

A Rational Approach to Tuning the pKa Values of Rhodamines for Living Cell Fluorescence Imaging

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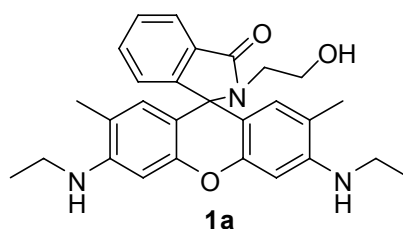
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Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Melting points of compounds were measured on a Beijing Taike XT-4 microscopy melting point apparatus, and all melting points were uncorrected; Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer from Thermo Finnigan or Agilent 1100 HPLC/MSD spectrometer; NMR spectra were recorded on an INOVA-400 spectrometer, using TMS as an internal standard; Electronic absorption spectra were obtained on a LabTech UV Power spectrometer; Photoluminescent spectra were recorded with a HITACHI F4600 fluorescence spectrophotometer; Cells imaging was performed with a Nikon Eclipse TE300 inverted microscope; TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from the Qingdao Ocean Chemicals.

Cell culture and fluorescent imaging: HeLa cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO₂ and 95% air at 37 °C. The cells were plated on 12-well plates and allowed to adhere for 24 h. Immediately before the experiments, the cells were washed with PBS buffer, and then the cells were incubated with probe **1e** (2 μM) for 30 min at 37 °C. Subsequently, the cells were washed in PBS medium of varying pH values (pH was adjusted by adding small amounts of 0.2 N solution of NaOH or 0.1 N solution of HCl, and 1 μg/mL of nigericin (Aldrich) was added to the medium to induce a rapid exchange of K⁺ for H⁺ for a fast equilibration of external and internal pH.¹⁻³ Then the fluorescence images were acquired through a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a cooled CCD camera, excitation with green light.

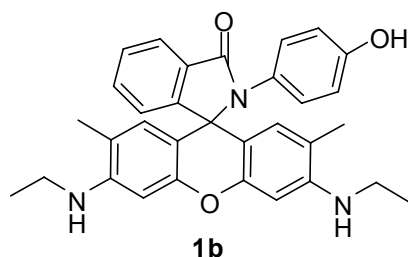
General approach for the synthesis of compounds **1a-e**:

To a solution of rhodamine 6G acid **3a** (225.1 mg, 0.5 mmol) in dry 1, 2-dichloroethane (8.0 ml) at room temperature, phosphorus oxychloride (0.25 mL) was added dropwise over a period of 5 min. After being refluxed for 8 h, the reaction mixture was cooled and concentrated under vacuum to give rhodamine 6G acid chloride. Without further purification, the resulting acid chloride was dissolved in dry THF (10 mL), and then was added dropwise to a solution of amines **4a-e** (0.6 mmol) in dry THF (10 ml) containing triethylamine (0.1 mL). After stirring for 8 h at room temperature, the mixture was concentrated under vacuum and the crude product was purified by column chromatography (CH₂Cl₂) to give compounds **1a-e** as a white solid.

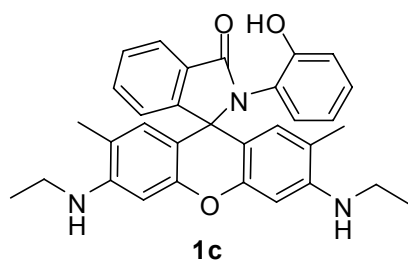


1a: (209.8 mg, 91.6% yield): m.p. 263~268 °C; UV-vis (pH 3.0 H₂O/DMF 4: 1, v/v) λ_{max} (log ε): 530 (4.87); ¹H NMR (400 MHz, CDCl₃): δ = 1.32-1.35 (t, 6H), 1.93 (s, 6H), 3.19-3.26 (m, 6H), 3.43-3.46 (t, 2H), 6.29 (s, 2H), 6.37 (s, 2H), 7.03-7.05 (m, 1H), 7.45-7.48 (m, 2H), 7.92-7.94 (m,

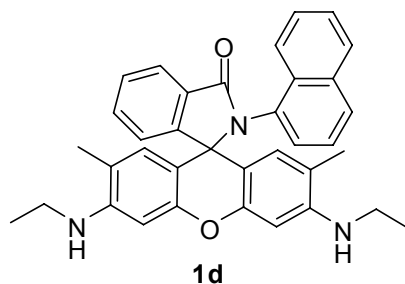
1H); ^{13}C NMR (100 MHz, CDCl_3): $\delta = 14.64, 16.76, 38.43, 44.69, 62.64, 65.98, 96.66, 118.25, 122.95, 123.79, 128.07, 128.20, 130.31, 132.82, 151.60, 153.87, 170.12$; ESI-MS m/z : 458.2 $[\text{M}+\text{H}]^+$; EI-MS m/z : 457.3 $[\text{M}]^+$; HRMS (EI): m/z calcd for $\text{C}_{28}\text{H}_{31}\text{O}_3\text{N}_3$ $[\text{M}]^+$ 457.2360; Found 457.2354.



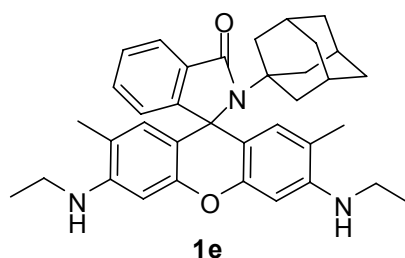
1b: (152.4 mg, 58.6% yield): m.p. 245~250 °C; UV-vis (pH 3.0 $\text{H}_2\text{O}/\text{DMF}$ 4: 1, v/v) λ_{max} (log ϵ): 532 (4.96); ^1H NMR (400 MHz, $d_6\text{-CD}_3\text{COCD}_3$): $\delta = 1.25\text{-}1.29$ (t, 6H), 1.97 (s, 6H), 3.15-3.19 (q, 4H), 6.19 (s, 2H), 6.43 (s, 2H), 6.52-6.55 (m, 5H), 6.76-6.78 (d, $J = 7.6$ Hz, 1H), 7.07-7.09 (dd, $J = 7.6$ Hz, 1H), 7.56-7.58 (m, 2H), 7.90-7.92 (dd, $J = 7.6$ Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3): $\delta = 14.11, 14.71, 16.77, 38.37, 68.10, 68.90, 96.66, 106.49, 115.89, 117.74, 123.34, 124.20, 127.58, 128.26, 128.55, 129.17, 129.73, 130.03, 131.65, 132.83, 147.31, 151.88, 152.82, 155.45, 167.89, 173.32$; ESI-MS m/z : 506.2 $[\text{M}+\text{H}]^+$. EI-MS m/z : 505.3 $[\text{M}]^+$; HRMS (EI): m/z calcd for $\text{C}_{32}\text{H}_{31}\text{O}_3\text{N}_3$ $[\text{M}]^+$ 505.2360; Found 505.2355.



1c: (186.2 mg, 71.6% yield): m.p. 256~260 °C; UV-vis (pH 3.0 $\text{H}_2\text{O}/\text{DMF}$ 4: 1, v/v) λ_{max} (log ϵ): 534 (4.97); ^1H NMR (400 MHz, CDCl_3): $\delta = 1.29\text{-}1.32$ (t, 6H), 1.92 (s, 6H), 3.14-3.19 (q, 4H), 3.51 (bs, 2H), 6.27 (s, 2H), 6.39-6.42 (dd, $J = 8.0, 2.0$ Hz, 1H), 6.45 (s, 2H), 6.55-6.59 (m, 1H), 6.75 (s, 1H), 6.88-6.90 (dd, $J = 8.0, 1.2$ Hz, 1H), 6.99-7.04 (m, 1H), 7.08-7.10 (m, 1H), 7.50-7.52 (m, 2H), 8.02-8.04 (m, 1H); ^{13}C NMR (100 MHz, CDCl_3): $\delta = 14.65, 16.74, 38.29, 69.11, 96.64, 106.06, 118.14, 119.78, 120.72, 123.43, 123.94, 124.99, 125.33, 127.97, 128.31, 128.40, 129.27, 133.30, 147.48, 151.29, 152.07, 154.98, 168.76$; ESI-MS m/z : 506.4 $[\text{M}+\text{H}]^+$. EI-MS m/z : 505.3 $[\text{M}]^+$; HRMS (EI): m/z calcd for $\text{C}_{32}\text{H}_{31}\text{O}_3\text{N}_3$ $[\text{M}]^+$ 505.2360; Found 505.2357.

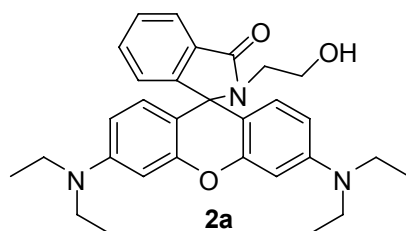


1d: (202.8 mg, 75.1% yield): m.p. 260~264 °C; UV-vis (pH 3.0 H₂O/DMF 4: 1, v/v) λ_{max} (log ϵ): 534 (4.96); ¹H NMR (400 MHz, CDCl₃): δ = 1.10-1.14 (t, 3H), 1.33-1.37 (t, 3H), 1.88 (s, 3H), 2.03 (s, 3H), 2.92-2.96(m, 2H), 3.19-3.23 (m, 2H), 6.26 (s, 1H), 6.28-6.30 (dd, J = 8.0, 2.0 Hz, 1H), 6.52 (s, 1H), 6.57 (s, 1H), 7.11-7.19 (m, 2H), 7.25-7.29 (m, 3H), 7.45-7.47 (d, J = 8.4 Hz, 1H), 7.62-7.67 (m, 4H), 8.11-8.14 (m, 1H); ¹³C NMR (100 MHz, CDCl₃): δ = 14.35, 14.63, 16.58, 16.89, 38.53, 68.74, 96.83, 118.17, 123.64, 124.34, 124.49, 125.09, 125.43, 126.04, 127.82, 128.44, 128.53, 128.75, 129.65, 130.74, 132.39, 132.66, 132.86, 134.07, 147.43, 151.96, 152.06, 152.76; ESI-MS m/z : 540.4 [M+H]⁺; EI-MS m/z : 539.3 [M]⁺; HRMS (EI): m/z calcd for C₃₆H₃₃O₂N₃ [M]⁺ 539.2567; Found 539.2560.

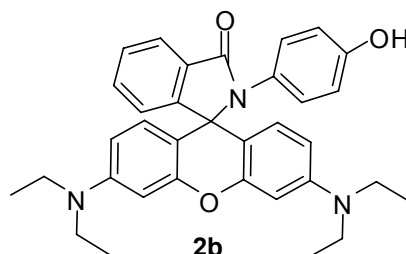


1e: (223.0 mg, 81.4 % yield): m.p. 272~275°C; UV-vis (pH 3.0 H₂O/DMF 4: 1, v/v) λ_{max} (log ϵ): 532 (5.03); ¹H NMR (400 MHz, CDCl₃): δ = 1.32-1.36 (t, 6H), 1.48-1.49 (d, J =2.8 Hz, 6H), 1.86 (s, 3H), 1.95 (s, 6H), 2.11 (d, J =2.0 Hz, 6H), 3.21-3.23 (q, 4H), 6.35 (bs, 2H), 6.457 (s, 2H), 6.74-6.76 (dd, J =6.4 2.0 Hz, 1H), 7.28-7.33 (m, 2H), 7.78-7.80 (m, 1H); ¹³C NMR (100 MHz, CDCl₃): δ = 14.73, 16.76, 29.85, 36.31, 38.59, 39.07, 59.31, 65.55, 97.04, 117.48, 122.17, 122.85, 127.27, 128.64, 129.93, 132.14, 146.89, 149.82, 155.61, 169.47 ppm. ESI-MS m/z : 548.5 [M+H]⁺. EI-MS m/z : 547.4 [M]⁺; HRMS (EI): m/z calcd for C₃₆H₄₁O₂N₃ [M]⁺ 547.3193; Found 547.3192.

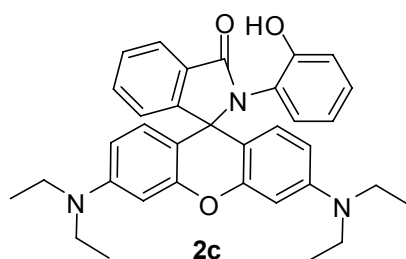
General approach for the synthesis of compounds 2a-e: To a solution of rhodamine B acid **3b** (239.1 mg, 0.5 mmol) in dry 1, 2-dichloroethane (10.0 ml) at room temperature, phosphorus oxychloride (0.25 mL) was added dropwise over a period of 5 min. After being refluxed for 8 h, the reaction mixture was cooled and concentrated under vacuum to give rhodamine B acid chloride. Without further purification, the resulting acid chloride was dissolved in dry THF (10 mL), and then was added dropwise to a solution of amines **4a-e** (0.6 mmol) in dry THF (10 ml) containing triethylamine (0.1 mL). After stirring for 8 h at room temperature, the mixture was concentrated under vacuum and the crude product was purified by column chromatography (CH₂Cl₂) to give compounds **2a-e** as a white solid.



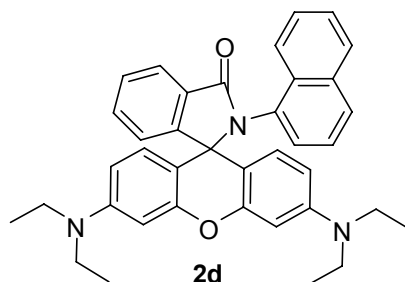
2a: (215.4 mg, 88.6% yield): m.p. 212~214 °C; UV-vis (pH 3.7 H₂O/DMF 4: 1, v/v) λ_{\max} (log ϵ): 560 (3.82); ¹H NMR (400 MHz, CDCl₃): δ = 1.16-1.20 (t, 12H), 3.28-3.37 (m, 10H), 3.46-3.47 (q, 2H), 6.29-6.30 (d, *J* = 8.0 Hz, 2H), 6.39 (s, 2H), 6.49-6.52 (d, *J* = 7.6 Hz, 2H), 7.06-7.10 (m, 1H), 7.44-7.48 (m, 2H), 7.89-7.93 (m, 1H); ¹³C NMR (100 MHz, CDCl₃): δ = 12.58, 44.38, 44.64, 62.62, 97.81, 108.27, 122.93, 123.80, 128.18, 128.52, 130.43, 132.72, 148.92, 153.24, 170.09; ESI-MS *m/z*: 486.2 [M+H]⁺.



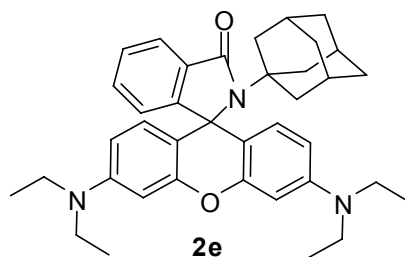
2b: (137.8 mg, 51.6% yield): m.p. 218~222 °C; UV-vis (pH 3.5 H₂O/DMF 4: 1, v/v) λ_{\max} (log ϵ): 562 (3.74); ¹H NMR (400 MHz, CDCl₃): δ = 1.11-1.15 (t, 12H), 3.29-3.31 (q, 8H), 6.19 (s, 2H), 6.22-6.24 (d, *J* = 8.0 Hz, 2H), 6.28-6.34 (m, 4H), 6.55-6.57 (d, *J* = 8.4 Hz, 2H), 7.11 (bs, 1H), 7.19-7.22 (m, 1H), 7.25 (s, 2H), 7.97-7.99 (d, *J* = 6.4 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): δ = 12.53, 44.28, 64.9, 97.87, 105.65, 108.32, 119.84, 120.76, 123.39, 123.97, 125.09, 125.47, 128.31, 128.39, 129.39, 133.21, 148.94, 152.14, 152.92, 154.94, 168.74; ESI-MS *m/z*: 533.4 [M+H]⁺.



2c: (170.4 mg, 63.8% yield): m.p. 190~195 °C; UV-vis (pH 3.5 H₂O/DMF 4: 1, v/v) λ_{\max} (log ϵ): 562 (3.91); ¹H NMR (400 MHz, CDCl₃): δ = 1.12-1.16 (t, 12H), 3.28-3.33 (q, 8H), 6.29-6.30 (m, 4H), 6.46-6.48 (d, *J* = 8.0 Hz, 1H), 6.59-6.66 (m, 3H), 6.79 (s, 1H), 6.89-6.92 (d, *J* = 7.6 Hz, 1H), 7.02-7.06 (m, 1H), 7.11-7.14 (m, 1H), 7.49-7.50 (m, 2H), 7.99-8.02 (m, 1H); ¹³C NMR (100 MHz, CDCl₃): δ = 12.52, 44.26, 69.07, 97.77, 105.53, 108.24, 119.87, 120.78, 123.38, 123.96, 125.03, 125.47, 128.29, 128.39, 129.29, 133.23, 148.88, 152.068, 152.88, 154.94, 168.77; ESI-MS *m/z*: 534.4 [M+H]⁺.



2d: (195.7 mg, 68.9% yield): m.p. 198~202 °C; UV-vis (pH 3.7 H₂O/DMF 4: 1, v/v) λ_{\max} (log ϵ): 564 (3.97); ¹H NMR (400 MHz, CDCl₃): δ = 0.93-0.97 (t, 6H), 1.14-1.18 (t, 6H), 3.11-3.14 (q, 4H), 3.32-3.36 (q, 4H), 5.85 (s, 1H), 6.19-6.23 (m, 2H), 6.32-6.33 (d, J = 7.6 Hz, 1H), 6.40-6.43 (dd, J = 8.8, 2.4 Hz, 1H), 6.68-6.70 (d, J = 8.8 Hz, 1H), 6.73-6.75 (d, J = 9.2 Hz, 1H), 7.08-7.15 (m, 2H), 7.21-7.25 (t, 1H), 7.34-7.37 (m, 2H), 7.58-7.66 (m, 4H), 8.09-8.11 (m, 1H); ¹³C NMR (100 MHz, CDCl₃): δ = 12.58, 44.38, 44.64, 62.62, 97.81, 104.79, 108.27, 122.93, 123.80, 128.18, 128.52, 130.43, 132.72, 148.92, 153.24, 153.86, 170.09. ESI-MS m/z : 568.5 [M+H]⁺.



2e: (208.6 mg, 72.4 % yield): m.p. 236~240°C; UV-vis (pH 5.5 H₂O/DMF 4: 1, v/v) λ_{\max} (log ϵ): 564 (4.79); ¹H NMR (400 MHz, CDCl₃): δ = 1.16-1.19 (t, 12H), 1.49-1.51 (m, 6H), 1.89 (s, 3H), 2.14 (s, 6H), 3.32-3.37 (q, 8H), 6.26-6.29 (dd, J = 8.8, 2.0 Hz, 2H), 6.34-6.35 (d, J = 2.0 Hz, 2H), 6.62-6.64 (d, J = 8.8 Hz, 2H), 6.79-6.82 (m, 1H) 7.28-7.30 (m, 2H) 7.75-7.78 (m, 1H); ¹³C NMR (100 MHz, CDCl₃): δ = 12.59, 29.78, 36.26, 38.93, 41.48, 44.27, 59.11, 97.97, 107.63, 108.55, 122.08, 122.82, 127.18, 128.69, 129.87, 132.05, 148.43, 151.37, 155.60, 169.65 ppm; ESI-MS m/z : 576.3 [M+H]⁺. EI-MS m/z : 575.3 [M]⁺; HRMS (EI): m/z calcd for C₃₈H₄₅O₂N₃ [M]⁺ 575.3506; Found 575.3497.

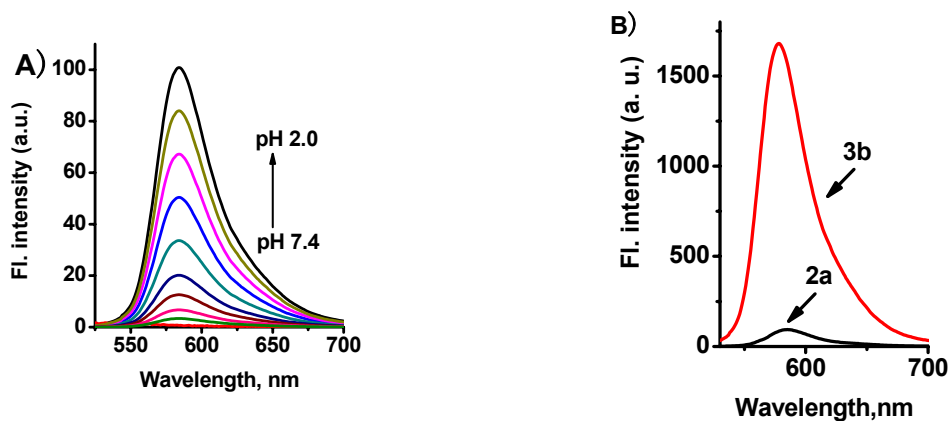


Fig. S1. A) pH-dependence of the fluorescence intensity of compound **2a** (2 μM) excited at 510 nm with the arrow indicating the change of the fluorescence intensities with pH decrease from 7.4 to 2.0. B) For comparison, the fluorescence spectra of compound **2a** (2 μM) and rhodamine B acid **3b** (2 μM) at pH 2.0 were displayed. Spectra were obtained with excitation at 510 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

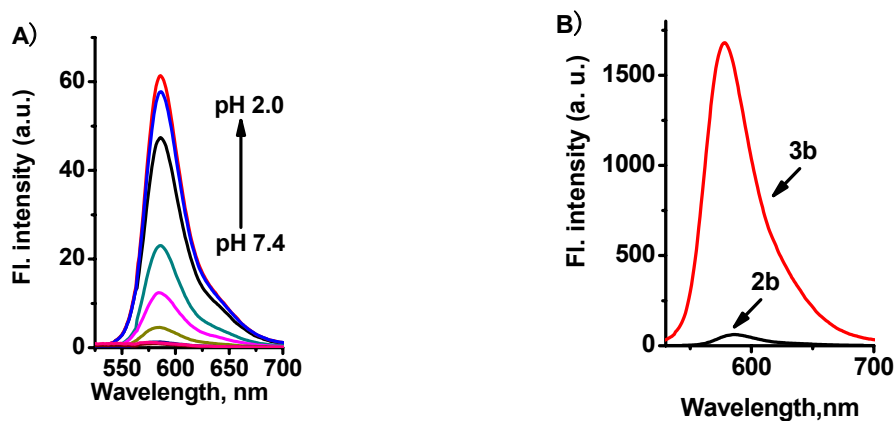


Fig. S2. A) pH-dependence of the fluorescence intensity of compound **2b** (2 μM) excited at 510 nm with arrows indicating the change of the fluorescence intensities with pH decrease from 7.4 to 2.0. B) For comparison, the fluorescence spectra of compound **2b** (2 μM) and rhodamine B acid **3b** (2 μM) at pH 2.0 were displayed. Spectra were obtained with excitation at 510 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

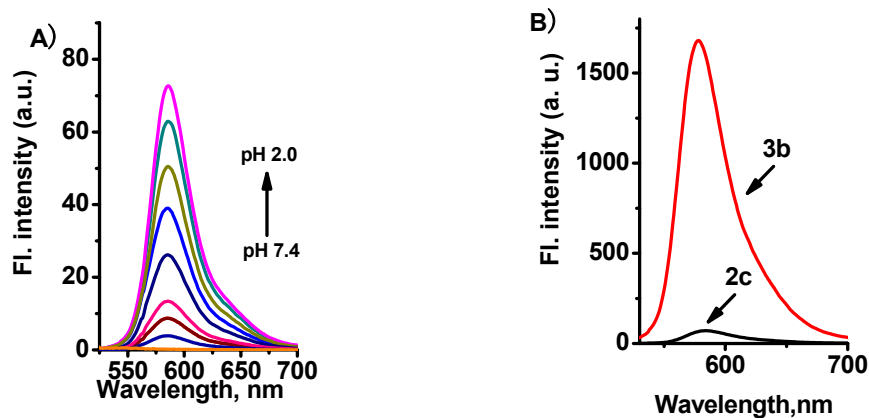


Fig. S3. A) pH-dependence of the fluorescence intensity of compound **2c** (2 μM) excited at 510 nm with arrows indicating the change of the fluorescence intensities with pH decrease from 7.4 to 2.0. B) For comparison, the fluorescence spectra of compound **2c** (2 μM) and rhodamine B acid **3b** (2 μM) at pH 2.0 were displayed. Spectra were obtained with excitation at 510 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

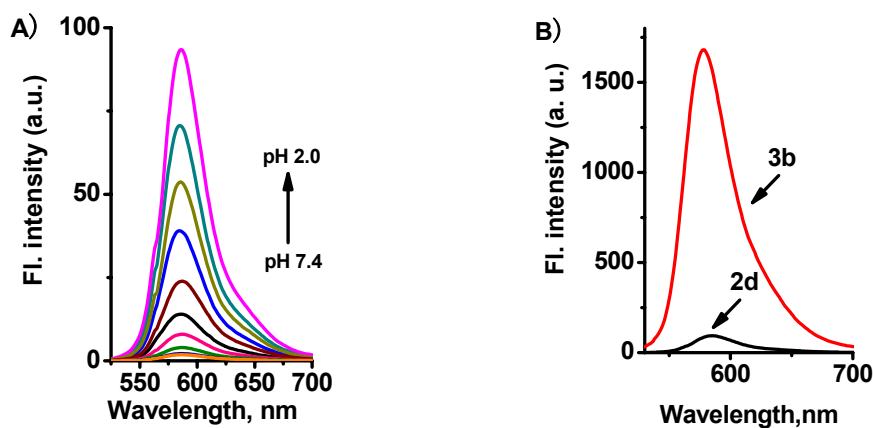


Fig. S4. A) pH-dependence of the fluorescence intensity of compound **2d** (2 μM) excited at 510 nm with arrows indicating the change of the fluorescence intensities with pH decrease from 7.4 to 2.0. B) For comparison, the fluorescence spectra of compound **2d** (2 μM) and rhodamine B acid **3b** (2 μM) at pH 2.0 were displayed. Spectra were obtained with excitation at 510 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

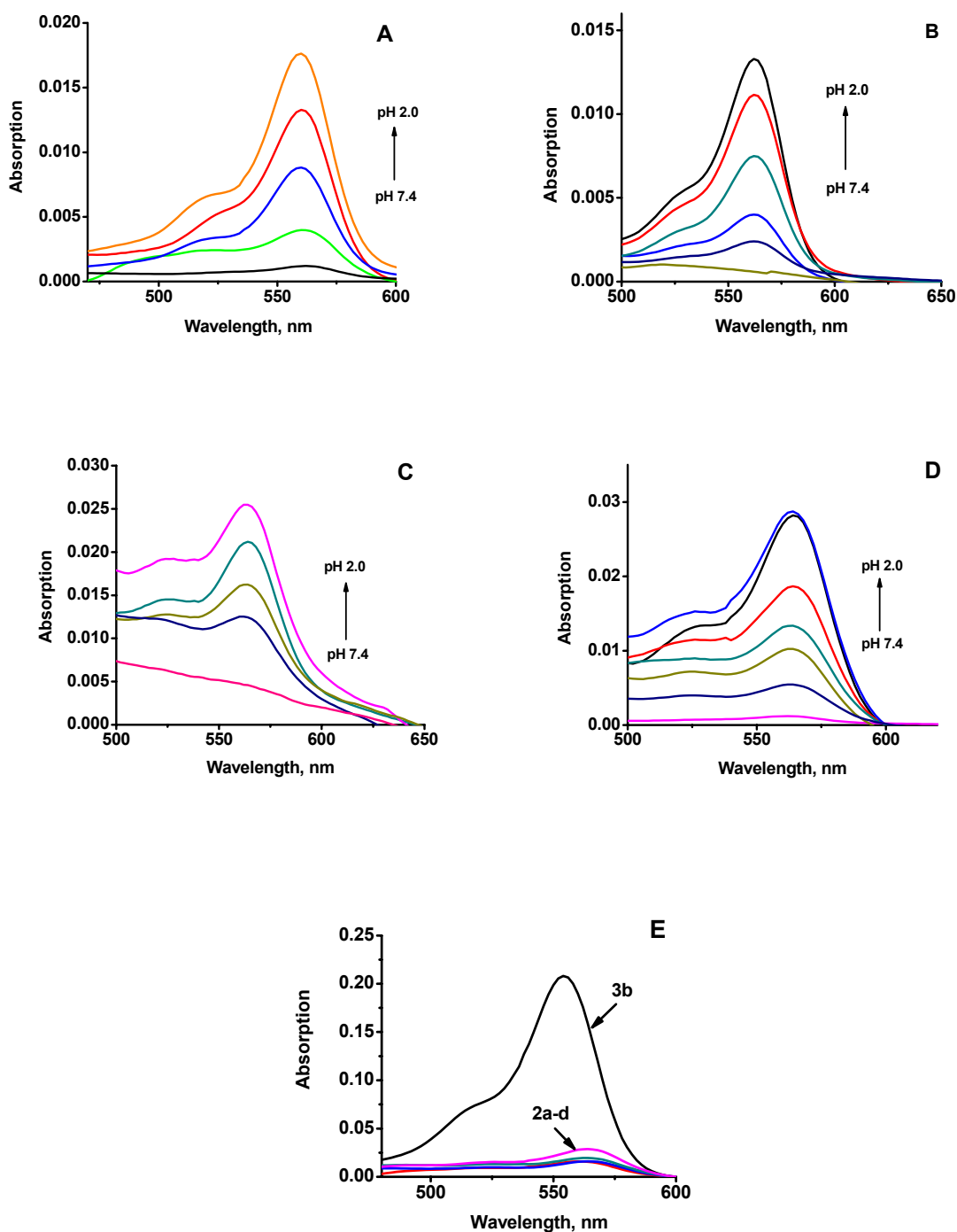


Fig. S5. pH-dependence of the absorption spectra of compounds **2a** (A), **2b** (B), **2c** (C), **2d** (D) (2 μ M) with the arrows indicating the change of absorbance with pH decrease from 7.4 to 2.0. E) For comparison, the absorption spectra of rhodamine B acid **3b** and compounds **2a-d** at pH 2.0 were displayed.

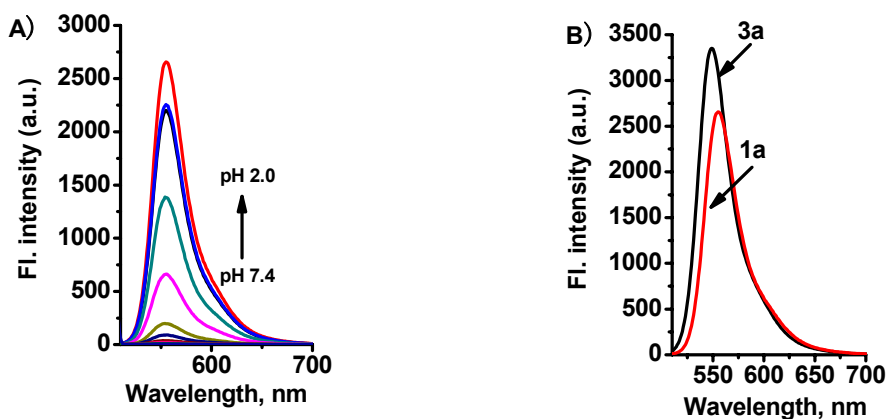


Fig. S6. A) pH-dependence of the fluorescence intensity of compound **1a** (2 μM) excited at 500 nm with arrows indicating the change of the fluorescence intensities with pH decrease from 7.4 to 2.0. B) For comparison, the fluorescence spectra of rhodamine 6G acid **3a** and compound **1a** (2 μM) at pH 2.0 were displayed. Spectra were obtained with excitation at 500 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

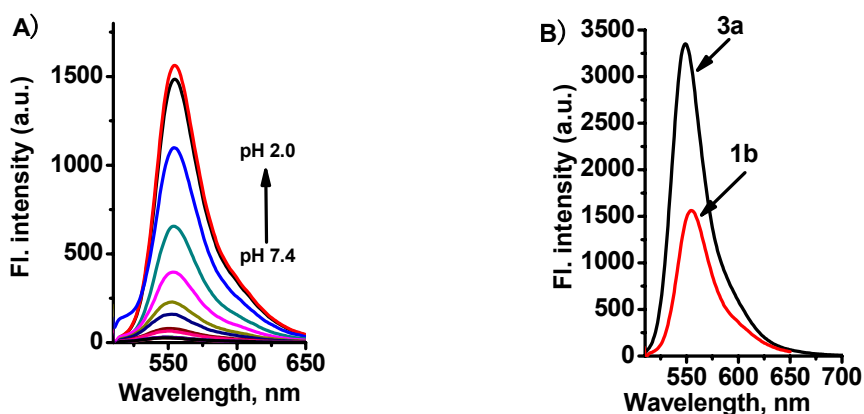


Fig. S7. A) pH-dependence of the fluorescence intensity of compound **1b** (2 μM) excited at 500 nm with arrows indicating the change of the fluorescence intensities with pH decrease from 7.4 to 2.0. B) For comparison, the fluorescence spectra of rhodamine 6G acid **3a** and compound **1b** (2 μM) at pH 2.0 were displayed. Spectra were obtained with excitation at 500 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

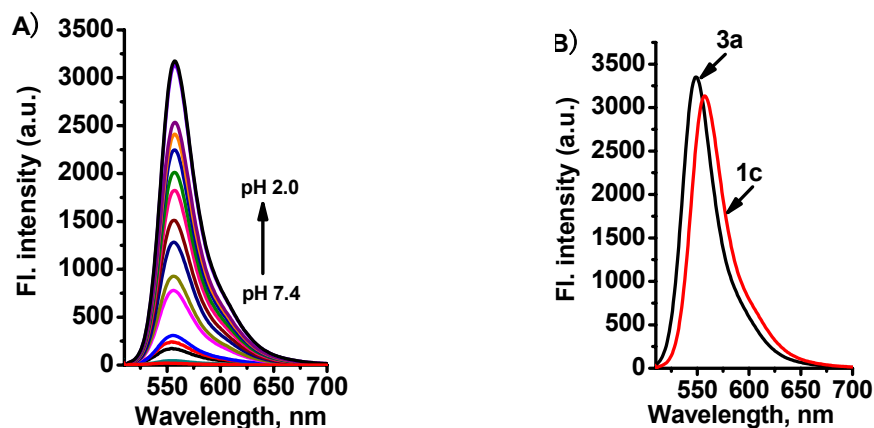


Fig. S8. A) pH-dependence of the fluorescence intensity of compound **1c** (2 μM) excited at 500 nm with arrows indicating the change of the fluorescence intensities with pH decrease from 7.4 to 2.0. B) For comparison, the fluorescence spectra of rhodamine 6G acid **3a** and compound **1c** (2 μM) at pH 2.0 were displayed. Spectra were obtained with excitation at 500 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

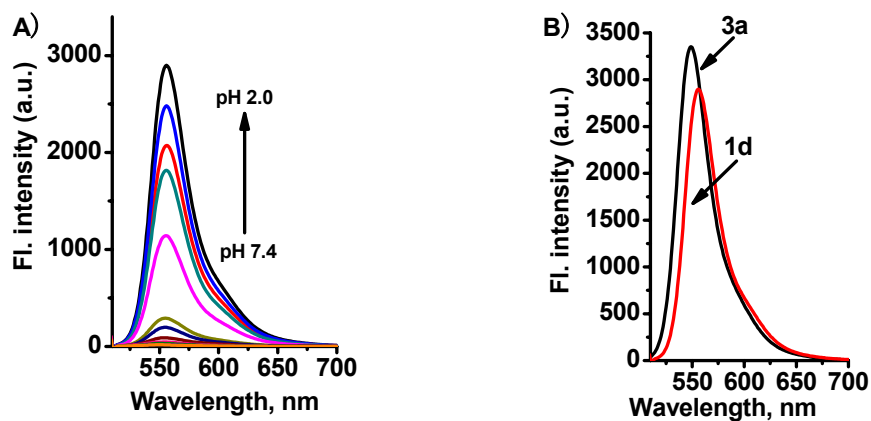


Fig. S9. A) pH-dependence of the fluorescence intensity of compound **1d** (2 μM) excited at 500 nm with arrows indicating the change of the fluorescence intensities with pH decrease from 7.4 to 2.0. B) For comparison, the fluorescence spectra of rhodamine 6G acid **3a** and compound **1d** (2 μM) at pH 2.0 were displayed. Spectra were obtained with excitation at 500 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

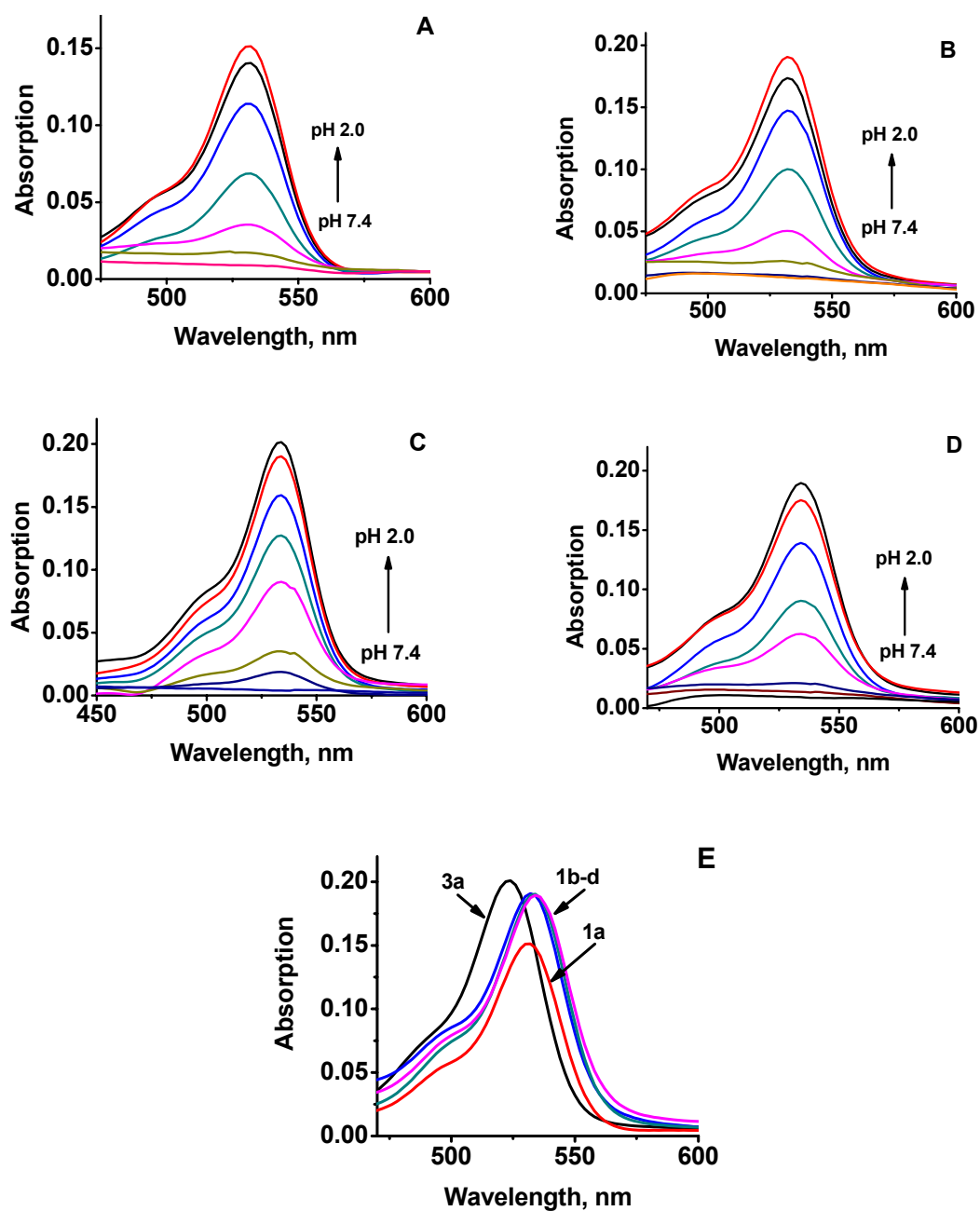


Fig. S10. pH-dependence of the absorption spectra of compounds **1a** (A), **1b** (B), **1c** (C), **1d** (D) (2 μM) with the arrows indicating the change of the absorption intensities with pH decrease from 7.4 to 2.0. E) For comparison, the absorption spectra of rhodamine 6G acid **3a** (2 μM) and compounds **1a-d** at pH 2.0 were displayed.

Table S1. The fluorescence quantum yields (Φ_f) of compound **1e** in different pH conditions. (Φ_f is the relative fluorescence quantum yield estimated by using Rhodamine 6G ($\Phi_f = 0.95$ in water) as a fluorescence standard.⁴).

pH	Φ_f	pH	Φ_f
9.0	0.006	6.0	0.57
7.49	0.08	5.5	0.65
7.04	0.17	5.1	0.78
6.55	0.38	4.5	0.80

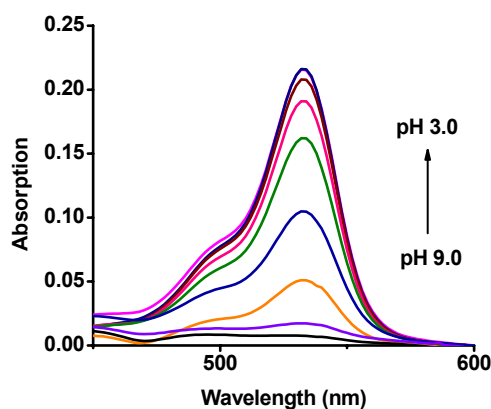


Fig. S11. pH-dependence of the absorption spectra of compound **1e** (2 μM) with the arrow indicating the change of the absorbance with pH decrease from 9.0 to 3.0.

Table S2. The fluorescence quantum yields of compound **2e** in different pH conditions. (Φ_f is the relative fluorescence quantum yield estimated by using Rhodamine B ($\Phi_f = 0.31$ in water) as a fluorescence standard.⁴).

pH	Φ_f	pH	Φ_f
8.5	0.003	6.0	0.12
7.49	0.004	5.6	0.18
7.04	0.01	5.1	0.23
6.55	0.03	4.5	0.28

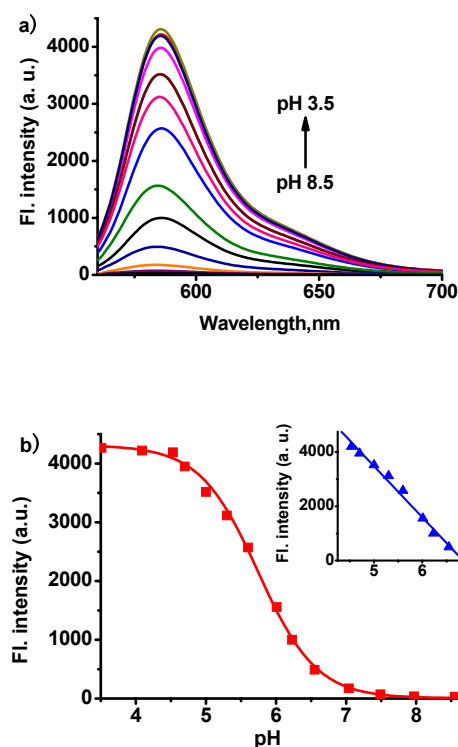


Fig. S12. (a) pH-dependence of the fluorescence intensity of compound **2e** (2 μM) with the arrow indicating the change of the fluorescence intensities with pH decrease from 8.5 to 3.5. Spectra were obtained with excitation at 550 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent). (b) Fluorescence responses (fluorescence intensity at 586 nm) of compound **2e** (2 μM) to different pH (8.5 to 3.5). The inset shows the linear relationship of fluorescence intensity at 556 nm and varying pH values from 4.5 to 6.5.

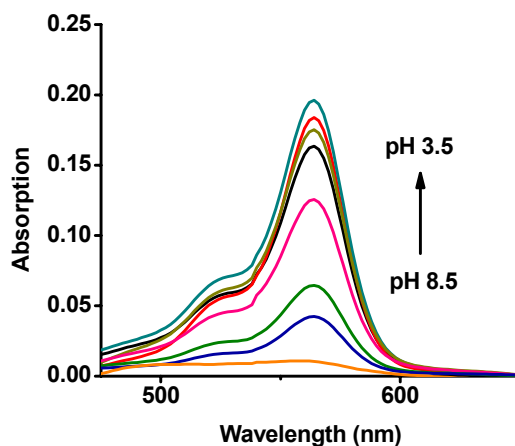


Fig. S13. pH-dependence of the absorption spectra of compound **2e** (2 μM) with the arrow indicating the change of the absorbance with pH decrease from 8.5 to 3.5.

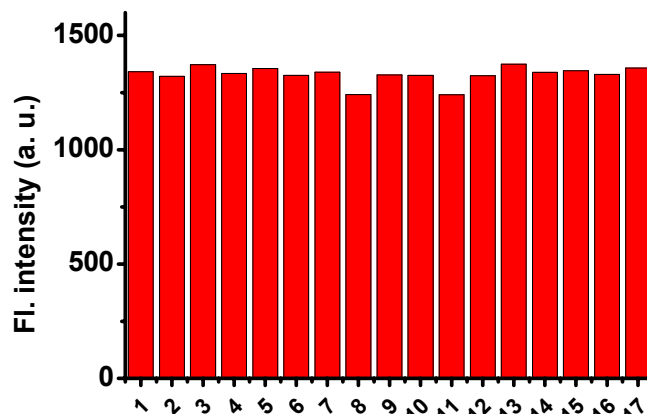


Fig. S14. Fluorescence intensity of compound **1e** (2 μ M) to biologically relevant species in PBS buffer (containing 20% DMF as a co-solvent, pH 6.5): 1, blank; 2, K⁺ (120 mM); 3, Na⁺ (120 mM); 4, Ca²⁺ (0.5 mM); 5, Mg²⁺ (0.5 mM); 6, Zn²⁺ (0.1 mM); 7, Fe³⁺ (0.1 mM); 8, Cu²⁺ (0.1 mM); 9, Co²⁺ (0.1 mM); 10, Glucose (0.1 mM); 11, Ser (0.1 mM); 12, Cys (0.1 mM); 13, Arg (0.1 mM); 14, Val (0.1 mM); 15, GSH (0.1 mM); 16, vitamin C (0.1 mM); 17, H₂O₂ (0.1 mM). The data were collected with emission at 556 nm and excitation at 500 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

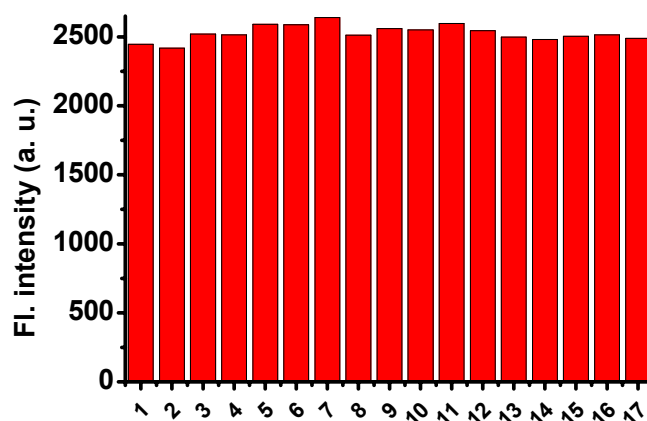


Fig. S15. Fluorescence intensity of compound **2e** (2 μ M) to biologically relevant species in PBS buffer (containing 20% DMF as a co-solvent, pH 5.6): 1, blank; 2, K⁺ (120 mM); 3, Na⁺ (120 mM); 4, Ca²⁺ (0.5 mM); 5, Mg²⁺ (0.5 mM); 6, Zn²⁺ (0.1 mM); 7, Fe³⁺ (0.1 mM); 8, Cu²⁺ (0.1 mM); 9, Co²⁺ (0.1 mM); 10, Glucose (0.1 mM); 11, Ser (0.1 mM); 12, Cys (0.1 mM); 13, Arg (0.1 mM); 14, Val (0.1 mM); 15, GSH (0.1 mM); 16, vitamin C (0.1 mM); 17, H₂O₂ (0.1 mM). The data were collected with emission at 586 nm and excitation at 550 nm in 25 mM PBS aqueous solution (containing 20% DMF as a co-solvent).

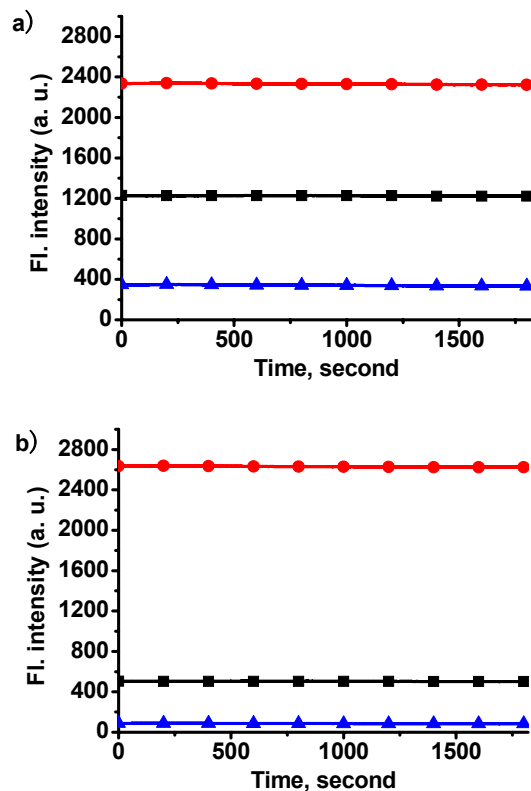


Figure S16. a) Fluorescence intensity of compound **1e** at 556 nm after irradiation with light (centered at 500 nm) (0-1800 second) at pH 7.4 (\blacktriangle), 6.5 (\blacksquare), and 5.5 (\bullet).
b) Fluorescence intensity of compound **2e** at 586 nm after irradiation with light (centered at 550 nm) (0-1800 second) at pH 7.4 (\blacktriangle), 6.5 (\blacksquare), and 5.5 (\bullet).

Cytotoxicity assays

Hela cells were grown in the modified Eagle's medium (MEM) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO₂ and 95% air at 37 °C. Immediately before the experiments, the cells were placed in a 96-well plate, followed by addition of increasing concentrations of probe **1e** (99% MEM and 1% DMSO). The final concentrations of the probe were kept from 5 μ M to 100 μ M (n = 3). The cells were then incubated at 37 °C in an atmosphere of 5% CO₂ and 95% air at 37 °C for 24, 48 or 72 h, followed by MTT assays. Untreated assay with MEM (n = 3) was also conducted under the same conditions.

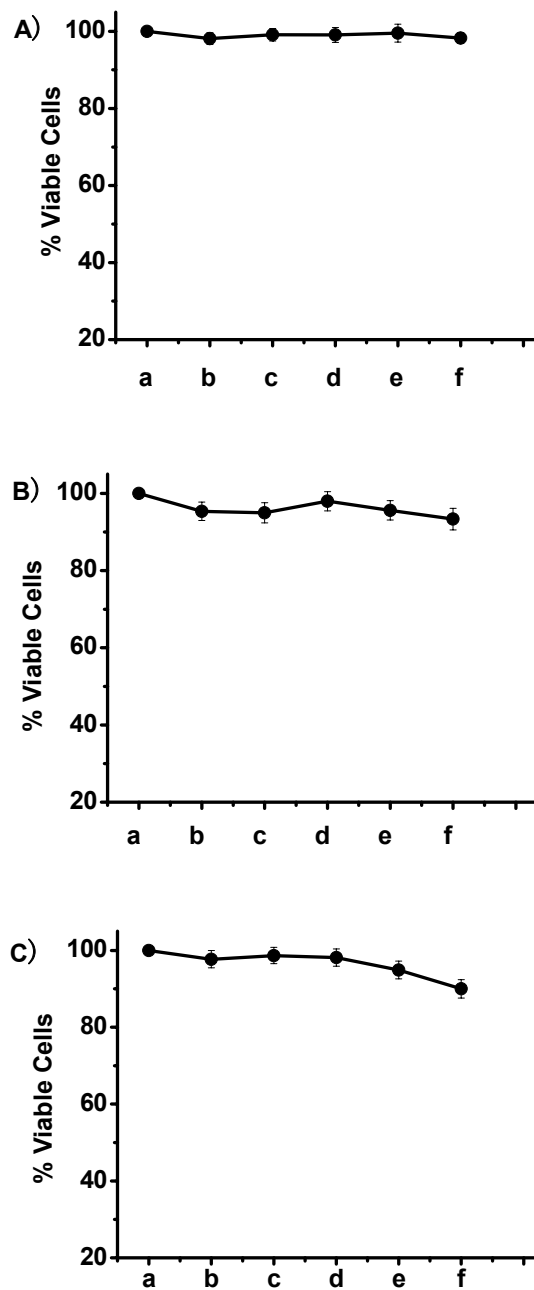


Fig. S17. Cytotoxicity studies of probe **1e** (a: 0; b: 5 μ M; c: 10 μ M; d: 20 μ M; e: 50 μ M; f: 100 μ M) for HeLa cells after 24 hours (A), 48 hours (B), and 72 hours (C).

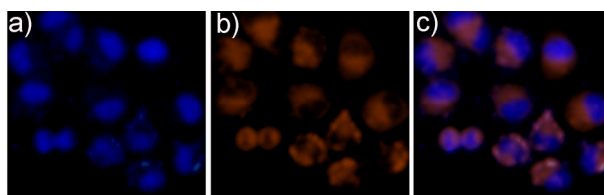


Fig. S18. HeLa cells co-incubated with **1e** (2 μM) and Hoechst 33258 (4.5 μM) for 30 min in PBS (pH 7.4, containing 2% DMSO as a co-solvent). Before imaging, the cells were washed in PBS medium (pH 6.5) with the addition of nigericin (1 $\mu\text{g}/\text{mL}$) to elicit a rapid exchange of K^+ for H^+ for a fast equilibration of external and internal pH.¹⁻³ a) emission from the blue channel (Hoechst 33258, nuclear stain); b) emission from the red channel (**1e**); c) overlay of the blue and red channels.

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