Electronic Supplementary Material (ESI) for Analyst.

A Benzimidazole-based Ratiometric Fluorescent Probe for Accurate and Rapid Monitoring of Lysosomal pH in Cell Autophagy and Anticounterfeiting

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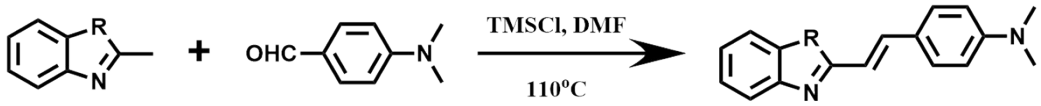
1. Synthesis Steps and Characterization of Probe BD, OD and ID



R =
- **N: 4-(2-(1*H*-benzo[*d*]imidazol-2-yl)vinyl)-*N*,*N*-dimethylaniline (BD)**
- **O: 4-(2-(benzo[*d*]oxazol-2-yl)vinyl)-*N*,*N*-dimethylaniline (OD)**
- **CMe_2: 4-(2-(3,3-dimethyl-3*H*-indol-2-yl)vinyl)-*N*,*N*-dimethylaniline (ID)**

Fig. S1 Schematic of the synthesis of probes **BD**, **OD** and **ID**

P-Dimethylaminobenzaldehyde and 2-methylbenzimidazole were mixed in N, N-dimethylformamide. After adding an appropriate amount of trimethylchlorosilane, the mixture was sealed and stirred at 110 °C for 24 hours. When cooling to room temperature, add an aqueous solution of saturated sodium carbonate to the mixture, adjust the pH to alkaline, and continue stirring for 30 minutes. Then, the reaction product was extracted with dichloromethane and saturated sodium chloride aqueous solution, the organic phase was collected, and the solvent was removed by evaporation under reduced pressure. Finally, the crude product was purified by silica

gel column chromatography (eluent PE/EA = 3/1, v/v) to obtain yellow powder **BD**. **BD** was characterized by ^{1}H NMR, ^{13}C NMR and MS (MALDI TOF), as Figure S2, S3 and S4. ^{1}H NMR (600 MHz, DMSO) δ 12.44 (s, 1H), 7.56 (s, 1H), 7.53 (s, 1H), 7.49 (s, 1H), 7.48 (s, 1H), 7.13 (d, *J* = 3.6 Hz, 2H), 6.94 (s, 1H), 6.91 (s, 1H), 6.76 (s, 1H), 6.74 (s, 1H), 2.96 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ: 152.37 – 152.34 (m), 151.35 – 151.06 (m), 135.42 – 135.37 (m), 128.74 – 128.72 (m), 123.82 – 123.80 (m), 122.13 – 122.10 (m), 112.69 – 112.68 (m), 112.62 – 112.61 (m), 40.30 – 40.30 (m). MS (MALDI TOF) m/z 264.1501 for $[M+H]^{+}$.

Probe **OD** and **ID** were synthesized similarly to **BD** except that 2-methylbenzimidazole was exchanged for an equal amount of 2-methylbenzoxazole or 2,3,3-trimethylindole.

OD was characterized by ^{1}H NMR, ^{13}C NMR, as Figure S5, S6. ^{1}H NMR (400 MHz, DMSO) δ 7.72 (s, 1H), 7.68 (s, 1H), 7.62 (s, 1H), 7.60 (s, 1H), 7.35 (d, *J* = 1.6 Hz, 1H), 7.33 (s, 1H), 6.98 (s, 1H), 6.94 (s, 1H), 6.76 (s, 1H), 6.74 (s, 1H), 2.99 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 163.95 – 163.91 (m), 151.91 – 151.88 (m), 150.23 – 150.16 (m), 142.50 – 142.46 (m), 140.62 – 140.61 (m), 129.89 – 129.88 (m), 125.19 – 125.17 (m), 124.94 – 124.93 (m), 122.78 – 122.75 (m), 119.48 – 119.47 (m), 112.37 – 112.35 (m), 110.70 – 110.69 (m), 108.15 – 108.14 (m), 40.19 – 40.18 (m).

ID was characterized by ^{1}H NMR, ^{13}C NMR, as Figure S7, S8. ^{1}H NMR (400 MHz, DMSO) δ 7.62 (dd, *J* = 19.3, 12.5 Hz, 3H), 7.44 (dd, *J* = 12.7, 7.4 Hz, 2H), 7.28 (td, *J* = 7.6, 1.1 Hz, 1H), 7.17 (td, *J* = 7.4, 0.8 Hz, 1H), 6.96 (d, *J* = 16.2 Hz, 1H), 6.75 (d, *J* = 8.8 Hz, 2H), 2.98 (s, 6H), 1.37 (d, *J* = 12.3 Hz, 6H). ^{13}C NMR (101 MHz, DMSO) δ 184.11 – 184.04 (m), 154.57 – 154.47 (m), 151.63 – 151.51 (m), 147.06 – 146.99 (m), 138.68 – 138.66 (m), 129.65 – 129.63 (m), 127.97 – 127.95 (m), 125.15 – 125.13 (m), 123.95 – 123.87 (m), 121.84 – 121.83 (m), 119.87 – 119.85 (m), 114.80 – 114.78 (m), 112.42 – 112.40 (m), 52.49 – 52.47 (m), 40.25 – 40.24 (m), 23.77 – 23.75 (m).

2. MTT Assay Procedure

SNU-423 cells in the exponential growth phase were seeded in a 96-well microtiter plate at a density of 5000/well, and incubated in a 37 ^{o}C incubator with a carbon dioxide concentration of 5%. After 24 hours, the **BD** solution with the concentration of 0 (control), 1, 10, 50, 100, 200 μM was prepared with fresh medium, and added to the microwells of different groups at 200 μL per

well to replace the original medium. After continued incubation for another 24 hours, cells were gently washed three times with phosphate buffered saline (PBS) and then injected into each well with 20μL MTT working solution (10 mg/mL in PBS). After 4 hours, the MTT solution in each well was discarded, and the cells were treated with 150 μL DMSO to dissolve the intracellular formazan crystals. The absorbance value of each well solution at the wavelength of 490 nm was determined using a microplate reader at room temperature. Cell viability was expressed by the percentage of OD values in the study group relative to the control group.

3. Supplementary Figures

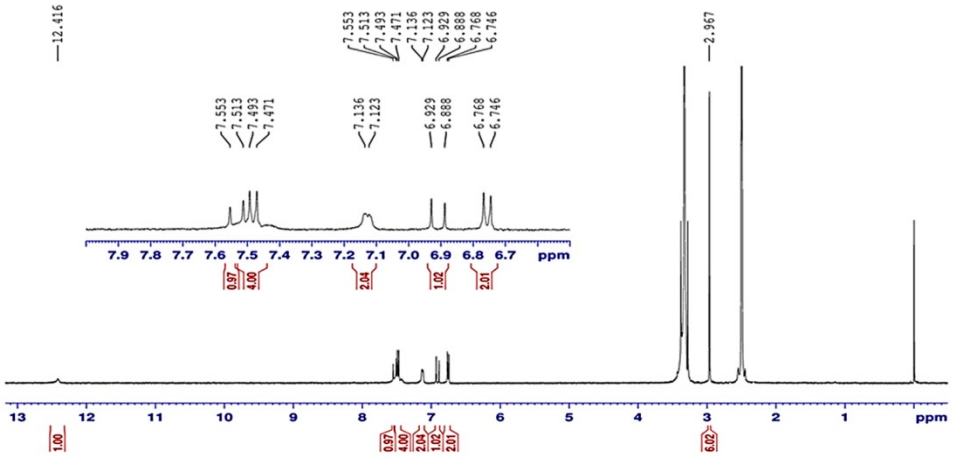


Fig. S2 ^{1}H NMR spectrum of **BD**

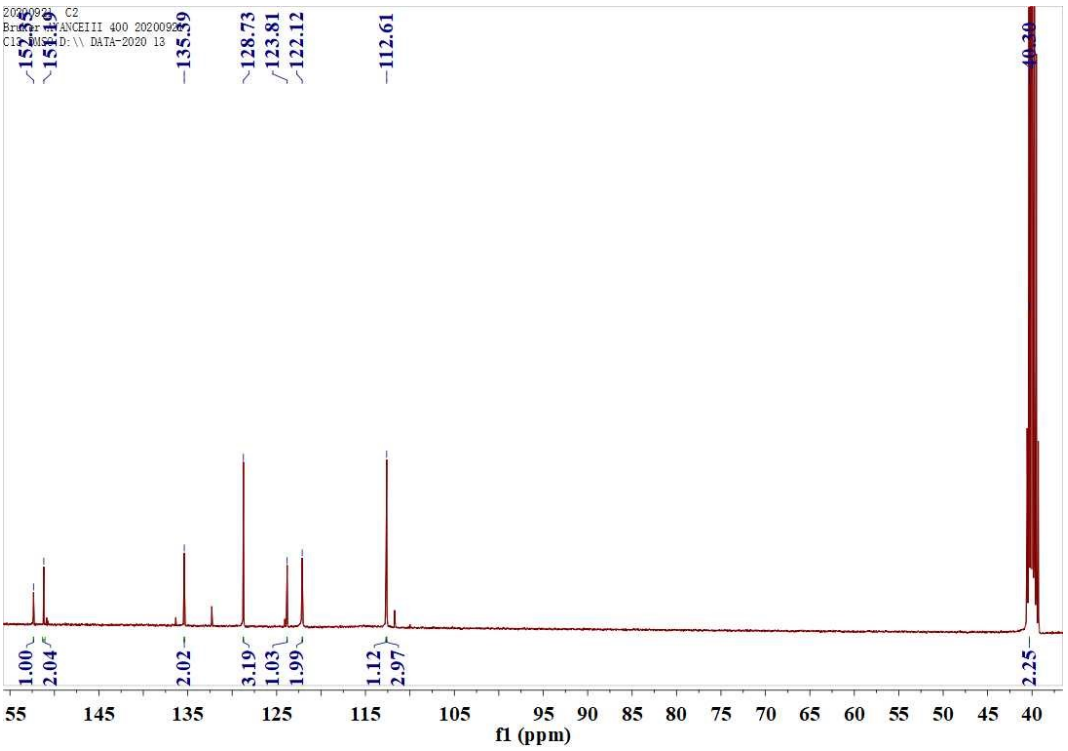


Fig. S3 ^{13}C NMR spectrum of **BD**

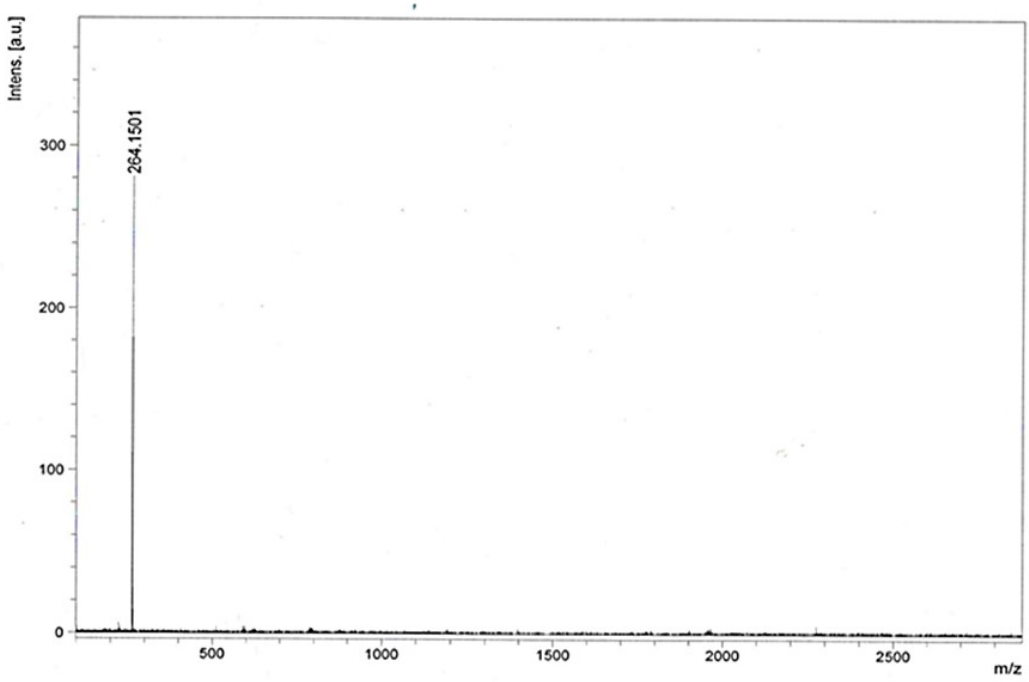


Fig. S4 HR-MS spectrum of **BD**

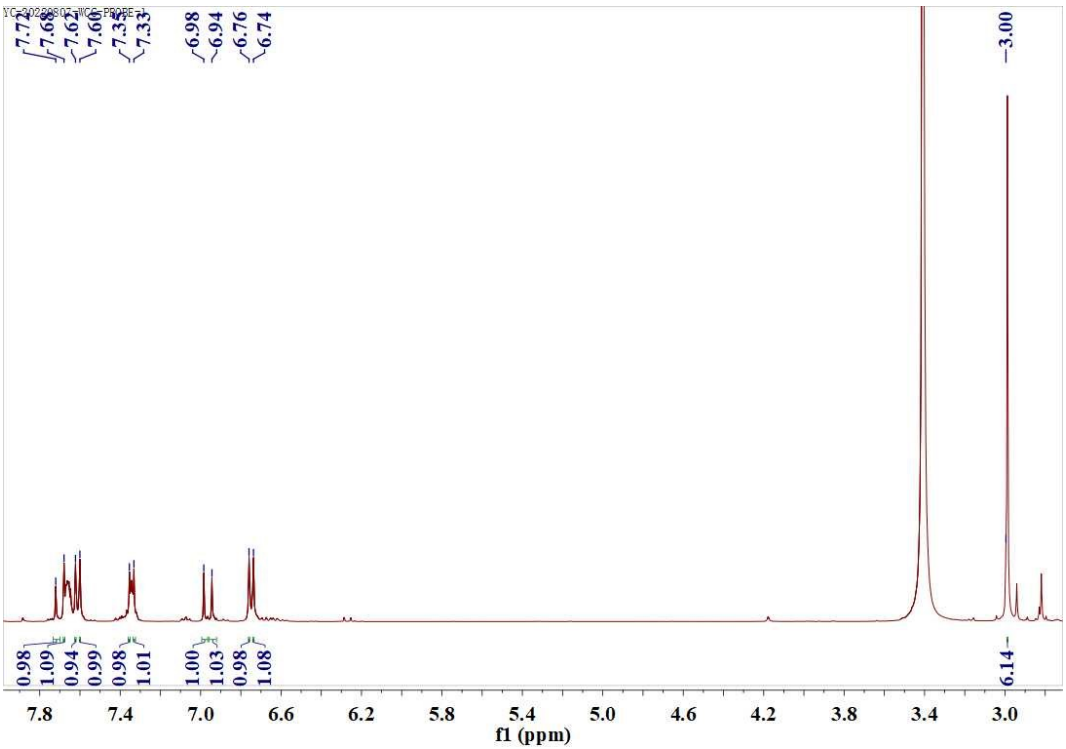


Fig. S5 ^{1}H NMR spectrum of **OD**

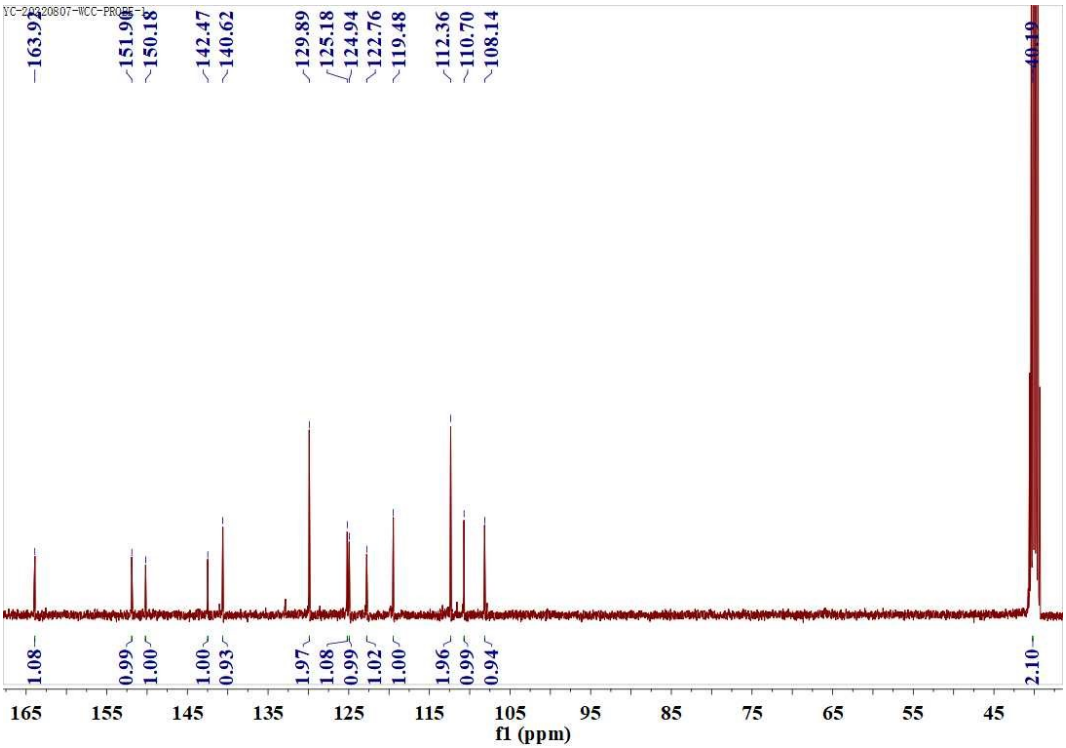


Fig. S6 ^{13}C NMR spectrum of **OD**

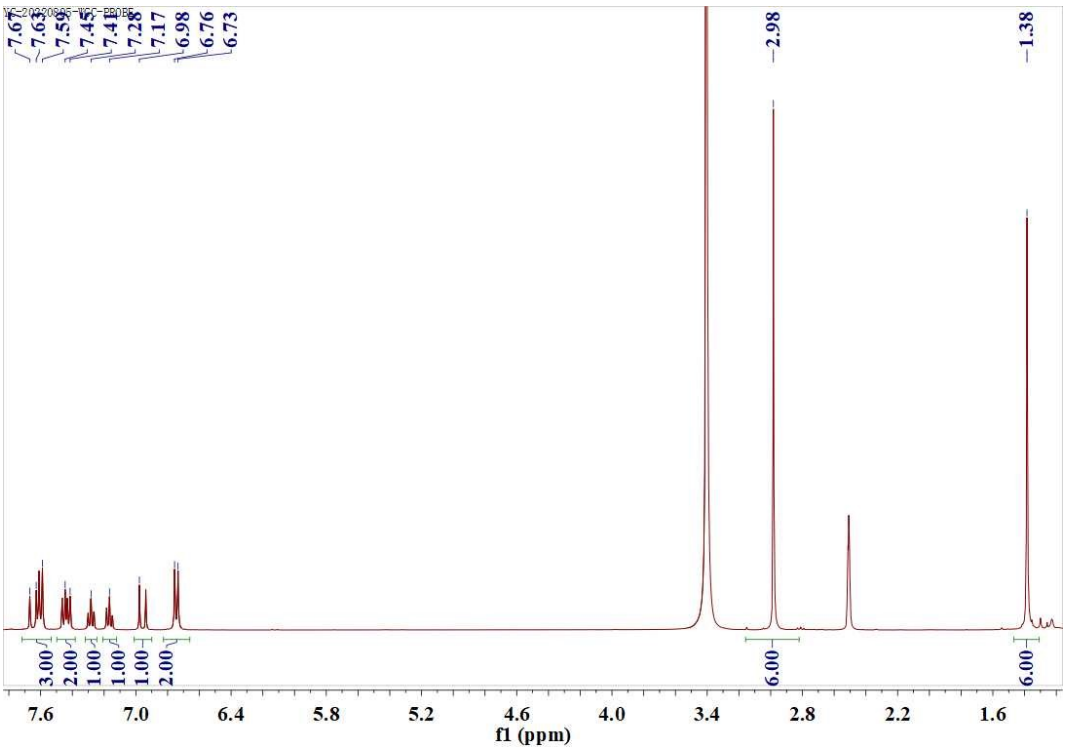


Fig. S7 ^{1}H NMR spectrum of **ID**

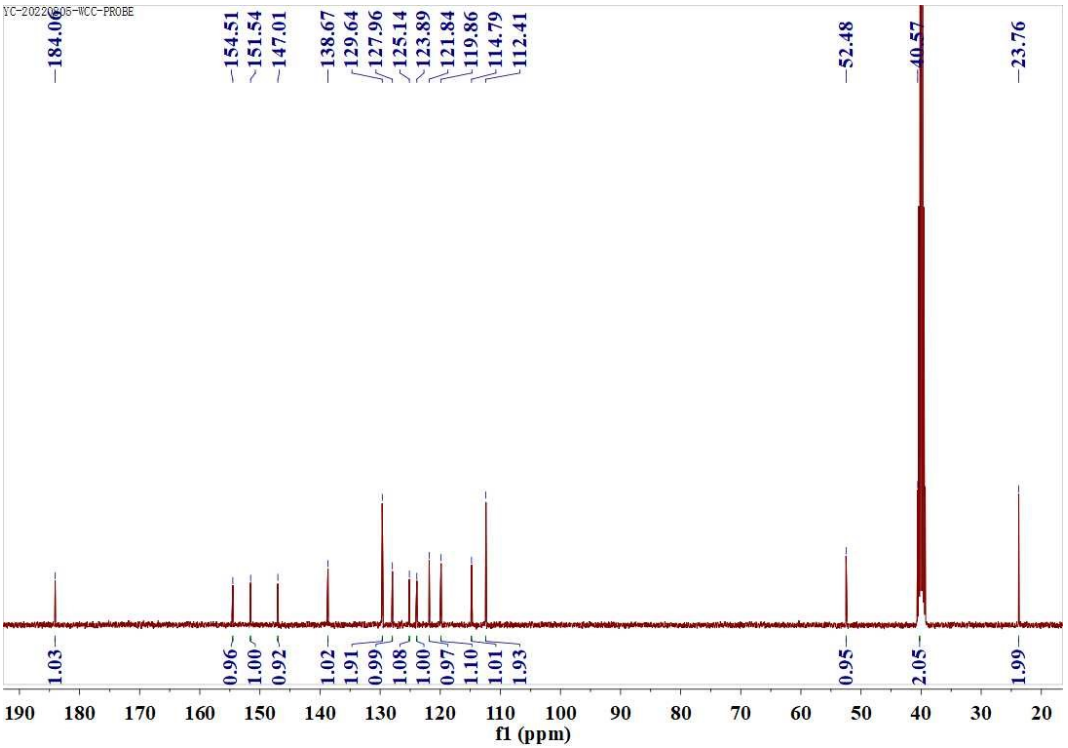


Fig. S8 ^{13}C NMR spectrum of **ID**

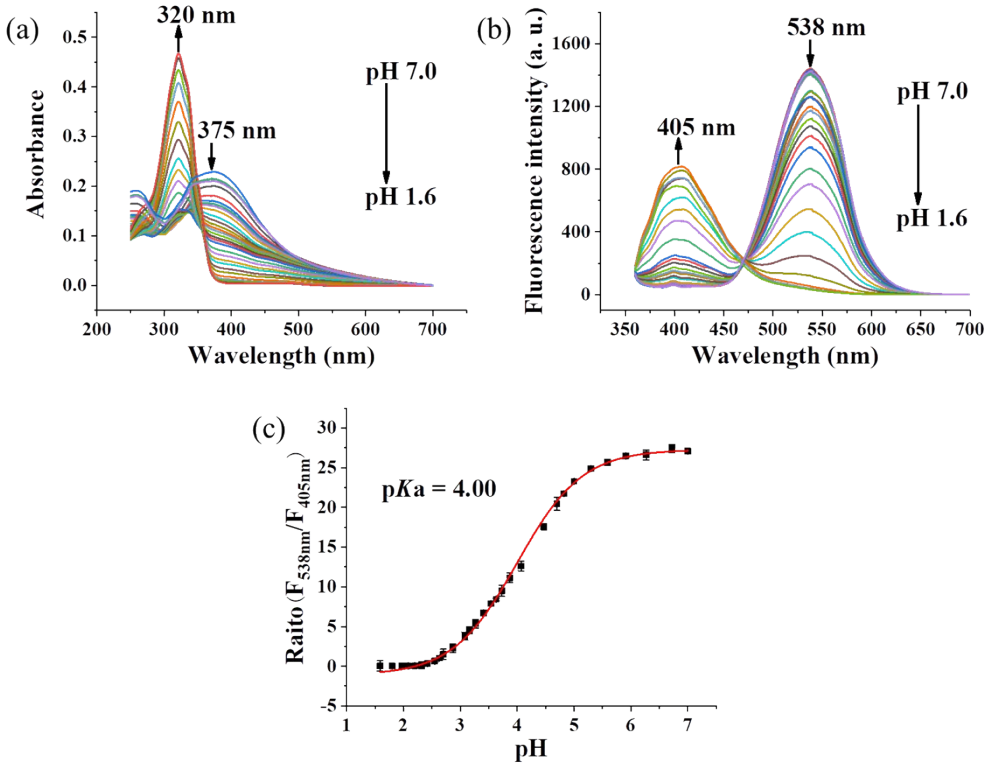


Fig. S9 The optical performance of **OD**. (a) The absorption spectra of **OD** (10 μM) in a solution of pH 7.0-1.6. (b) The fluorescence spectra of **OD** (10 μM) in a solution of pH 7.0-1.6, λ_{ex} = 350 nm. (c) The pH (7.0-1.6) dependent Raito (F_{538nm}/F_{405nm}) sigmoidal fitting graph. Error bars represent standard deviation, n=5.

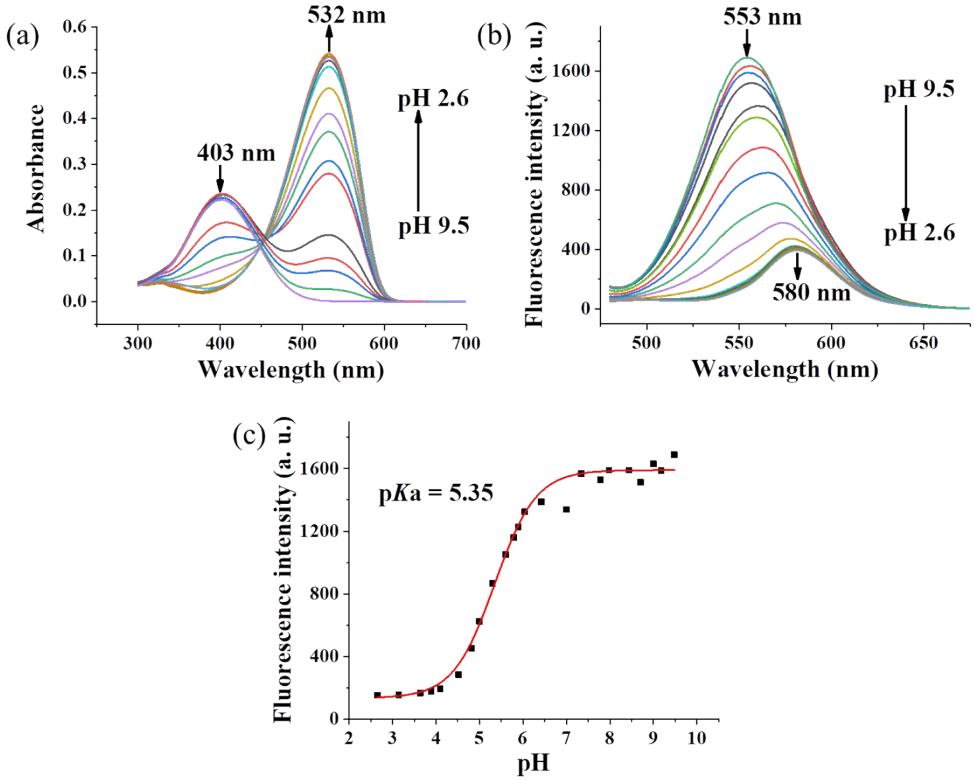


Fig. S10 The optical performance of **ID**. (a) The absorption spectra of **ID** (10 μM) in a solution of pH 9.5-2.6. (b) The fluorescence spectra of **ID** (10 μM) in a solution of pH 9.5-2.6, λ_{ex} = 450 nm. (c) The pH (9.5-2.6) dependent fluorescence intensity (553 nm) sigmoidal fitting graph. Error bars represent standard deviation, n=5.

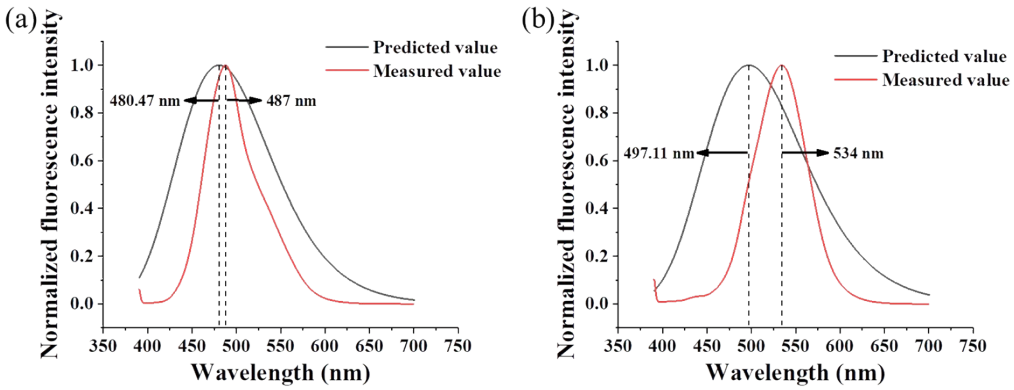


Fig. S11 Predicted emission wavelength compared to measured emission wavelength. (a) Before protonation. (b) After protonation.

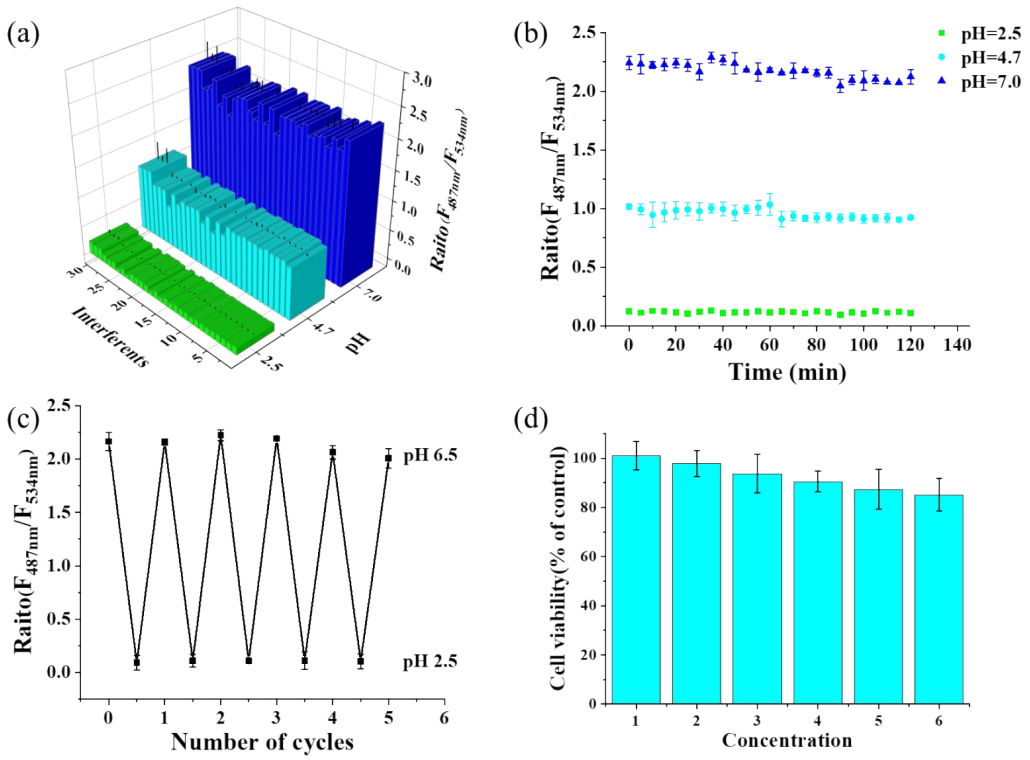


Fig. S12 (a) Raito (F_{487nm}/F_{534nm}) of **BD** (10 μM) in the presence of several cations, anions, and biomolecular interferences at pH 2.5, 47, and 7.0. 1, blank; 2, Na^{+} (10 mM); 3, K^{+} (10 mM); 4, Mg^{2+} (10 mM); 5, Cd^{2+} (0.5 mM); 6, Ca^{2+} (0.5 mM); 7, Co^{2+} (0.5 mM); 8, Ba^{2+} (0.5 mM); 9, Cu^{2+} (0.5 mM); 10, Zn^{2+} (0.5 mM); 11, Fe^{3+} (0.5 mM); 12, Al^{3+} (0.5 mM); 13, NH^{4+} (0.5 mM); 14, chloroquine (0.5 mM); 15, hydrogen peroxide (0.5 mM); 16, N-acetylcysteine (0.5 mM); 17, glutamate (0.5 mM); 18, leucine (0.5 mM); 19, valine (0.5 mM); 20, serine (0.5 mM); 21, cysteine (0.5 mM); 22, aspartate (0.5 mM); 23, phenylalanine (0.5 mM); 24, vitamin C (0.5 mM); 25, Cl^{-} (100 mM); 26, SO_4^{2-} (10 mM); 27, NO_3^{-} (0.5 mM); 28, PO_4^{3-} (0.5 mM); 29, CO_3^{2-} (0.5 mM); 30, $C_2H_3O^{2-}$ (0.5 mM). (b) The photostability of **BD** (10 μM) in pH 2.5, 4.7, and 7.0 solutions within 2 hours. (c) The pH reversibility of **BD** (pH=2.5 and 7.0). (d) Cytotoxicity of different concentrations of **BD** to SNU-423 cells. 1, Control; 2, 1 μM; 3, 10 μM; 4, 50 μM; 5, 100 μM; 6, 200 μM. Error bars represent standard deviation, n=5 (a-c) or n=6 (d).

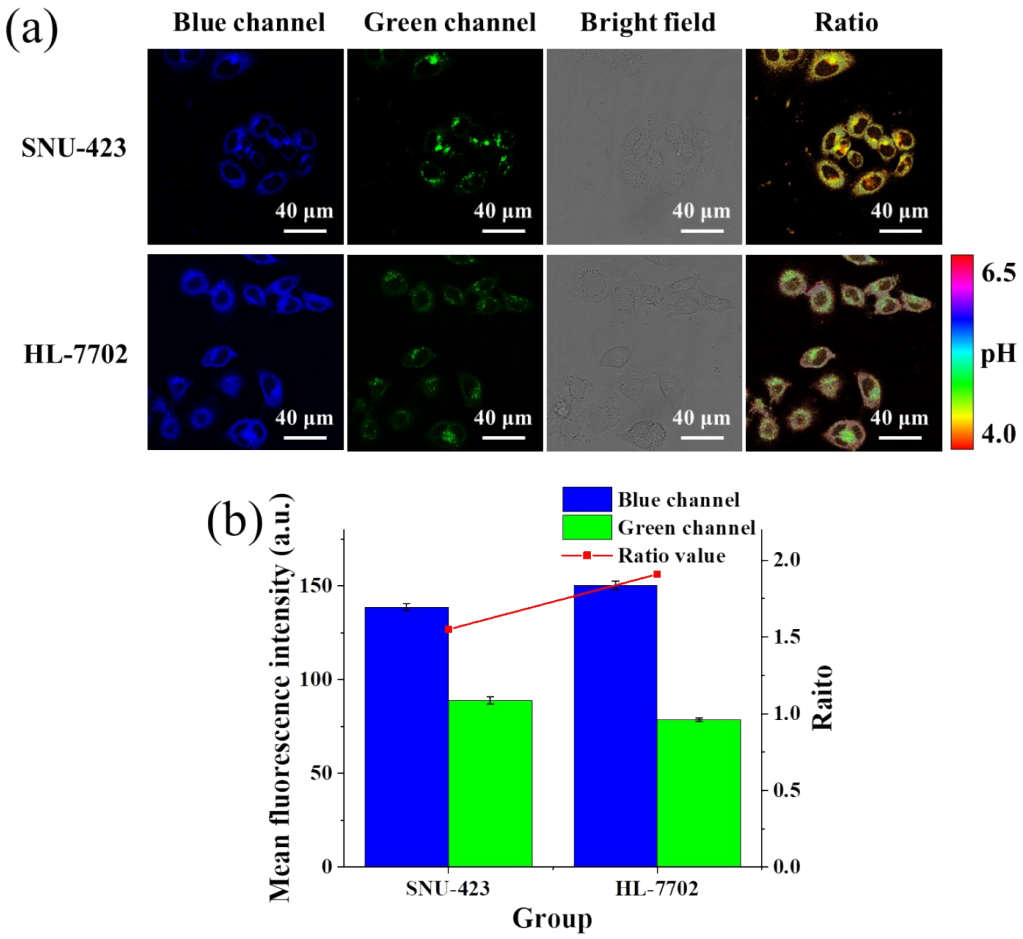


Fig. S13 (a) Confocal images of probe **BD** on SNU-423 and HL-7702 cells. λ_{ex} = 408 nm, the blue channel λ_{em} = 460-500 nm, the green channel λ_{em} = 510-560 nm. (b) The mean fluorescence intensities of the blue and green channels and the corresponding ratiometric fluorescence values. Error bars represent standard deviation, n=5.