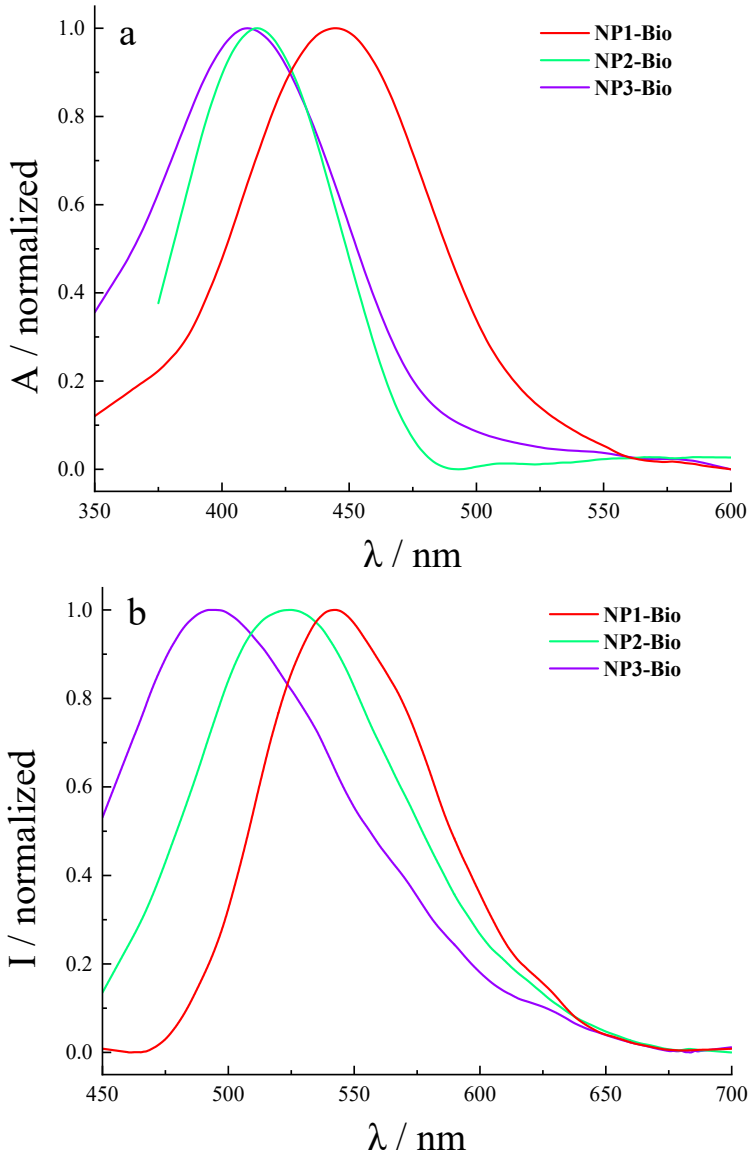


Fig. S4 Normalized absorption and emission spectra of the probes in PBS (100 mM, pH 7.4). [probe] = 10 μM, λ_{ex} = 425 nm.

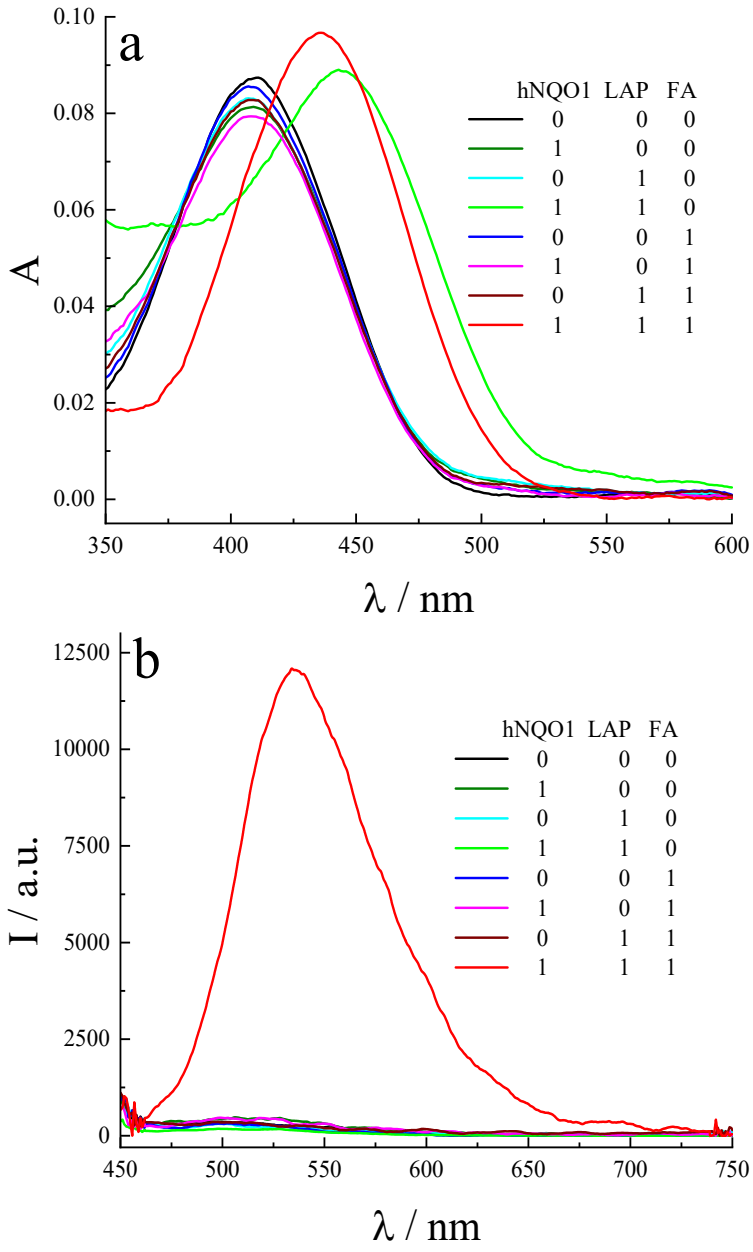


Fig. S5 The absorption (a) and emission (b) spectra of **NP3-Bio** under different conditions. [**NP3-Bio**] = 10 μM, [hNQO1] = 10 μg/mL, [LAP] = 0.5 U/mL, [FA] = 200 μM, [NADH] = 20 μM, λ_{ex} = 425 nm.

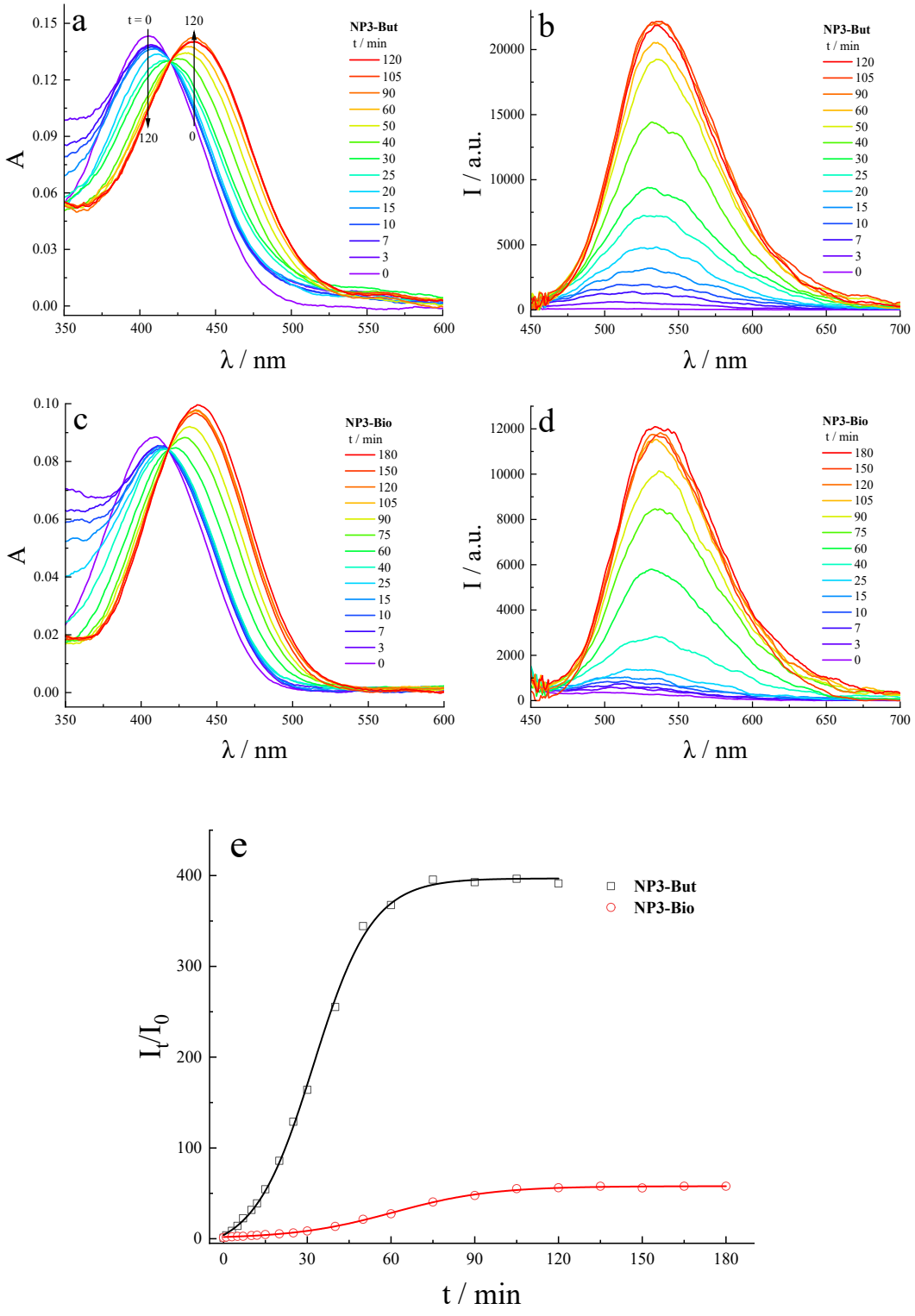


Fig. S6 Time-dependent absorption (a, c) and emission (b, d) spectra of the quaternary systems (**NP3-But/NP3-Bio** + FA + LAP + hNQO1); (e) plots of the fluorescence intensity ratios of the quaternary systems as a function of time. [**NP3-But/NP3-Bio**] = 10 μM, [FA] = 200 μM, [LAP] = 0.5 U/mL, [hNQO1] = 10 μg/mL, [NADH] = 20 μM, λ_{ex} = 425 nm, λ_{em} = 530 nm.

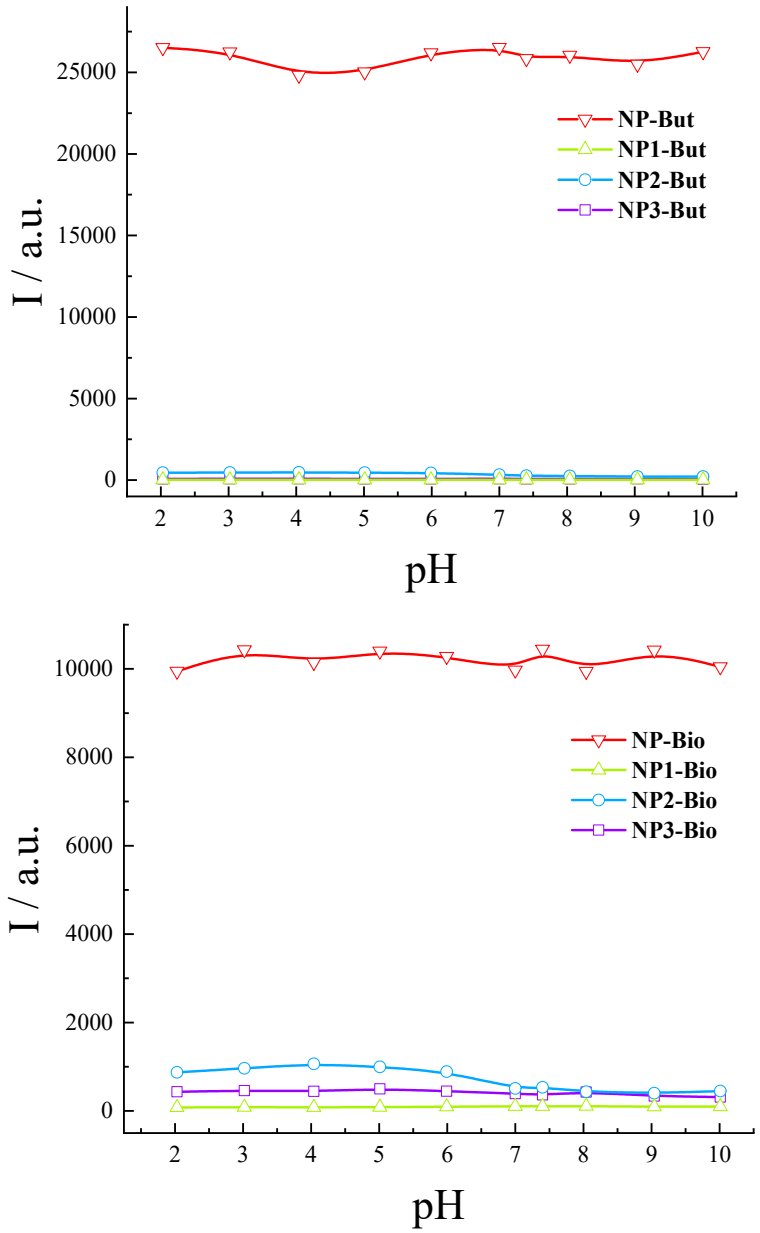


Fig. S7 Plots of the fluorescence intensities of different compounds as a function of pH. [probe] = 10 μM, λ_{ex} = 425 nm, λ_{em} = 530 nm.

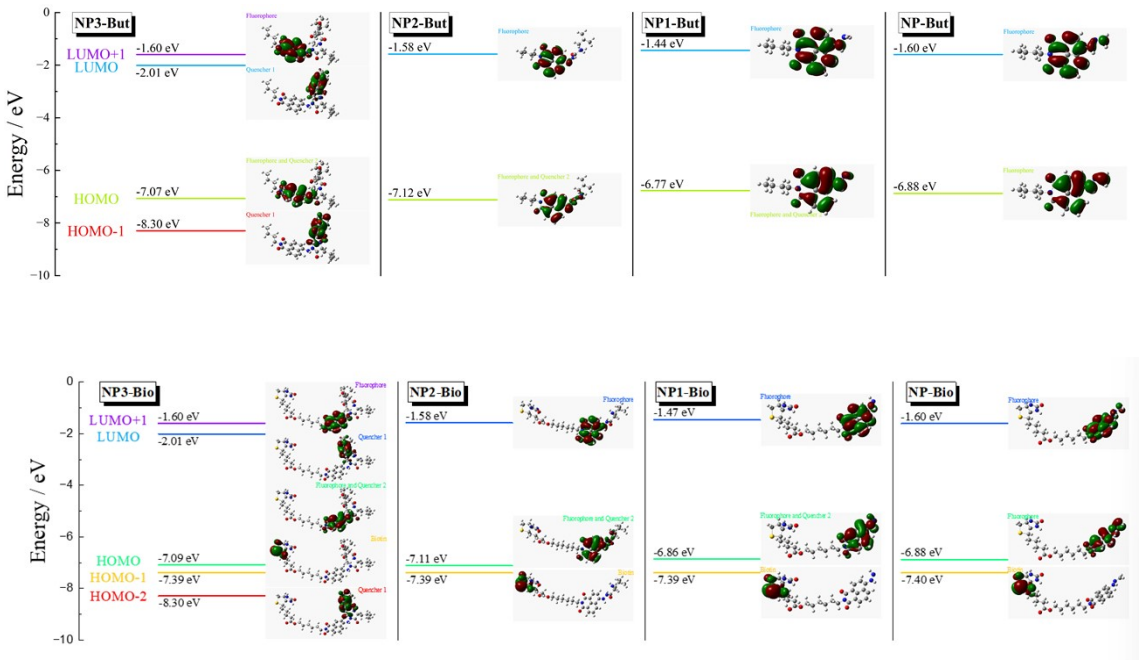


Fig. S8 Frontier molecular orbitals of **NP3-But/Bio** and their reaction products at different stages (**NP2-But/Bio**, **NP1-But/Bio** and **NP-But/Bio**).

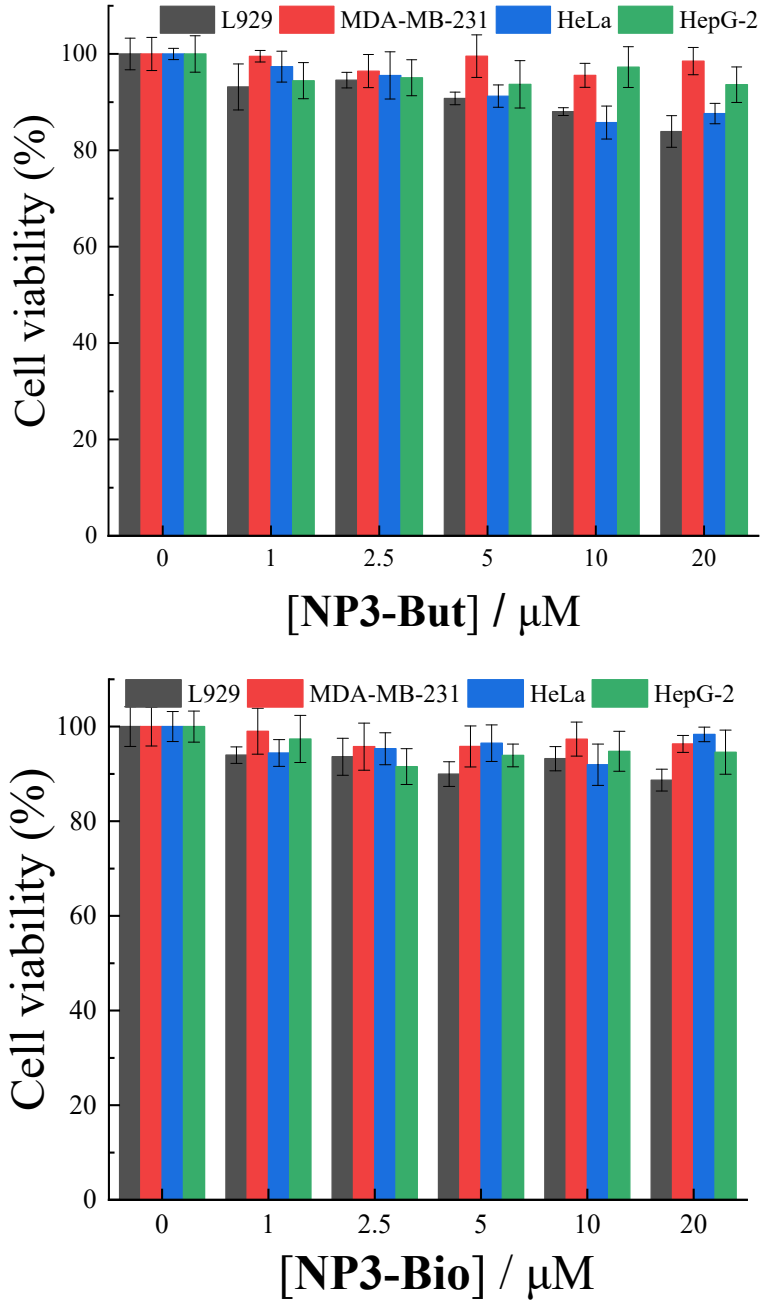


Fig. S9 The viabilities of various cell lines treated with **NP3-But** and **NP3-Bio**.

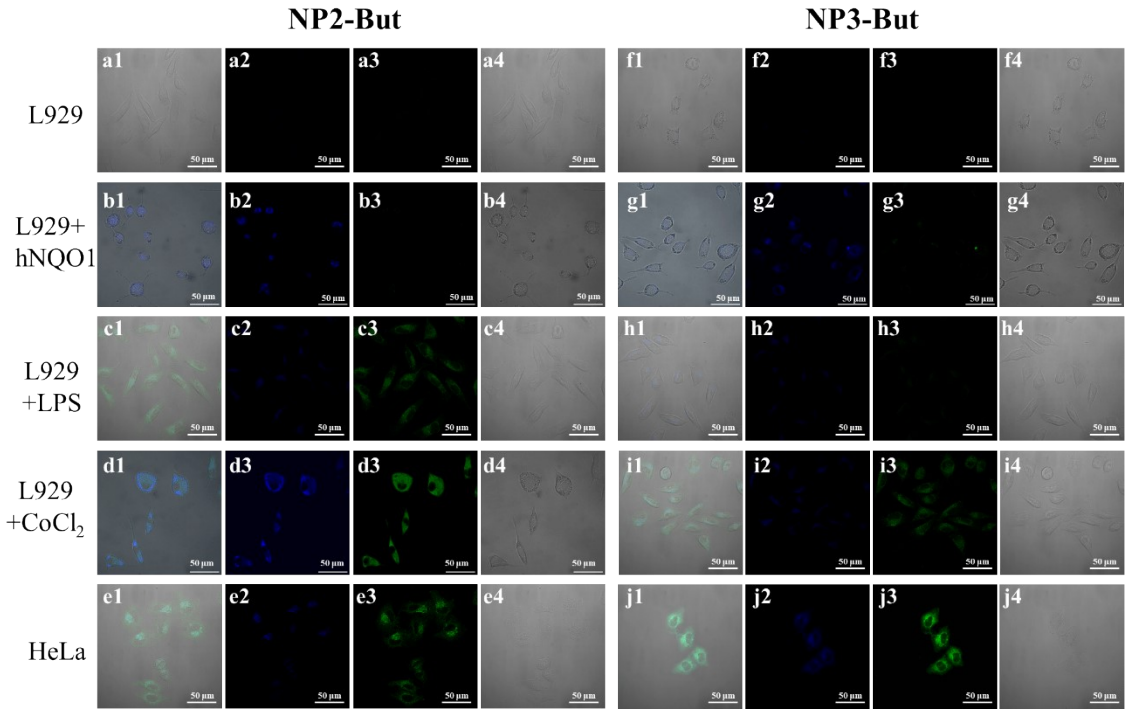


Fig. S10 Confocal fluorescence imaging of L929 (a-d, f-i) and HeLa (e, j) cells stained with 10 μM **NP2-But** (a-e) and **NP3-But** (f-j); pre-treated with 1 μg/mL hNQO1 for 30 min (b, g), 1 μg/mL LPS for 12 h (c, h) or 1 mM $CoCl_2$ for 24 h (d, i). (1) Merged of (2), (3) and (4); (2) blue channel; (3) green channel; (4) bright field.

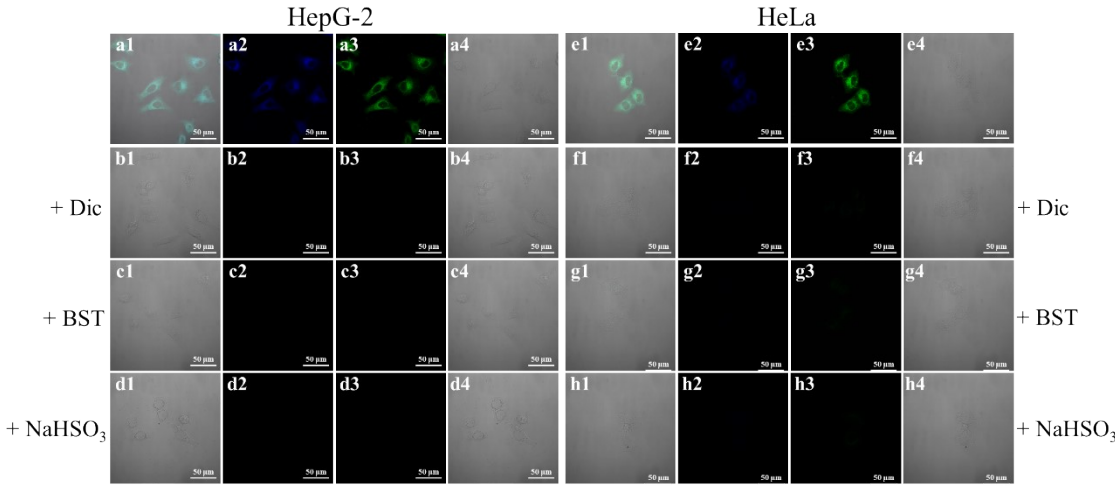


Fig. S11 Confocal fluorescence imaging of HepG-2 (a-d) and HeLa (e-h) cells stained with 10 μM **NP3-But**. Pre-treated with 500 μM Dic for 6 h (b, f), 100 μM BST for 1 h (c, g) or 200 μM $NaHSO_3$ for 30 min (d, h). (1) Merged of (2), (3) and (4); (2) blue channel; (3) green channel; (4) bright field.

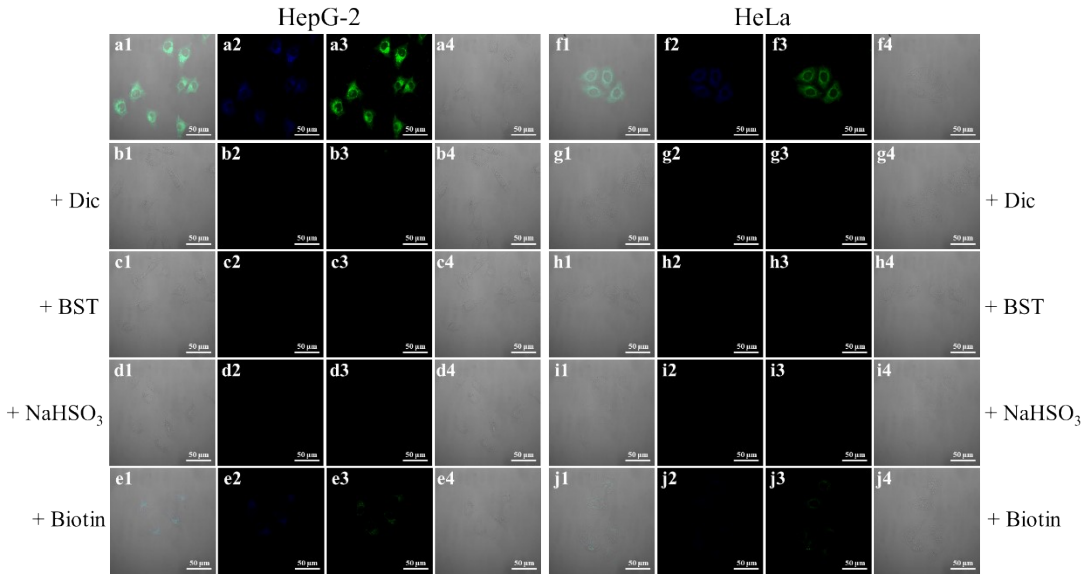
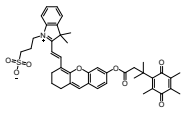
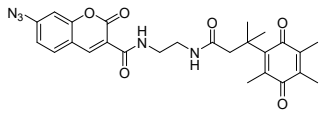
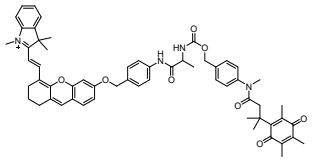
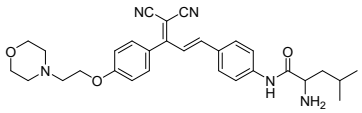
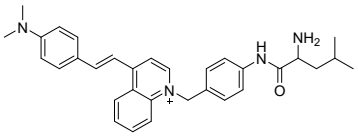
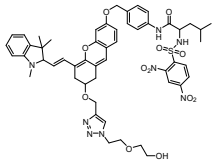
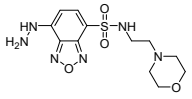
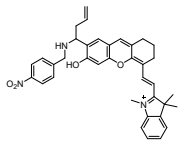
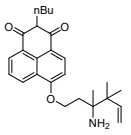


Fig. S12 Confocal fluorescence imaging of HepG-2 (a-e) and HeLa (f-j) cells stained with 10 μM **NP3-Bio**. Pre-treated with 500 μM Dic for 6 h (b, g), 100 μM BST for 1 h (c, h) or 200 μM $NaHSO_3$ for 30 min (d, i) or 1 mM biotin for 30 min (e, j). (1) Merged of (2), (3) and (4); (2) blue channel; (3) green channel; (4) bright field.

Table S1 Comparison of **NP3-But** and **NP3-Bio** with some reported probes

Analytes	**Chemical structure**	**Distinguish between cells**	**Literature**
hNQO1		normal cells (H596) vs cancer cells (HT-29, OVCAR-3)	[2]
hNQO1 + H_2S		normal cells (HEK293T cells) vs colorectal cancer cells (HT29, HCT116 cells)	[3]
hNQO1 + APN		normal cells (L02, Hek293, L929, LX-2 cells) vs cancer cells (A549, HepG-2, MCF-7 cells)	[4]
LAP		normal liver cells (L02 cells) vs liver cancer cells (HepG-2 cells)	[5]
LAP + viscosity + polarity		different types of inflammatory cells (L02 cells induced by different drugs)	[6]
LAP + GSH		normal liver cells (L02 cells) vs liver cancer cells (HepG2, Hepa1-6 cells)	[7]
FA		Lysosomal formaldehyde in EC1 cells	[8]
FA + viscosity		mitochondrial viscosity and FA in HeLa cells	[9]
FA + pH		Lysosomal formaldehyde in HeLa cells	[10]
FA + LAP + hNQO1	**NP3-But**	inflammatory L929 / MDA-MB-231 cells vs HeLa/HepG-2 cells	This work
FA + LAP + hNQO1	**NP3-Bio**	hypoxic L929 / MDA-MB-231 cells vs HeLa/HepG-2 cells	

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