Supplementary Information

A viscosity-activated and tumor-specific NIR probe for precise imaging of cancer cells

Xue Wu,[a†] Lingling Cao,[a†] Qiuyue Zhao,[a] Junjie Kou,[a] Ying Li,[a*] Fanpeng Kong,[a*] and Bo Tang [a,b]

[a] College of Chemistry, Chemical Engineering and Materials Science, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Normal University, Jinan 250014, People's Republic of China. [b] Laoshan Laboratory, Qingdao 266237, Shandong, People's Republic of China.

1. General Experimental Section

All chemical/biological reagents and solvents were obtained commercially and used as supplied. Bruker 400NMR spectrometers were used for NMR. High-resolution mass spectrometry (HR-MS, ESI). spectra were obtained on a Bruker maxis UHRTOF instrument. A Shimadzu UV-1700 vis spectrophotometer and a HITACHI F-4600 fluorescence spectrophotometer were used to record absorption and fluorescence spectra. Cell imaging and in vivo imaging experiments were measured on a Leica SP8 confocal fluorescence spectrophotometer. A549 cells, HeLa cells, BEAS-2B cells, and 4T1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All the animal experiments were carried out under the relevant laws and guidelines issued by the Ethical Committee of Shandong Normal University (No. AEECSDNU2024063). After experiments, the mice carcasses were sealed and stored in a special refrigerator at -20 °C. Then, the carcasses were deeply buried after burning by Yunshui Tengyue Environmental Technology Co., Ltd. (Jinan, China).

Measurement of Probe Signal-to-Noise Ratio

The fluorescence spectra and signal-to-noise ratio of the probe were measured in high-viscosity glycerol and low-viscosity PBS solutions using a fluorescence spectrophotometer. Probe solutions at a concentration of 10 μM were prepared in PBS buffer and 99% glycerol, respectively. The excitation-emission spectra of the probe in both solutions were recorded, with the maximum emission intensity in PBS (I_{PBS}) and in glycerol (I_{Gly}) documented. Instrument parameters were set as follows: excitation and emission slits at 5 nm, voltage at 900 V. These parameters remained consistent throughout all chemical system experiments.

Fluorescence Properties of the Probe in Solvents of Different Polarities

Solvents exhibit distinct dielectric constants and varying polarities. Therefore, this experiment employed eight solvents with different polarities: PBS buffer, methanol (MeOH), acetonitrile (ACN), N, N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethanol (EtOH), dichloromethane (DCM), and glycerol (Gly) to characterize the UV absorption and fluorescence

spectra of the probe. The probe concentration was maintained at 10 μM, with an excitation wavelength set at 725 nm.

Fluorescence Properties of the Probe in Solvents of Different Viscosities

A glycerol-PBS system was employed to investigate the viscosity sensitivity of the probe. Solutions with varying viscosities (ranging from 0% to 99% glycerol) were prepared by mixing PBS and glycerol at different volume ratios. The glycerol volume fractions were 0%, 10%, 20%, 30%, 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, and 99%. The probe stock solution was added to each viscosity solution, thoroughly mixed, and the fluorescence spectra were recorded using a fluorescence spectrophotometer. Measurements were conducted with an excitation wavelength of 725 nm and a probe concentration of 10 μM.

Selectivity Assay of the Probe Toward Interfering Species

To validate the stability and specificity of the probe, a series of interfering substances including oxidizing agents, reducing agents, amino acids, metal ions, and halide ions were selected. The fluorescence emission spectra of the probe were measured in PBS buffer and 80% (v/v) glycerol solutions. Specifically, aliquots of the probe stock solution were mixed with PBS buffer or 80% glycerol, followed by the addition of different interfering ion solutions. The final probe concentration was consistently maintained at 10 μM, with interfering substances at the following concentrations: 1 mM: GSH, Cys, Hcy, DTT; 200 μM: Glu, NaHS, Na_2SO_3, H_2O_2, HClO, $ONOO^-$, KO_2, metal ions, and anions. After thorough mixing, the solutions were incubated at 37°C for 30 minutes in a constant-temperature incubator. Fluorescence emission spectra were then recorded for each interference group in both PBS and 80% glycerol systems, using an excitation wavelength of 725 nm and a probe concentration of 10 μM.

pH Stability Assay of the Probe

The pH stability of the probe was evaluated by measuring its fluorescence properties in 40% and 80% (v/v) glycerol-PBS solutions at varying pH levels. First, glycerol solutions (40% and 80% v/v) were adjusted to pH values across the range of 3-9 using HCl/NaOH under pH meter monitoring. The probe stock solution was then dissolved in these pre-adjusted solutions. After vortex mixing,

the solutions were incubated at 37°C for 30 minutes in a constant-temperature incubator. Fluorescence spectra were subsequently recorded at each pH value with an excitation wavelength of 725 nm and a probe concentration of 10 μM.

Cell Culture

This study utilized cancer cell lines (HeLa, A549) and a normal cell line BEAS-2B (human normal bronchial epithelial cells) for cellular experiments. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Culture flasks were maintained at 37°C in a humidified incubator with 5% CO_2. Upon reaching 80-90% confluency, cells were passaged using trypsinization. For imaging experiments, cells were seeded in glass-bottom dishes 24 hours prior, incubated with probes or drugs, and rinsed three times with PBS before analysis.

MTT Cytotoxicity Assay

An *in vitro* cytotoxicity analysis of the probe was performed using HeLa, A549, and BEAS-2B cells. Confluent cells were trypsinized, diluted in culture medium to a density of 1×10^5 cells/mL, and seeded into 96-well plates for 24-hour incubation. After adherence, cells were treated with probe solutions at concentrations of 0, 5, 10, 15, 20, 25, and 30 μM for 24 hours. The supernatant was then aspirated, and 100 μL of MTT solution (0.5 mg/mL) was added to each well for 4 hours. Following MTT removal, 100 μL of DMSO was introduced to dissolve the formed purple formazan crystals. Absorbance at 490 nm was measured using a microplate reader. Cell viability was calculated as:

Cell viability (%) = $(A_1 / A_2) \times 100$

where A_1 = absorbance of probe-treated groups and A_2 = absorbance of control groups.

Biotin Competition Cell Imaging Assay

Biotin receptors are overexpressed in cancer cells (e.g., breast/lung cancers) but exhibit low expression levels in normal cells. Leveraging biotin's structural simplicity, low cytotoxicity, and tumor-targeting specificity, it is widely used in tumor-targeted research. To determine whether the probe enters cancer cells via specific binding to biotin receptors, A549, HeLa, and HepG2 cells were selected for study. Cells were

divided into two groups: Probe-only group**:** Incubated with culture medium containing 10 μM probe for 30 min. Competition group**:** Pre-treated with 1 mM D-biotin for 1 h, followed by incubation with 10 μM probe for 30 min. After incubation, probe-containing medium was removed, and cells were rinsed thrice with PBS. Subsequently, 1 mL fresh culture medium was added, and confocal imaging was immediately performed using 633 nm excitation with fluorescence collection at 660-795 nm.

Cell Viscosity Imaging under Drug Stimulation

Lipopolysaccharide (LPS) effectively induces cellular inflammatory responses, which are frequently accompanied by increased intracellular viscosity. To investigate LPS-induced changes in cellular viscosity and assess the probe's capability to detect such alterations, A549 and HeLa cells were pretreated with LPS to trigger inflammation. Trypsinized cells of each line were seeded into three glass-bottom dishes and cultured until full adherence. One dish per cell line served as the control group, while the other two comprised experimental groups. The experimental groups were pretreated with 10 μg/mL LPS for 60 min and 120 min, respectively, whereas the control group received no treatment. After incubation, all dishes were thoroughly rinsed with PBS buffer. Subsequently, all three groups were incubated with probe-containing medium (10 μM) for 30 min. Following probe removal and PBS rinsing, 1 mL of fresh culture medium was added, and confocal imaging was immediately performed.

Discriminatory Imaging of Tumor and Normal Cells

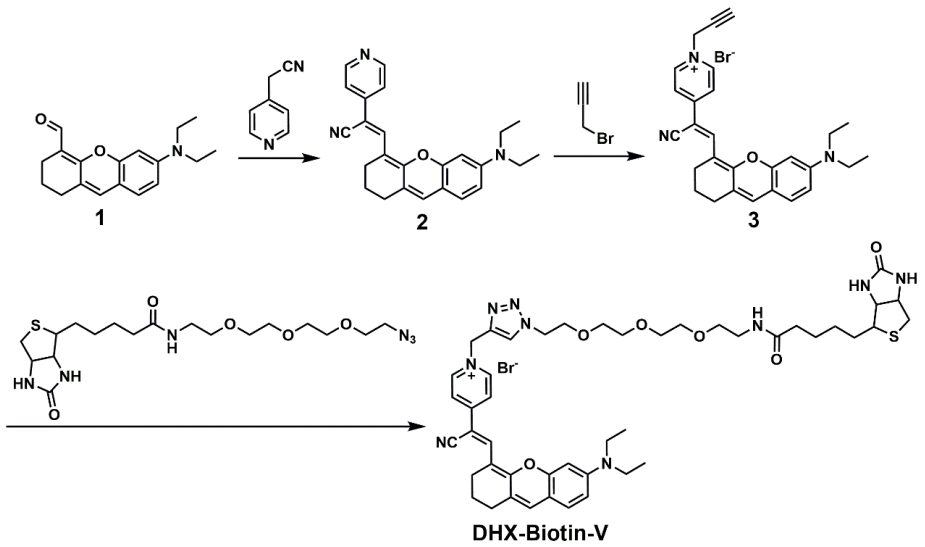
To investigate the probe's ability to discriminate between normal cells and biotin receptor-positive tumor cells-leveraging the overexpression of biotin receptors in cancer cells-the lung cancer cell line A549 and normal lung epithelial cell line BEAS-2B were selected as model systems. Trypsinized A549 and BEAS-2B cells were seeded in glass-bottom dishes and cultured until full adherence. Cells were then incubated with probe-containing medium (10 μM) for 40 min. Following incubation, the probe medium was aspirated, and cells were washed thrice with PBS. Subsequently, 1 mL of fresh culture medium was added, and confocal imaging was immediately performed.

In vivo Imaging

All 5-week-old female kunming mice were housed in the microbarrier unit (HH-MMB-I) to adapt

to the environment for one week. The model of tumor-bearing mice was established by subcutaneous injection of 4T1 cells at a density of 1×107 cells per mouse. Two weeks later, tumor-bearing mice were used for imaging experiments. **DHX-Biotin-V** (10 μM, 50 μL) was intravenously injected into the tumor-bearing or normal mice. All mice were transferred to IVIS Lumina II in vivo imaging system equipped with 680 nm and 790 ± 10 nm filters for in vivo imaging and image-guided lesion resection. The mice were anesthetized prior to injection and during imaging. After a broken neck death, a laparotomy was performed on mice to remove organ tissues and tumors, then washed with PBS three times. Images were gathered using IVIS Lumina II in vivo imaging system with an excitation of 680 nm and an open filter (790 ± 10 nm). All in vivo experiments, including hepatic surgery in mice, were approved by the Ethical Committee of Shandong Normal University (Approval No. AEECSDNU2024063) and conducted in compliance with national regulations, international guidelines, and institutional standards at Shandong Normal University. After experiments, the mice carcasses were sealed and stored in a special refrigerator at -20 °C. Then, the carcasses were deeply buried after burning by Yunshui Tengyue Environmental Technology Co., Ltd. (Jinan, China).

2. Synthesis of probe DHX-Biotin-V



Scheme S1. The synthesis route of probe **DHX-Biotin-V**.

Synthesis of Compound 2

In a 25 mL round-bottom flask, 4-pyridineacetonitrile (331 mg, 2.8 mmol) and Compound **1** (397 mg, 1.4 mmol) were combined with 5 mL of anhydrous ethanol and catalytic piperidine. The

mixture was heated under reflux with continuous stirring until reaction completion, monitored by TLC. Upon reaction termination, the solution turned purple. The mixture was concentrated under reduced pressure and purified by column chromatography using petroleum ether/ethyl acetate (2:1 v/v) as eluent. A blue-black solid was obtained (350 mg, 65% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.57 (d, *J* = 5.6 Hz, 2H), 8.18 (s, 1H), 7.53 (d, *J* = 6.1 Hz, 2H), 7.01 (d, *J* = 8.6 Hz, 1H), 6.62 (s, 1H), 6.44 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.36 (d, *J* = 2.1 Hz, 1H), 3.42 (q, *J* = 7.1 Hz, 4H), 3.02 (t, *J* = 6.0 Hz, 2H), 2.58 - 2.53 (m, 2H), 1.87 - 1.79 (m, 2H), 1.22 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 156.55, 154.87, 149.50, 145.13, 138.86, 127.63 (d, *J* = 15.3 Hz), 123.82, 119.71, 119.30, 110.74, 108.92, 108.17, 97.90, 96.96, 44.60, 29.46, 26.14, 21.23, 12.66. HRMS: (ESI, m/z) Calcd for $C_{25}H_{25}N_3O$ $[M+H]^+$: 384.2070, found 384.2065.

Synthesis of Compound 3

Compound **2** (153 mg, 0.4 mmol) and propargyl bromide (476 mg, 4.0 mmol) were dissolved in 6 mL of anhydrous acetonitrile under an argon atmosphere. The reaction mixture was stirred at 50°C for 6 h, during which precipitation occurred. After completion, the mixture was concentrated in vacuo. Purification was achieved via column chromatography using a gradient elution: initially dichloromethane/methanol (20:1 v/v), followed by dichloromethane/methanol (5:1 v/v). The title compound was obtained as a purified solid (140 mg, 70% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.59 (d, *J* = 6.9 Hz, 2H), 8.39 (s, 1H), 7.97 (d, *J* = 6.9 Hz, 2H), 7.55 (s, 1H), 7.43 (d, *J* = 8.9 Hz, 1H), 6.99 (s, 1H), 6.86 (d, *J* = 8.9 Hz, 1H), 5.39 (s, 2H), 3.98 (s, 1H), 3.52 (dd, *J* = 13.5, 6.5 Hz, 4H), 2.96 (s, 2H), 2.67 (s, 2H), 1.80 (s, 2H), 1.18 (t, *J* = 6.9 Hz, 6H); ^{13}C NMR (101 MHz, DMSO-d_6): δ 162.86, 153.18, 151.95, 142.45, 140.05, 137.47, 129.77, 122.85, 119.60, 112.53, 112.30 - 112.15 (m), 111.90 (d, *J* = 48.8 Hz), 96.93, 88.97, 80.75, 76.91, 47.80, 44.71, 28.34, 25.92, 21.11, 13.04. HRMS: (ESI, m/z) Calcd for $C_{28}H_{28}N_3O^+$ $[M]^+$: 422.2226, found 422.2278.

Synthesis of probe DHX-Biotin-V

Compound 4 (113 mg, 0.22 mmol) and azide-PEG_3-biotin conjugate (100 mg, 0.22 mmol) were dissolved in 5 mL methanol under argon atmosphere. Sodium ascorbate (7.9 mg, 0.04 mmol) and $CuSO_4 \cdot 5H_2O$ (5 mg, 0.02 mmol) were sequentially added to the solution. The reaction mixture was stirred at room temperature for 20 h under light protection. Upon completion, the mixture was

diluted with 50 mL water to remove residual inorganic salts, then dried over anhydrous sodium sulfate. After concentration, purification by column chromatography (dichloromethane/methanol = 8:1 v/v) afforded the probe as a solid (100 mg, 48% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.98 (d, *J* = 5.7 Hz, 2H), 8.75 (s, 1H), 8.36 (s, 1H), 7.87 (s, 2H), 7.52 (s, 1H), 7.20 (d, *J* = 8.9 Hz, 1H), 7.11 (s, 1H), 6.87 (s, 1H), 6.71 (s, 1H), 6.59 (s, 1H), 6.01 (s, 2H), 5.30 (s, 1H), 4.60 (t, *J* = 4.9 Hz, 2H), 4.52 - 4.46 (m, 1H), 4.36 - 4.30 (m, 1H), 3.91 (t, *J* = 5.0 Hz, 2H), 3.71 - 3.57 (m, 10H), 3.53 (d, *J* = 6.9 Hz, 4H), 3.43 (s, 2H), 3.11 (dd, *J* = 11.6, 7.0 Hz, 1H), 3.06 (t, *J* = 5.9 Hz, 2H), 2.91 - 2.69 (m, 5H), 2.68 - 2.62 (m, 2H), 2.25 (dd, *J* = 16.0, 8.4 Hz, 2H), 1.91 - 1.82 (m, 2H), 1.66 (dd, *J* = 14.3, 7.2 Hz, 2H), 1.43 (d, *J* = 6.2 Hz, 2H), 1.26 (t, *J* = 6.9 Hz, 7H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 173.72, 163.88, 162.79, 156.12, 153.18, 142.48, 140.56, 140.28, 135.66, 128.70, 126.71, 119.62, 119.26, 111.88, 111.19, 70.76 - 70.21 (m), 70.06, 69.78, 69.20, 61.88, 60.21, 55.77, 53.46, 53.09, 50.34, 45.21, 40.61, 39.12, 35.73, 29.70, 28.93, 28.11 (d, *J* = 3.5 Hz), 25.90, 25.63, 21.02, 12.72. HRMS: (ESI, m/z) Calcd for $C_{46}H_{60}N_9O_6S^+$ $[M]^+$: 866.4381, found 866.4332.

3. Supplemental figures

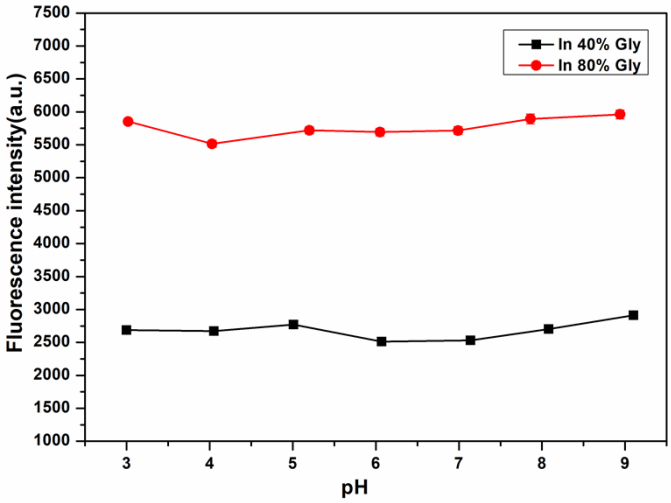


Fig. S1. Stability of **DHX-Biotin-V** (10 μM) at different pH

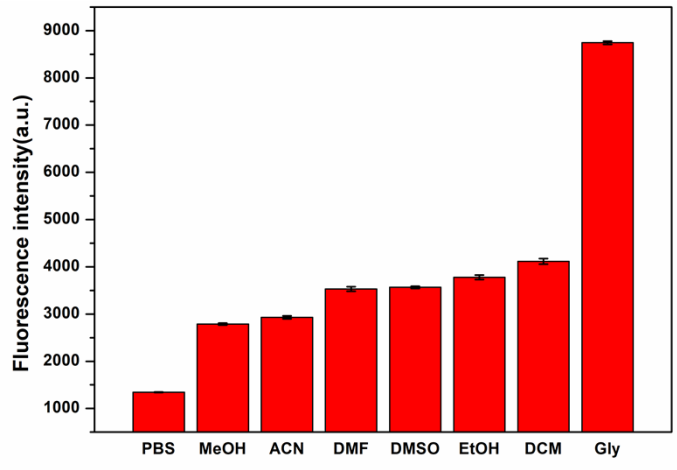


Fig. S2. Fluorescence of **DHX-Biotin-V** (10 μM) in different solvents with various polarities.

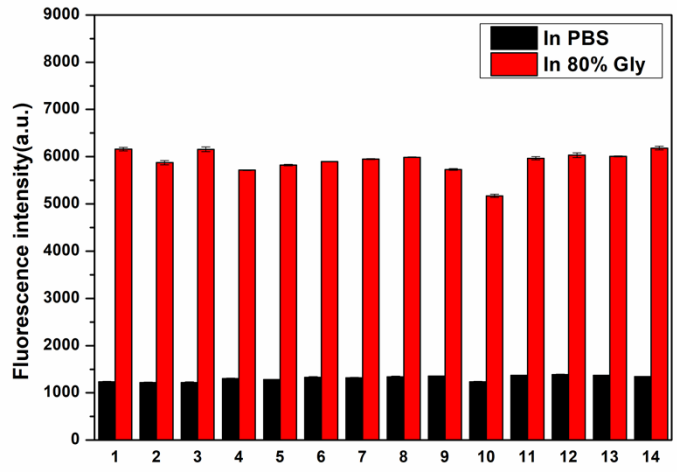


Fig. S3. Fluorescence response of **DHX-Biotin-V** (10 μM) in the presence of various analytes in PBS and 80 % glycerol. λ_{ex} = 725 nm. 1-14：Fe^{3+} (200 μM), Fe^{2+} (200 μM), Na^{+} (200 μM), K^{+} (200 μM), Cu^{2+} (200 μM), Ca^{2+} (200 μM), Mg^{2+} (200 μM), Zn^{2+} (200 μM), Ni^{3+} (200 μM), Ag^{+} (200 μM), Br^{-} (200 μM), F^{-} (200 μM), I^{-} (200 μM), Blank.

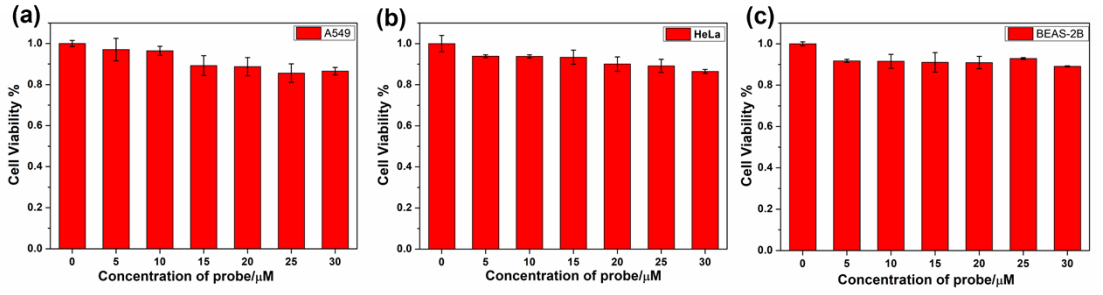


Fig. S4. MTT assay of A549 cells (a), HeLa cells (b), and BEAS-2B cells (c) in the presence of different concentrations of **DHX-Biotin-V.**

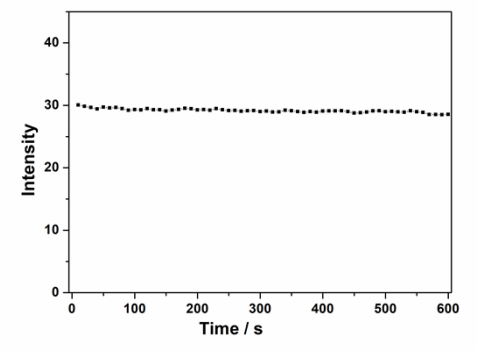


Fig. S5. Test of photo-stability. Fluorescence images were achieved by means of time-sequential scanning of the **DHX-Biotin-V**-loaded HeLa cells for 10 min. Normalized fluorescence intensity from three regions from 0 to 600 s of time-sequential scanning.

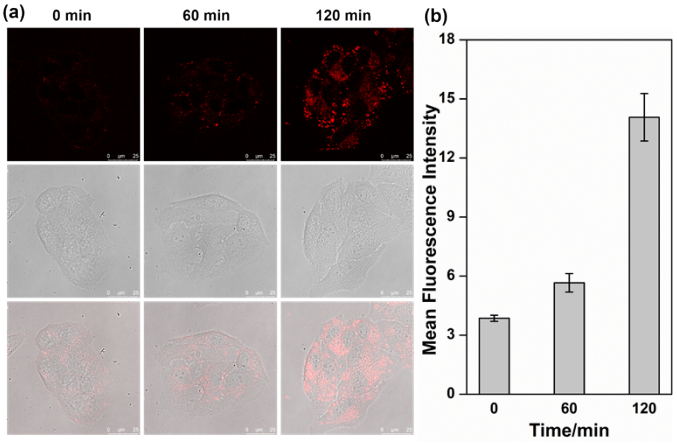


Fig. S6 (a)Time-dependent fluorescence intensity changes of **DHX-Biotin-V** (10 μM) in HeLa cells induced by nystatin (10 μM). (b) Relative fluorescence intensities of the images in Fig a. λ_{ex} = 633 nm, collected 660-795 nm, Scale bar: 25 μm.

4. ^{1}H NMR, ^{13}C NMR and HR MS spectra

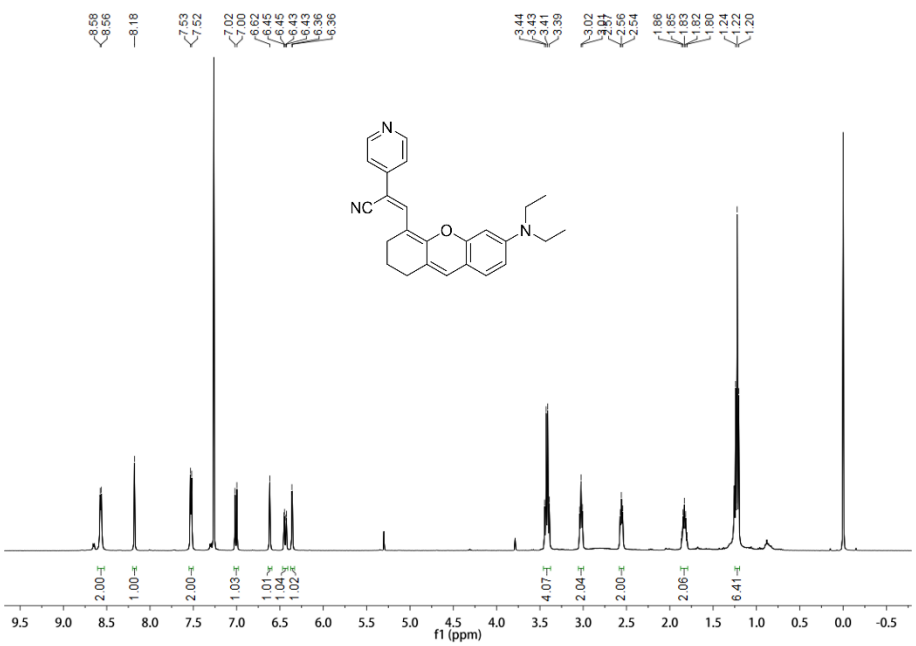


Fig. S7 ^{1}H NMR spectrum of compound **2** in $CDCl_3$ (400 MHz).

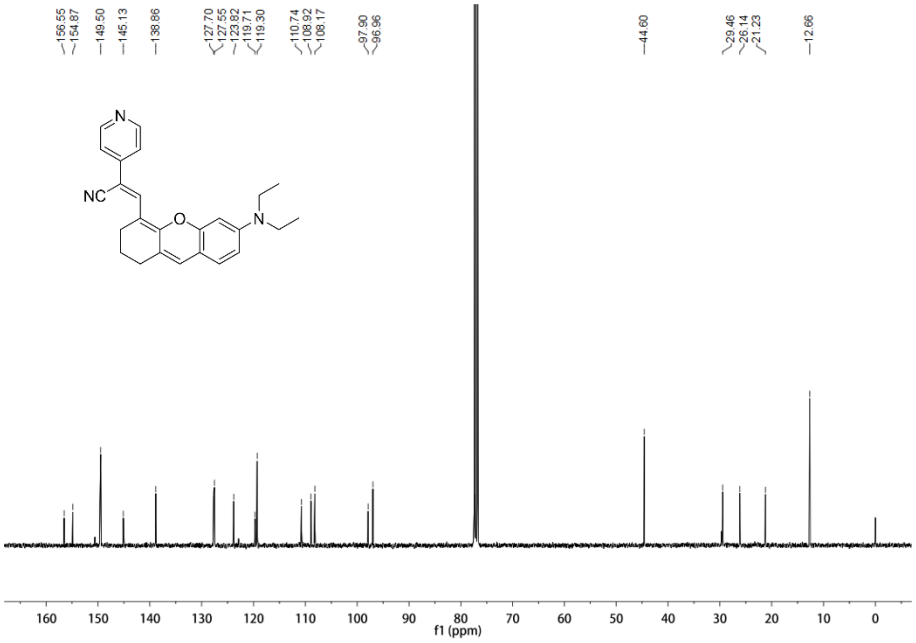


Fig. S8 ^{13}C NMR spectrum of compound **2** in $CDCl_3$ (100 MHz).

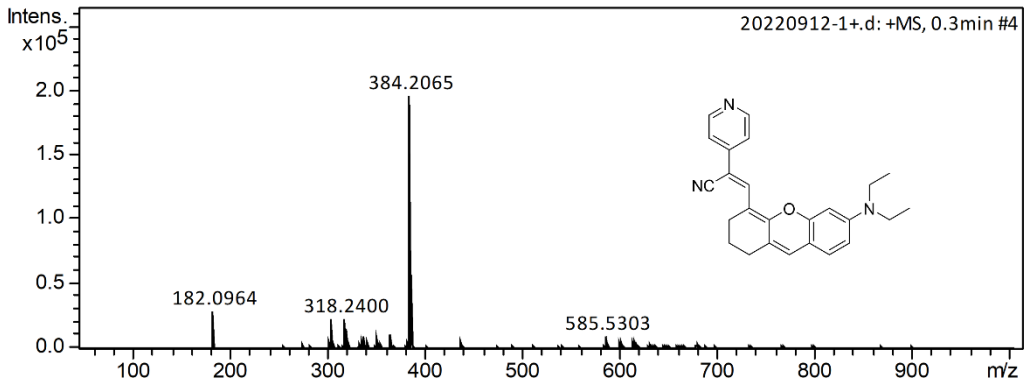


Fig. S9 HR MS spectrum of compound **2**

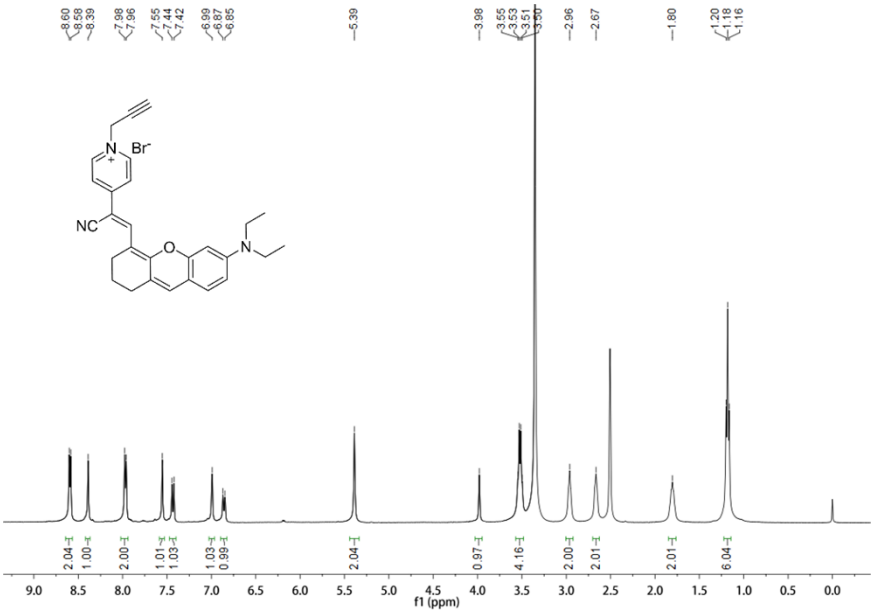


Fig. S10 ^{1}H NMR spectrum of compound **3** in $CDCl_3$ (400 MHz).

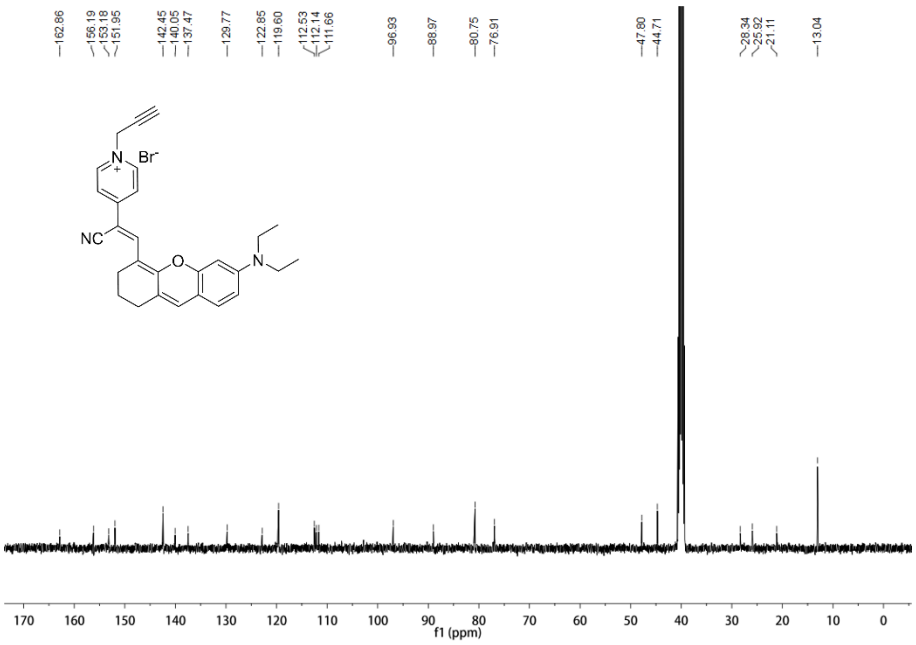


Fig. S11 ^{13}C NMR spectrum of compound **3** in $CDCl_3$ (100 MHz).

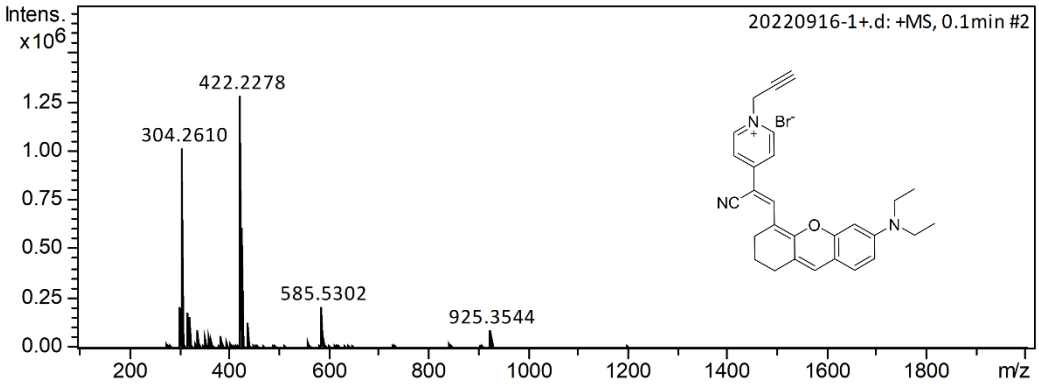


Fig. S12 HR MS spectrum of compound **3**

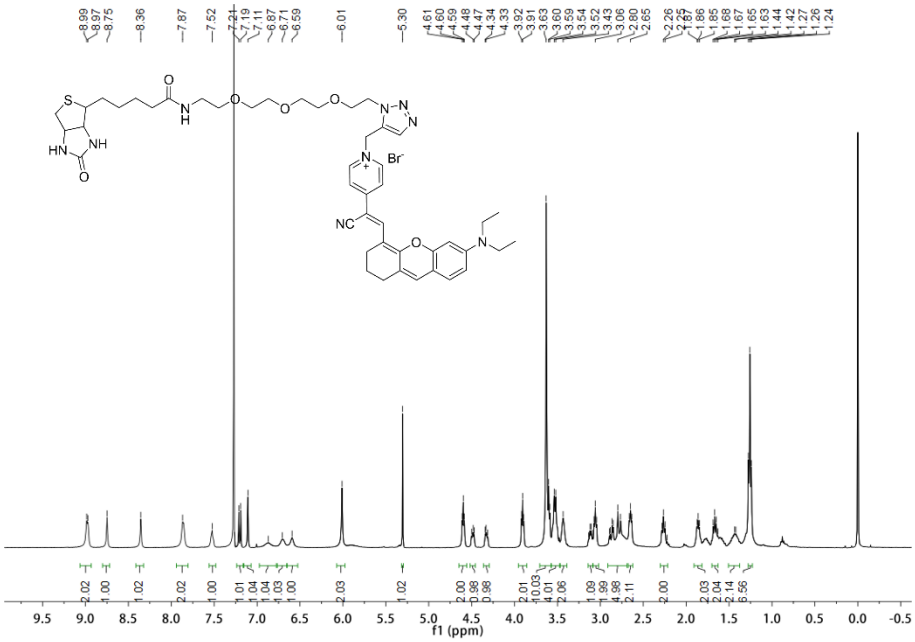


Fig. S13 ^{1}H NMR spectrum of compound **DHX-Biotin-V** in $CDCl_3$ (400 MHz).

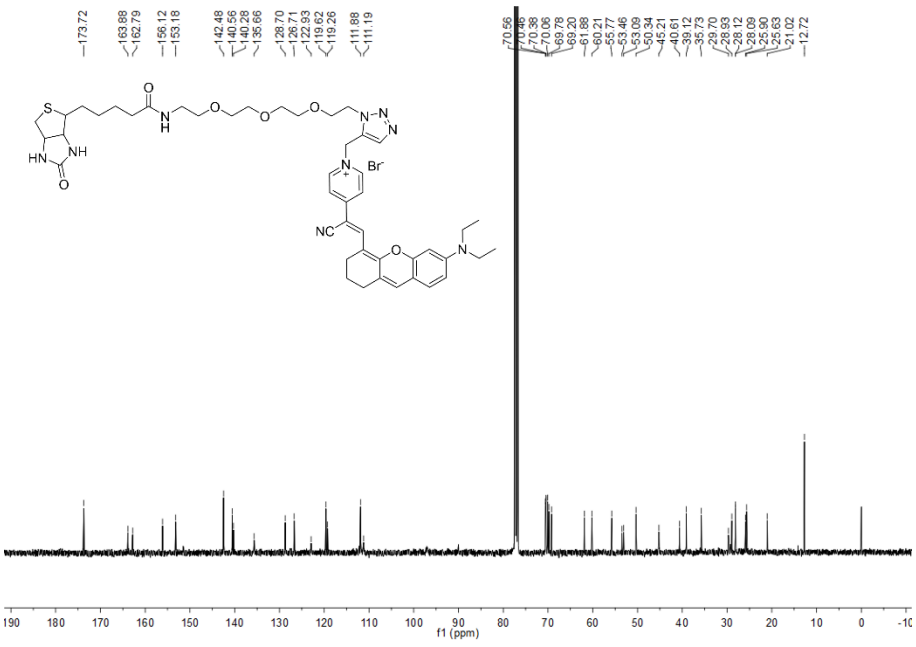


Fig. S14 ^{13}C NMR spectrum of compound **DHX-Biotin-V** in $CDCl_3$ (100 MHz).

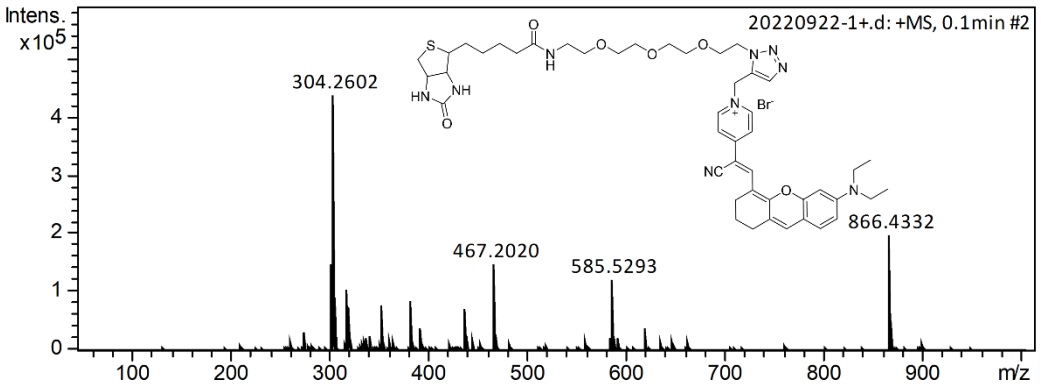


Fig. S15 HR MS spectrum of **DHX-Biotin-V**