Supporting Information

A Highly Sensitive Naphthalimide-Based Fluorescence Polarization Probe for Detecting Cancer Cells

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1. Synthetic experiments

Material and apparatus

All chemical reagents and solvents were purchased from commercial sources and used without further purification. Thin–layer chromatography (TLC) was performed on silica gel plates. Column chromatography was performed using silica gel (Hailang, Qingdao) 200–300 mesh. ^{1}H and ^{13}C NMR spectra were recorded employing a Bruker AV–400 spectrometer with chemical shifts expressed in parts per million (in deuteriochloroform, Me_4Si as internal standard). Glass fiber membrane was purchased from Shanghai Jiening Biotechnology companies. Electrospray ionization (ESI) mass spectrometry was performed in a HP 1100 LC-MS spectrometer. The scanning electron microscopic (SEM) images were obtained by a ZEISS-ULTRA 55 scanning electron microscope. IR spectra were recorded on a Thermo Nicolet Avatar 370 FT-IR spectrometer. UV absorption spectra were recorded using a Hitachi UV–visible spectrophotometer (U–3900). Samples for UV absorption were contained in 1 cm × 1 cm quartz cuvettes (3.5 mL of volume). Zeta potential of samples was measured with MALVERN Nano ZS90. The fluorescence lifetimes of probes were tested by single-photon counting technique with a hydrogen-filled flash lamp or a nitrogen lamp as the excitation source (Edinburgh FL 900). Data were analyzed through a non-linear least-squares fitting program with deconvolution method. The temporal resolution after deconvolution of the exciting pulse is ~ 200 ps. [18b, S1]

IR spectra

By comparing IR spectrums (Fig. 2d) of HA, BIO2 and probe BIO, aromatic compounds characteristic peaks of BIO2 containing the C−H out-of-plane bending vibration at 772 cm^{-1}

and C=C stretching vibration at 1575 cm^{-1}, 1545 cm^{-1}, 1401 cm^{-1} and 1384 cm^{-1} can be found on the probe BIO IR spectrum, N−H bending vibration at 1679 cm^{-1} and C−N stretching vibration at 1245 cm^{-1} of terminal primary amine groups disappeared with the formation of Amide bond between small molecule fluorophore BIO2 and HA. The BIO spectrum also shows the characteristic vibration band of HA C−O−C at 1040 cm^{-1}. Thus, satisfactory analysis data corresponding to its expected molecular structure were obtained.

Zeta potential

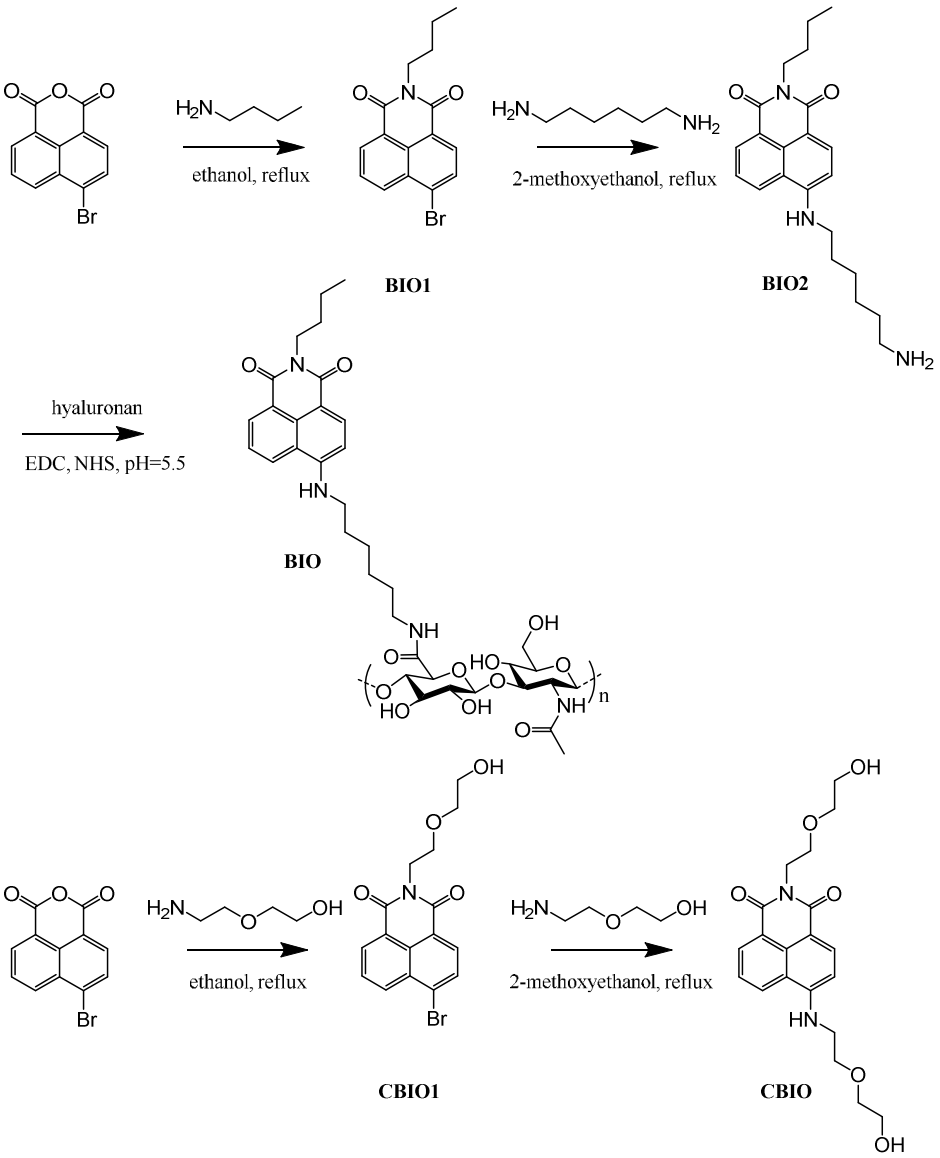
As we know, there are a number of exposed carboxyl groups on the HA chain, which makes the HA itself negatively charged. However, when we attached BIO2 fluorophores to HA chains by covalent bonds, due to the reducing of exposed carboxyl groups on HA, so the potential of BIO moving forward positively, that is, the zeta potential of probe BIO decreased from -66.7 mV to -42.9 mV in water in Fig. 2e.

UV absorption spectra and interpolation analysis

The interaction between HA and BIO2 was further verified by UV absorption spectra (Figure 2f). BIO featured one pair of conspicuous adsorption bands belong to cyclic conjugated system of aromatic compounds attributes to compound BIO2 which had been successfully labeled on the HA chain at the vicinity of 255 nm together with an intense characteristic band of BIO2 at longer wavelength of 451 nm. In order to further study the chemical properties of the probe BIO quantitatively, we have evaluated the efficiency of labeling by interpolation method. In short, UV absorbance of BIO2 samples on different concentrations were measured to get the standard line of UV absorbance-concentration ($Y = 0.0072 + 0.0081\ X$, R^2 =

0.9997), then UV absorbance of probe BIO sample on a certain concentration were measured and the corresponding concentration of BIO2 on the BIO chain was obtained upon the aforesaid standard line. By calculation, the efficiency of labeling of BIO reaches about 9 %.

Synthetic procedures



Scheme S1. Synthesis of fluorescence polarization probe **BIO** and its control probe **CBIO**.

N–butyl–4–bromo–1,8–naphthalimide (**BIO1**)

BIO1 was prepwered according to the literature procedure[S2]. To a stirred solution of 4–bromo–1,8–naphthalic anhydride (8.05 g, 29 mmol) dissolved in absolute ethanol (150 mL), was added n–butylamine (75 mL, 30 mmol). The reaction mixture was stirred under refluxing condition for 4 h under nitrogen atmosphere. After cooling to room temperature, the solid was filtered off, washed with ethanol and then solvent was evaporated. The crude product was then purified by chromatography on a silica gel column (DCM: methanol, 50:1, v/v) to give **BIO1** as a pale yellow solid (2.36 g, 29% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.83 (d, J = 8.7 Hz, 1H), 8.74 (d, J = 7.3 Hz, 1H), 8.69 (d, J = 8.0 Hz, 1H), 8.41 (d, J = 8.0 Hz, 1H), 7.99 (t, J = 8.0 Hz, 1H), 7.28 (s), 4.23–4.17 (m, 2H), 1.79–1.69 (m, 2H), 1.52–1.42 (m, 2H), 1.00 (t, J = 7.4 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.28, 162.45, 149.53, 132.38, 129.91, 129.74, 129.23, 129.07, 127.04, 123.88, 123.66, 123.06, 40.65, 30.09, 20.33, 13.79.

N–butyl–4–(6'–aminohexyl)amino–1,8–naphthalimide (**BIO2**)

N–butyl–4–bromo–1,8–naphthalimide (1.38 g, 4.17 mmol) was added to a 100 mL round bottomed flask containing 50 mL of 2–methoxyethanol, and to the solution was added 1,6–hexanediamine (3 mL). The mixture was heated under reflux for about 12 h under dark, and the reaction was monitored by TLC 5:1 CH_2Cl_2:methanol (R_f 0.3). When all of the material **BIO1** had been consumed, the mixture was cooled to room temperature, and solvent was was concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using 5:1 CH_2Cl_2:methanol as the eluent, which yielded the desired product **BIO2** as a orange–yellow powder (430 mg, 31% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.55

(d, J = 7.3 Hz, 1H), 8.43 (d, J = 8.4 Hz, 1H), 8.13 (d, J = 8.4 Hz, 1H), 7.65–7.53 (m, 1H), 6.69 (d, J = 8.5 Hz, 1H), 5.49 (t, J = 4.9 Hz, 1H), 4.91 (d, J = 1.3 Hz, 2H), 4.22–4.10 (m, 2H), 3.39 (dd, J = 12.4, 7.0 Hz, 2H), 2.72 (t, J = 6.8 Hz, 2H), 1.89–1.74 (m, 2H), 1.70 (dd, J = 12.3, 4.8 Hz, 2H), 1.51 (d, J = 6.5 Hz, 2H), 1.45–1.37 (m, 4H), 1.30 (dd, J = 24.0, 8.4 Hz, 2H), 0.96 (t, J = 7.3 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.74, 164.21, 149.54, 134.47, 131.07, 129.81, 125.97, 124.61, 123.12, 120.20, 110.13, 104.27, 63.64, 43.61, 41.85, 39.99, 33.18, 30.33, 28.87, 26.98, 20.44 , 13.90. IR (KBr, cm^{-1}): 3382, 2932, 2859, 1679, 1639, 1614, 1580, 1550, 1466, 1430, 1397, 1382, 1360, 1301, 1245, 1107, 1078, 775, 759. HRMS (ES^+): calc. for $C_{22}H_{30}N_3O_2$ $[M+H]^+$ 368.2338, found 368.2338.

Fluorescence polarization probe **BIO**

45 mg (123 μmol) of N–butyl–4–(6'–aminohexyl)amino–1,8–naphthalimide (**BIO2**) was dissolved in DMSO (5 mL). Sodium hyaluronate (HA, 74.50 mg) was stirred with a solution (40 mL) of phosphate buffer solution (pH = 7.4) in an ice bath in another bottomed flask. When HA has been dissolved completely, EDC (111.75 mg) and NHS (37.25 mg) were added to the jellied solution. The pH of the resulting solution was adjusted to 6.8 by adding HCl, and then keeping activating for about 30 min. At last the two solutions were mixed and ice bath was removed. The mixture was stirred at 25 ^{o}C overnight. The crude product obtained was dialyzed with ultrapure water to wash out unreacted reagents until dialyzed ultrapure water becomes colorless, and then drying at a low temperature to get thin–layered orange–yellow solid. IR (KBr, cm^{-1}): 3389, 2923, 1640, 1575, 1545, 1401, 1384, 1316, 1154, 1078, 1039, 947, 772.

Synthesis of control probe **CBIO**

N-[2-(2-hydroxyethoxy)ethyl]-4-bromo-1,8-naphthalimide (**CBIO1**)

To a solution of 4–bromo–1,8–naphthalic anhydride (1.39 g, 5 mmol) dissolved in absolute ethanol (30 mL), was added 2-(2-aminoethoxy)ethanol (0.52 mL, 5.05 mmol). The mixture was stirred at 80^{o}C to reflux for 1 h under argon atmosphere. Then, the result mixture was cooled to room temperature and a pale yellow crystal was formed, N-[2-(2-hydroxyethoxy)ethyl]-4-bromo-1,8-naphthalimide (**CBIO1**, 1.06 g, 58% yield) was obtained as a pale yellow solid by filtration and washed with ethanol three times. **CBIO1** was pure enough for the next synthesis.

CBIO1 (500 mg, 1.37 mmol) and 2-(2-aminoethoxy)ethanol (0.14 mL, 1.39 mmol) were dissolved in 2-Methoxyethanol (12 mL), and to the mixture was heated up to reflux to stir overnight darkly under argon atmosphere. The reaction was monitored by TLC 2:1 CH_2Cl_2:petroleum ether (60-90 ^{o}C) (Rf 0.3). The solvent was then removed, and the residue was purified by column chromatography using 2:1 CH_2Cl_2:petroleum ether (60-90 ^{o}C) as the eluent, which yielded the desired product **CBIO** as a yellow powder (138 mg, 28% yield). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.65 (d, J = 8.3 Hz, 1H), 8.40 (d, J = 7.2 Hz, 1H), 8.23 (d, J = 8.6 Hz, 1H), 7.72 (t, J = 5.1 Hz, 1H), 7.69 – 7.61 (m, 1H), 6.80 (d, J = 8.7 Hz, 1H), 4.20 (t, J = 6.6 Hz, 2H), 3.73 (t, J = 5.7 Hz, 2H), 3.62 (t, J = 6.6 Hz, 2H), 3.56 (dd, J = 11.2, 5.8 Hz, 2H), 3.51 (dt, J = 7.6, 3.4 Hz, 4H), 3.33 (ddd, J = 17.3, 10.8, 5.7 Hz, 4H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.48, 163.58, 151.33, 134.89, 131.38, 130.09, 129.25, 124.96, 122.46, 120.77,

108.39, 104.64, 73.00, 72.78, 68.80, 67.81, 60.92, 43.55, 39.06. HRMS (ES^+): calc. for $C_{20}H_{24}N_2O_6Na$ $[M+Na]^+$ 411.1532, found 411.1530.

2. Detailed protocol for in vitro and in living cells assay

Material and apparatus

Double distilled water was used to prepare all aqueous solutions. DMEM (High glucose) medium were from Hyclone. Foetal Bovine Serum (FBS) was from Gibco. Other biological reagents were of analytical grade and commercially available. All spectroscopic measurements were performed in 0.05 M PBS buffer (pH = 7.4). All fluorescence polarization experiments were made with Varian spectrophotometer (Cary Eclipse). Samples for fluorescence polarization measurements were contained in 1 cm × 1 cm quartz cuvettes. All cell images were taken by Leica TCS SP5 II Confocal Laser Scanning Microscope. 1.215 mg mL^{-1} of **BIO** stock solution, 24 micro liter of stock solution was added into 2 mL of PBS buffer to make the final detection system.

2.1. pH effect on the fluorescence intensity of BIO in water

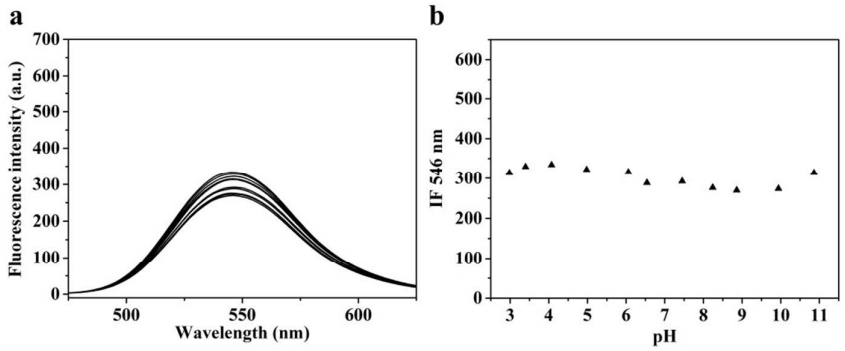


Figure S1. pH effect on the fluorescence intensity of probe **BIO** in water with different pH values. (a) Fluorescence spectra of probe **BIO** with different pH values (λ_{ex} = 450 nm, slit = 10 nm). (b) Fluorescence intensity plots of probe **BIO** at 546 nm with different pH values.

DLS (Dynamic light scattering) analysis of BIO in water solution

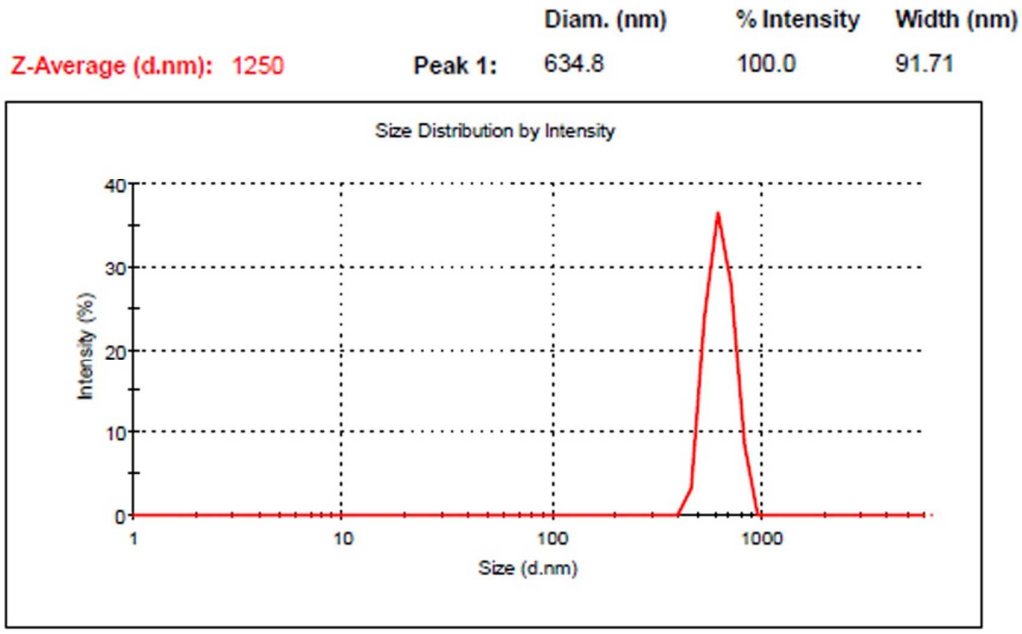


Figure S2. DLS (Dynamic light scattering) analysis of **BIO** in water solution. **BIO** was disolved in pure water. Temperature for detection was 25 ^{o}C. This assay was conducted on a dynamic light ccattering detection system NANO-ZS90 (England, Malvern).

Cell culture

Hela cells, BxPC-3 cells, A549 cells, ROS1728 cells, MCF–7 cells and HEK-293T cells were obtained from American Type Culture collection, and grown in DMEM (High glucose) medium supplemented with 10% FBS. Hela cells, BxPC-3 cells, A549 cells, ROS1728 cells

and HEK-293T cells were incubated in a 5% CO_2 humidified incubator at 37 ^oC and typically passaged with sub–cultivation ratio of 1:4 every two days. MCF–7 cells were passaged with every three days.

MTT assay

The cytotoxicity of fluorescence polarization probe **BIO** against Hela cells were measured by using a standard methyl thiazolyl tetrazolium (MTT) assay. The cells were seeded into 96–well cell culture plate at 10^4 $well^{-1}$ in complete medium, the medium contained DMEM and 10% fetal bovine serum (FBS), the cells was incubated for 12 h at 37 ^oC under 5% CO_2 to allow the cells attach. After the medium was removed, Hela cells were incubated with fresh medium containing various concentrations of probe **BIO** for 12h and 24h respectively. After incubating, the **BIO** containing medium was replaced with PBS buffer, MTT was then added to each well (final concentration 1 mg mL^{-1}) for 4 h at 37°C, and formazan crystals formed through MTT metabolwasm by viable cells were dissolved in DMSO. After shaking for 15 min, the cell viability was determined by measuring the light absorbance at 490 nm with a microplate reader (Thermo Multiskan Spectrum).

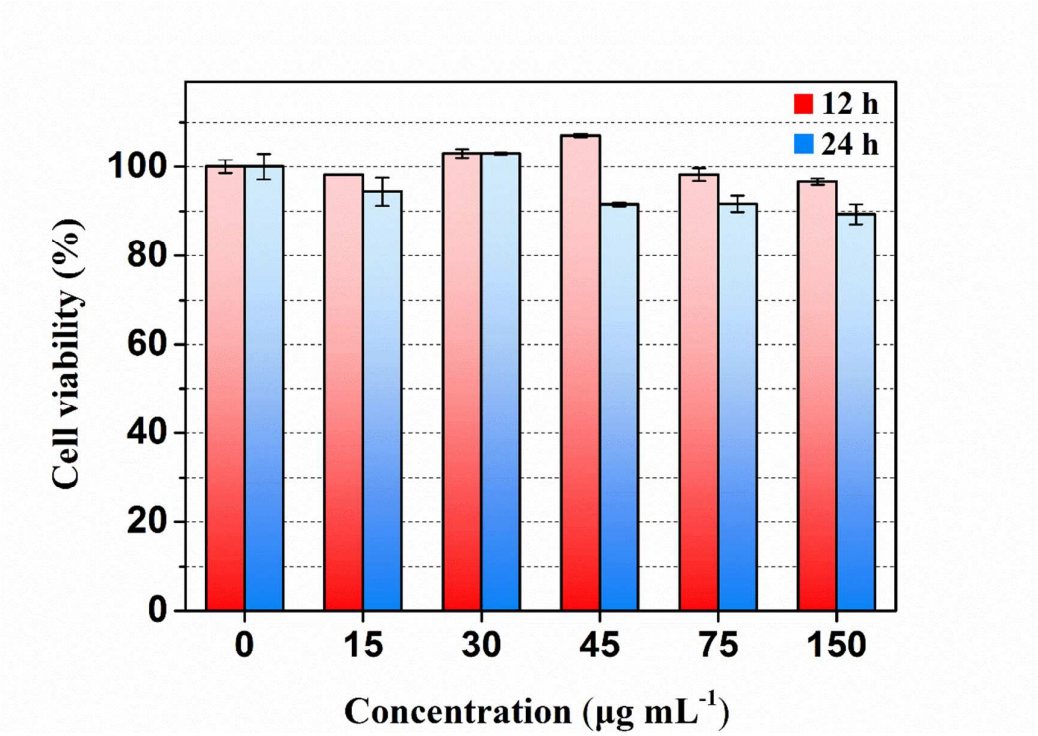


Figure S3. Cell viability of probe **BIO** (0, 15, 30, 45, 75 and 150 μg mL^{-1}) at 12 and 24 h.

Cell specific experiment

The Hela cells (BxPC-3 cells, A549 cells, ROS1728 cells, MCF–7 cells and HEK-293T similarly) were seeded into confocal petri dish in complete medium (90% DMEM and 10% FBS), and then incubated for 12 h under standard culture conditions (atmosphere of 5% CO_2 and 95% air at 37°C) to allow the cells attach. The cells were washed three times with DMEM, and then were incubated with 2 mL of probe **BIO** (14.56 μg mL^{-1}) under standard culture conditions. Probe **BIO** was first prepwered as a water solution with a concentration of 1.215 mg mL^{-1} and was diluted with DMEM for cell incubation. Cells were washed thrice with 2 mL of PBS, and 2 mL of DMEM culture medium was added and then observed under confocal microscope.

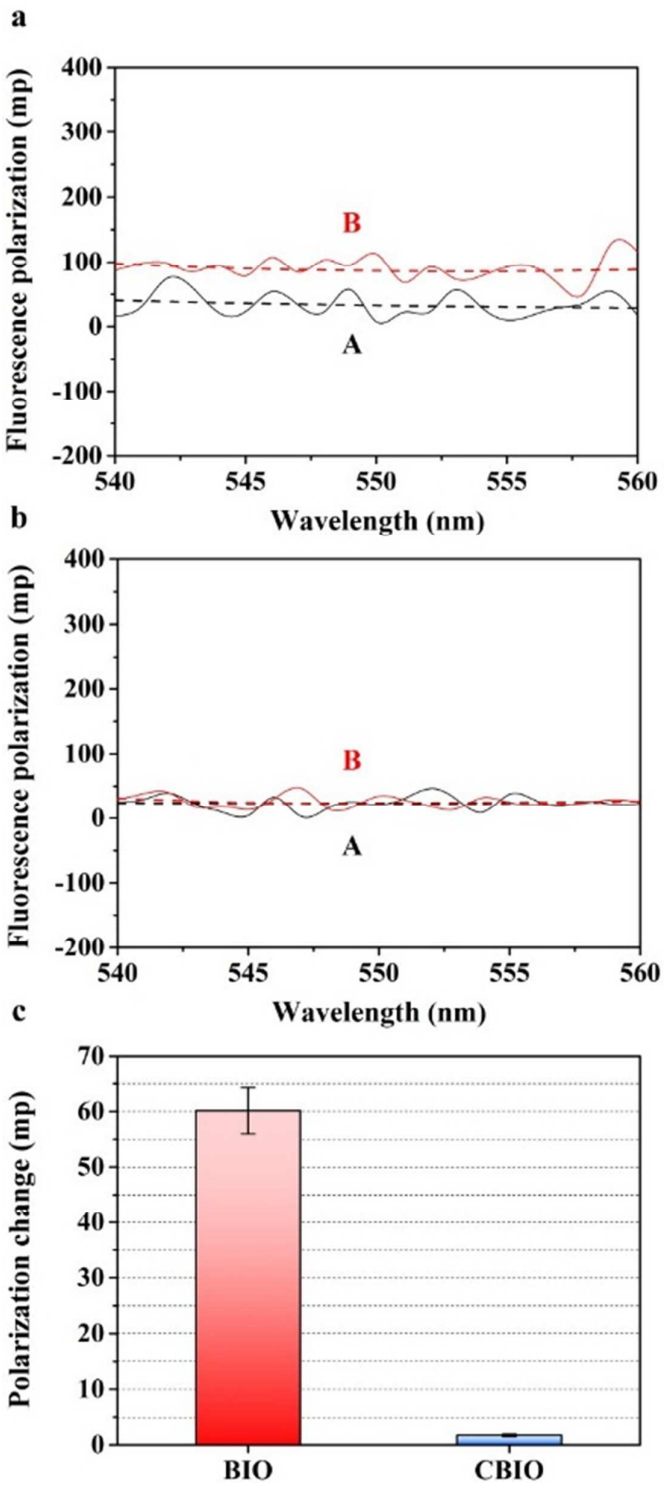


Figure S4. Specific targeted test based on Hela cells (CD44 -positive) of (a) fluorescence polarization probe **BIO** and (b) non-specific control probe **CBIO** by fluorescence polarization assay. (A) before the addition of cells and (B) after the HA-CD44 specific recognition. (c) Comparison of cell-specific targeting of fluorescence polarization probe **BIO** and control probe **CBIO**.

Table S1. Comparisons of the proposed fluorescence polarization probe with other reported sensors for Cancer cell detection

Detection technique	Materials for construction of probes (or sensors)	Detection limit (cell mL^{-1})	Linear range (cell mL^{-1})	Type of cancer cells	References
Chemiluminescence	G-Quadruplex Aptamers	6000	2×10^{3}-6×10^{5}	Hela cells	Li et al. 2009[48]
Electrochemical (impedance)	Grapheme based aptasensor	794	1×10^{3}-1×10^{6}	Hela cells	Feng et al. 2011 [49]
Electrochemical (AC impedimetric approach)	gold nanoparticles deposited on boron-doped diamond electrode	10	1×10^{1}-1×10^{5}	Hela cells	Weng et al. 2011 [50]
Electrochemical (DPV)	PEI modified single-wall carbon nanotubes	10	1×10^{1}-1×10^{6}	Hela cells	Liu et al. 2013 [51]
Electrochemical (impedance)	folate conjugated PEI modified carbon nanotubes	90	2.4×10^{2}-2.4×10^{5}	Hela cells	Wang et al. 2013 [52]
Fluorescence polarization	Naphthalimide modified HA	85	2.5×10^{2}-1×10^{6}	Hela cells	This work
Photoelectrochemical (Photocurrent)	CdS-polyamidoamine nano-composite film	5000	5×10^{3}-1×10^{7}	SMMC-7721 cells	Qian et al. 2010 [53]
Electrochemical (impedance)	Carboxymethyl chitosan-functionalized graphene	500	5×10^{2}-5×10^{6}	HL60 cells	Yang et al. 2013 [54]
Electrochemical (impedance)	polystyrene/polyaniline/Au nanocomposite	730	1.6×10^{3}-1.6×10^{8}	HL60 cells	Gu et al. 2009 [55]
Electrochemical (impedance and Square-wave voltammograms)	CdS nanoparticles labeled anti-CEA	330	1×10^{4}-1×10^{7}	MCF-7 cells	Li et al. 2010[56]
Electrochemical (cyclic voltammetry and impedance)	P-gp mouse monoclonal antibody bound on an epoxysilane monolayer	7100	5×10^{4}-1×10^{7}	K562A cells	Hao et al. 2007 [57]

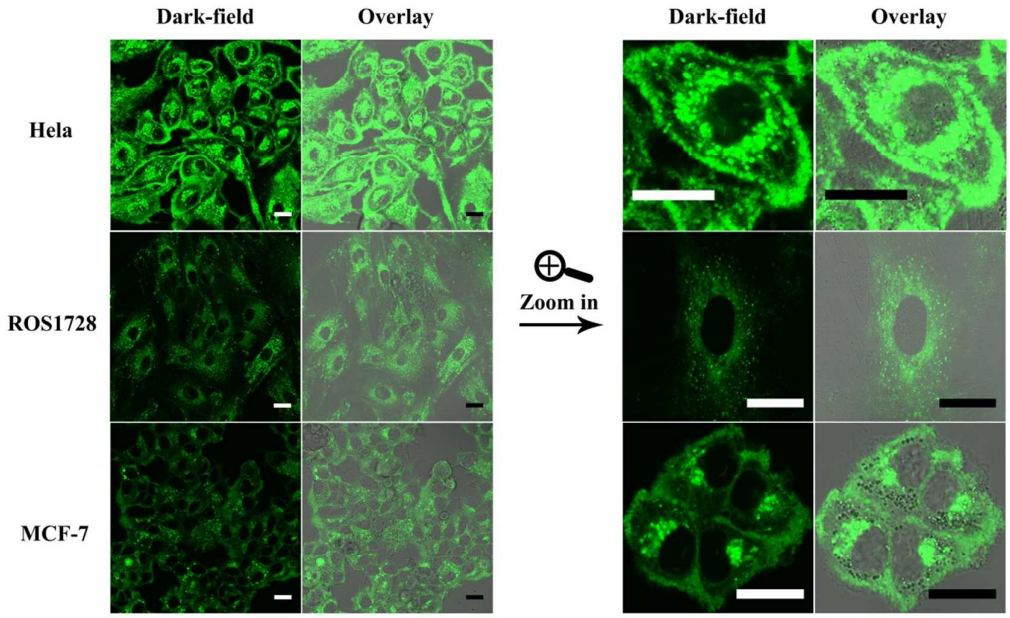


Figure S5. Specific experiment of fluorescence polarization probe **BIO** based on three types of cells. Scale bars are 20 μm.

Co–localization of probe BIO with commercial lysosome–probe Red DND–99

The Hela cells were seeded into confocal petri dish in complete medium, and then incubated for 12 h under standard culture conditions. After the cells had attached, the cells were washed three times with DMEM, and then were incubated with 2 mL of lysosome–probe Red DND–99 (50 nM) under standard culture conditions for 20 min. Lysosome–probe Red DND–99 was first prepared as a DMSO stock solution with a concentration of 10^{-3} M and was diluted with DMEM for cell incubation. At the end of incubation with lysosome–probe Red DND–99, the medium was removed, the cells were washed three times with DMEM and then were incubated with fresh medium containing probe **BIO** (14.56 μg mL^{-1}) for 30 min. Finally, cells were washed thrice with 2 mL of PBS, and 2 mL of DMEM culture medium was added and then observed under confocal microscope.

Fluorescence polarization detection

The blank control group containing only probe **BIO** was prepared by dispersing the probe **BIO** in 2 mL of PBS buffer, the fluorescence polarization measurements of the resulting solution were performed using the fluorescence spectrofluorometer at a constant temperature (25 ^{o}C). Then, the samples with Hela cells (the cell concentration was from 2.5×10^2 to 1.0×10^6 cells mL^{-1}) were obtained by dispersing different concentration cancer cells into the forenamed mixture of probe **BIO** and PBS buffer. To avoid the natural deposition of cell, the mild shaking was required during the reaction process. After the reaction of 30 min, the obtained mixture solutions were transferred to quartz cuvettes and the fluorescence polarization of the mixture solution was again detected at fluorescence spectrofluorometer. The measurements were taken using excitation filter: 460 nm (10 nm bandwidth), emwassion filter: 540–560 nm (10 nm bandwidth).

3. NMR spectrum and HRMS

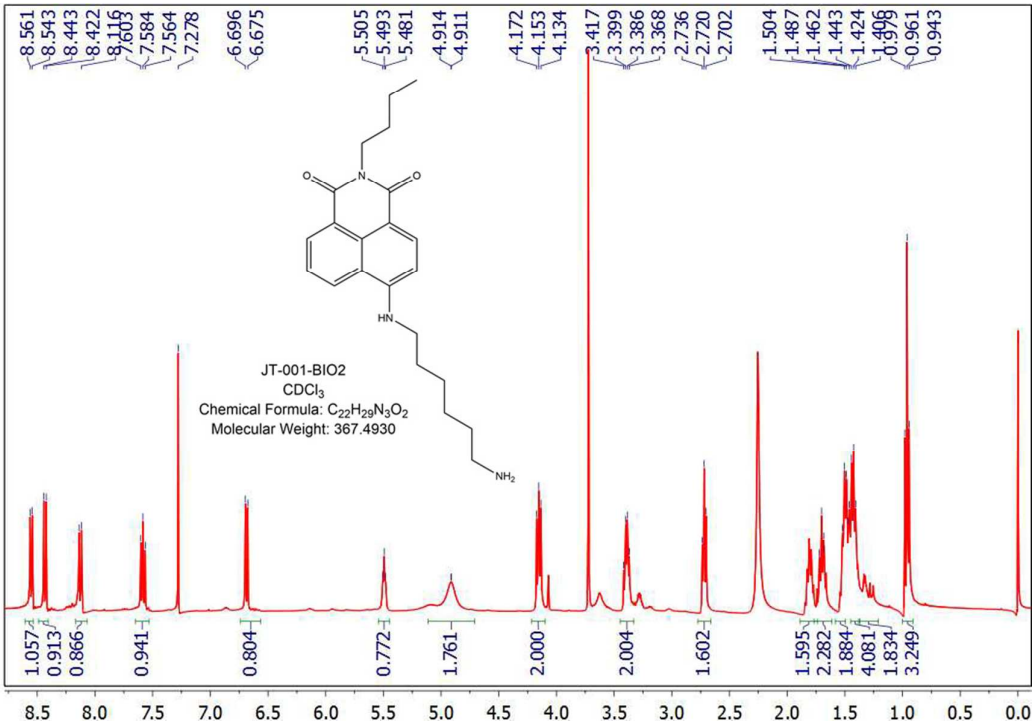


Figure S6. ^{1}H NMR spectrum of compound **BIO2**.

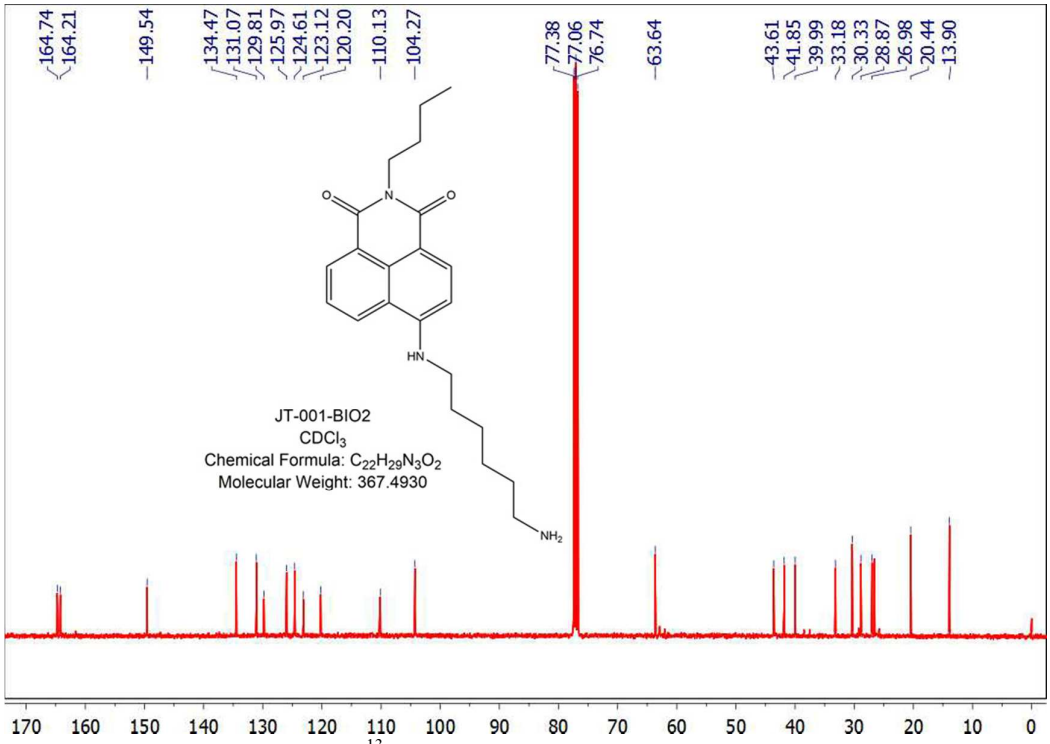


Figure S7. ^{13}H NMR spectrum of compound **BIO2**.

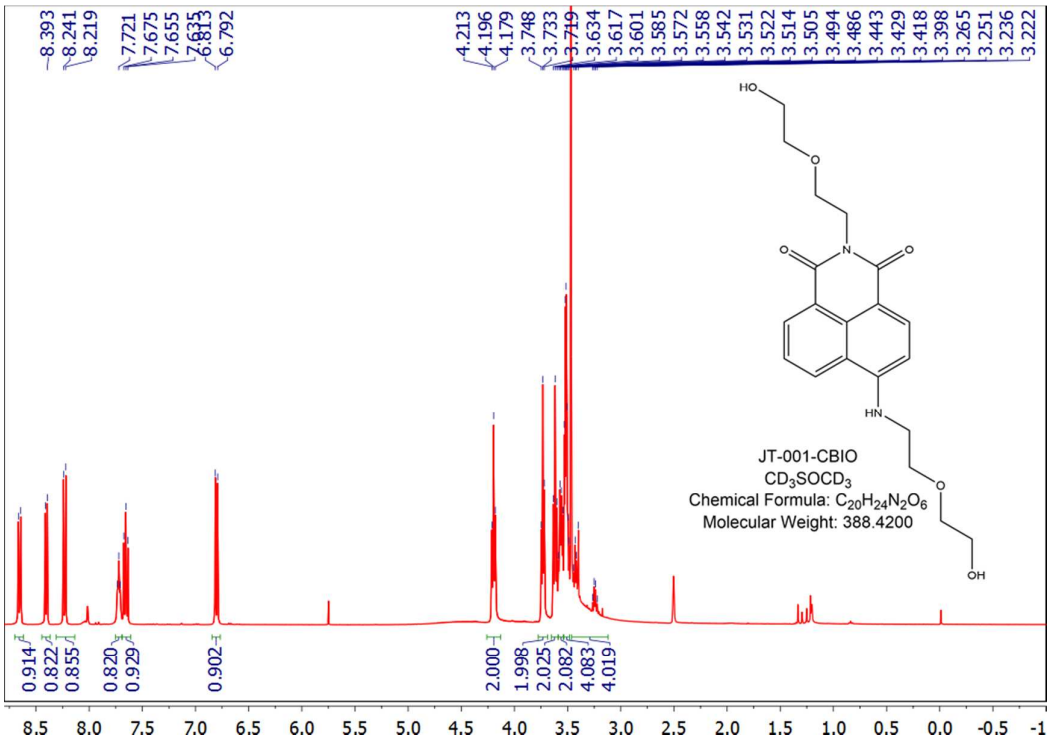


Figure S8. ^{1}H NMR spectrum of control probe **CBIO**.

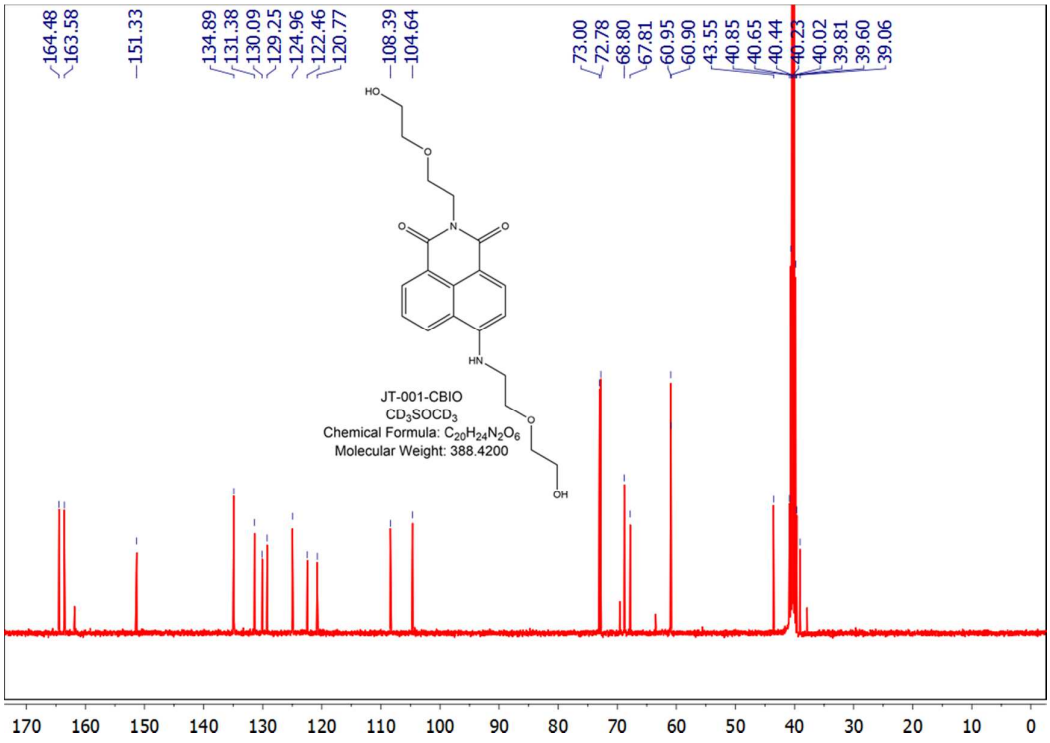


Figure S9. ^{13}C NMR spectrum of control probe **CBIO**.

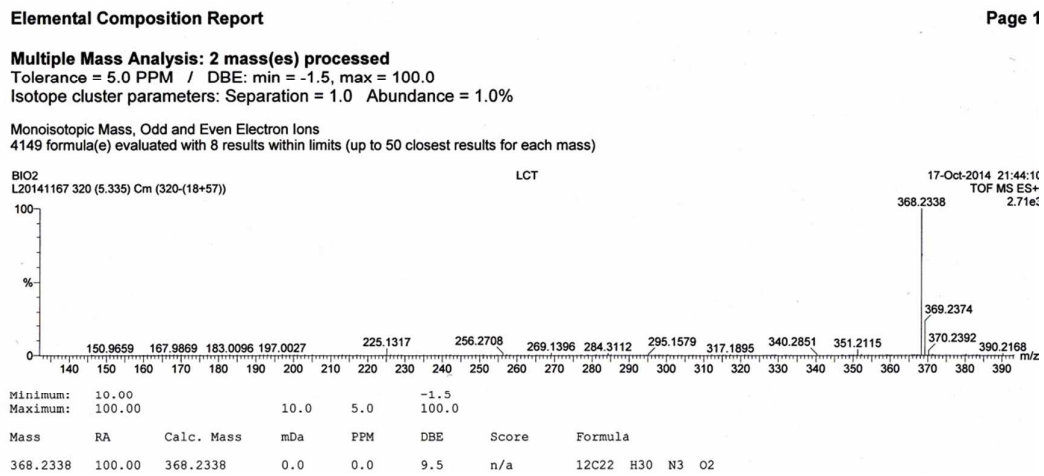


Figure S10. HRMS of compound **BIO2**.

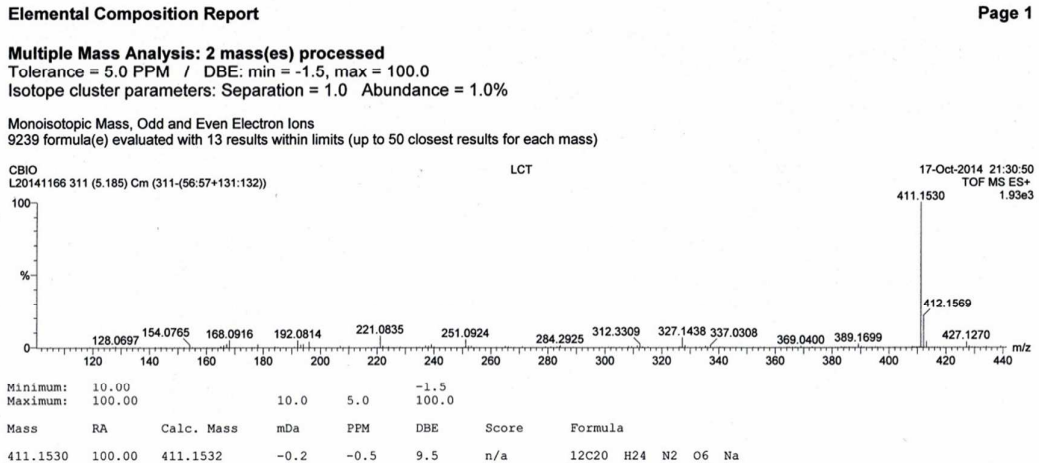


Figure S11. HRMS of control probe **CBIO**.

4. References

[S1] Liu, P.; Tian, H.; Chang, C. Luminescence Properties of Novel Soluble Quinacridones. *J. Photoch. Photobio.* A **2000,** *137*, 99-104.

[S2] Lou, Z.; Li, P.; Pan, Q.; Han, K. A Reversible Fluorescent Probe for Detecting Hypochloric Acid in Living Cells and Animals: Utilizing a Novel Strategy for Effectively Modulating the Fluorescence of Selenide and Selenoxide. *Chem. Commun.* **2013,** *49*, 2445-2447.