Unusual Fluorescent Responses of Morpholine-Functionalized Fluorescent Probes to pH via Manipulation of BODIPY's HOMO and LUMO Energy Orbitals for Intracellular pH Detection

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Synthesis:

Probe A: To a solution of compound **2** (0.9 mmol, 113 mg) in anhydrous THF (1 mL) was added ethylmagnesium bromide (0.9 mmol, 0.9 mL of 1.0 M solution). When the mixture was heated at 60°C for 2 hours and cooled down to room temperature, this freshly made Grignard reagent was transferred to a Schlenk flask containing a solution of BODIPY dye **1** (100 mg, 0.15 mmol) in anhydrous THF (2 mL) via cannula under nitrogen protection. The resulting mixture was stirred at 60°C overnight until complete consumption of the starting material which was monitored by TLC plates. When water (10 mL) was added to the mixture, the resulting mixture was extracted with CH_2Cl_2 (25 mL). The organic layer was washed with water (50 mL) and brine solution (50 mL), dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure and the crude product was purified by column chromatography using CH_2Cl_2/MeOH (9:0.5, v/v) as eluent to yield fluorescent probe **A** as orange oil (29 mg, 23%).

BODIPY dye 4: A mixture of DMF (10.0 mL) and $POCl_3$ (10.0 mL) was stirred in an ice bath for ten minutes under an argon atmosphere. The mixture was warmed to room temperature and further stirred for 35 minutes. After adding BODIPY dye **3** (600 mg, 0.93 mmol) in $ClCH_2CH_2Cl$ (70 mL) to the reaction mixture, the resulting mixture was stirred at 50 ^oC for two hours. The reaction mixture was cooled down to room temperature and then was slowly poured into saturated $NaHCO_3$ aqueous solution at 0 ^oC in an ice bath. The mixture was warmed to room temperature and was further stirred for 2 hours until no more bubble was generated. The mixture was then washed with water and brine solution. The organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give crude intermediate BODIPY dye. This crude intermediate BODIPY dye was used directly for the next step reaction without any purification. The BODIPY dye **4** was prepared from the crude product in the last step by the similar procedures but was run at higher temperature of 60 ^oC for 4 hours instead of 50 ^oC for 2 hours. The crude product was purified by column chromatography using hexanes/CH_2Cl_2/acetone/EtOH (4/2/2/0.5, v/v/v/v) as eluent to yield BODIPY **4** as deep red oil (295 mg, overall yield: 45%). ^1H NMR (400 MHz, $CDCl_3$): δ 9.97 (s, 2H), 6.92 (d, J = 8 Hz, 1H), 6.63 – 6.60 (m, 2H), 4.13 (t, J = 4.8 Hz, 2H), 4.02 (t, J = 4.8 Hz, 2H), 3.84 (t, J = 4.8 Hz, 2H), 3.71 – 3.58 (m, 8H), 3.50 – 3.47 (m, 2H), 3.44 – 3.28 (m, 11H), 3.25 (s, 3H), 2.27 (s, 6H), 1.78 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.8, 162.0, 159.9, 156.4, 148.0, 145.2, 132.5, 129.4,

127.7, 114.9, 107.0, 100.9, 70.6, 69.6, 67.8, 59.0, 58.9, 13.4, 11.6. HRMS (ESI): calculated for $C_{35}H_{47}BF_2N_2O_{10}Na$ $[M+Na]^+$,727.3184; found, 727.3196.

Fluorescent probe B: A mixed solution of BODIPY **4** (100 mg, 0.14 mmol) and morpholine (75 mg, 0.84 mmol) in 30 mL dry $ClCH_2CH_2Cl$ was stirred at 50 ^oC for 4 hours. When $NaBH(OAc)_3$ (118 mg, 0.56 mmol) and acetic acid (1 drop) were added to the flask at room temperature, the mixture was stirred overnight at room temperature, diluted with CH_2Cl_2 and washed with water and brine solutions. The organic layer was collected, dried over Na_2SO_4 and concentrated in reduced pressure. The crude product was purified by column chromatograph using CH_2Cl_2/MeOH, (9/0.5, v/v) as eluent to obtain fluorescent probe **B** as deep orange oil (72 mg, 61%).

BODIPY dye 6: When BODIPY dye **4** (88 mg, 0.125 mmol), compound **5** were dissolved in a mixture of toluene (30 mL), piperidine (0.2 mL) and acetic acid (0.2 mL), the reaction mixture was refluxed at 120 ^oC for 3 hours. Any water formed during the reaction was removed azeotropically by using a Dean-Stark apparatus. After the reaction was quenched by water (5 mL) at room temperature, the mixture was concentrated under reduced pressure and re-dissolved in CH_2Cl_2 (100 mL). It was then washed sequentially with saturated NH_4Cl solution (100 mL), water (100 mL) and brine solution (100 mL), dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure and the crude product was purified by column chromatography using hexane/CH_2Cl_2/acetone/EtOH (3/3/2/0.7, v/v/v/v) as eluent to obtain BODIPY dye **6** as violet color oil (35 mg, 10.4%). ^1H NMR (400 MHz, $CDCl_3$): δ 9.88 (s, 2H), 7.10 (d, *J* = 8 Hz, 1H), 6.64 – 6.58 (m, 5H), 6.52 (d, *J* = 16 Hz, 2H), 6.43 (d, *J* = 2.4 Hz, 1H), 5.96 (d, *J* = 16 Hz, 2H), 4.14 (t, *J* = 4.8 Hz, 4H), 4.07 (t, *J* = 4.8 Hz, 4H), 3.94 – 3.40 (m, 64H), 3.38 – 3.32 (m, 18H), 3.30 (s, 3H), 2.91 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 186.9, 161.9, 159.8, 157.5, 150.0, 148.9, 147.5, 142.0, 140.0, 132.2, 131.1, 129.5, 126.4, 120.7, 116.9, 115.0, 114.0, 112.6, 107.2, 100.2, 71.9, 71.7, 70.8, 70.7, 70.6, 70.5, 70.3, 69.6, 69.5, 68.7, 58.9. HRMS (ESI): calculated for $C_{77}H_{111}BF_2N_2O_{26}Na$ $[M+Na]^+$, 1551.7378; found, 1551.7388.

Fluorescent probe C: A mixed solution of BODIPY **6** (30 mg, 0.02 mmol) and morpholine (15 mg, 0.17 mmol) in 8 mL of dry $ClCH_2CH_2Cl$ was stirred at 50 ^oC for 8 hours. When $NaBH(OAc)_3$ (30 mg, 0.112 mmol) and acetic acid (1 drop) were added to the flask at room temperature, the mixture was stirred for 24 hours at room temperature, diluted with CH_2Cl_2 and washed with water and brine solution. The organic layer was collected, dried over anhydrous

Na_2SO_4 and concentrated in reduced pressure. The crude product was purified by preparative TLC plate (CH_2Cl_2/MeOH, 9/0.5, v/v) to obtain fluorescent probe **C** (15 mg, 45%).

Optical Measurement:

The UV-Vis absorption spectra of fluorescent probes **A**, **B** and **C** for pH dependency, selectivity, photostability and solvent effect measurements were collected in the range from 300 to 800 nm with increments of 1 nm. Their corresponding fluorescence spectra were collected at the excitation wavelength of 470 nm, 490 nm, and 580 nm for fluorescent probes **A**, **B** and **C** with increments of 1 nm, respectively. The excitation and emission slit widths were set up to 3 nm. The concentration of the dye in each sample is 5 μM. Sulforhodamine 101 dye (Φ_f = 95% with excitation wavelength at 577 nm in ethanol)[1] was used as a reference standard to determine the fluorescence quantum yields of fluorescent probe **C** in ethanol and buffer solutions. Rhodamine 6G (Φ_f = 95% in ethanol)[2, 3] was used as a reference standard to determine the fluorescence quantum yields of fluorescent probes **A** and **B** in ethanol and buffer solutions. Both samples and references were freshly prepared under identical conditions. The fluorescence quantum yields were calculated using the following equation:

$$\Phi_X = \Phi_{st}(Grad_X/Grad_{st})(\eta_X^2/\eta_{st}^2)$$

Where the subscripts '*st*' and '*X*' stand for standard and test, respectively, Φ is the fluorescence quantum yield, "*Grad*" represents the gradient from the plot of integrated fluorescence intensity versus absorbance and η is the refractive index of the solvent.

Determination of pK_a by fluorometric titration

The constants K_a of fluorescent probes **A**, **B** and **C** were determined in buffer solutions by fluorometric titration as a function of pH using the fluorescence spectra. The expression of the steady-state fluorescence intensity *F* as a function of the proton concentration has been extended for the case of a n: 1 complex between H^+ and a fluorescent probe, which expressed by the equation as below:[4]

$$F = \frac{F_{min}[\mathrm{H}^+]^n + F_{max}K_a}{K_a + [\mathrm{H}^+]^n}$$

F_{min} and F_{max} stand for the fluorescence intensities at maximal and minimal H^+ concentrations, respectively, and *n* is apparent stoichiometry of H^+ binding to the probe which

affects the fluorescent change. Nonlinear fitting of equation expressed above to the fluorescence titration data recoded as a function of H^+ concentration with K_a and n as free adjustable parameters yields the estimated apparent constant of K_a.

MTS assay:

MTS assay was performed with HUVEC-C cells (from ATCC). The cells were plated at a density of 5,000 cells/well on a 96-well cell culture plate and incubated at 37 ^oC in 5% CO_2 incubator overnight. After incubation, the media was removed and the cells were washed with 1X PBS. Fresh media with 0 μM, 5 μM, 15 μM, 25 μM, or 50 μM of fluorescent probe **C** dissolved in DMSO (with less than <0.5% DMSO final concentration in media) were added to the wells and measured in 6 replicates for each dye concentration. Blanks used for background subtraction had everything else except the cells and were prepared at the same time. The plates were incubated at 37 ^oC in 5% CO_2 incubator for 48 h. After the 48-hour incubation, 20 μL of MTS solution (from CellTiter 96 Aqueous Non-Radioactive Cell proliferation Assay (MTS) kit, Promega) was added to each well. The absorbance at 490 nm was acquired after 4 h incubation at 37 ^oC, using an ELISA plate reader (BioTek Instruments, Inc.). Plots were normalized to control wells containing media and cells only.

Table S1. Optical properties of fluorescent probes **A**, **B** and **C**.

	solvent	**λ_{abs} (nm)**	**λ_{em} (nm)**	**ε_{max} (10^4 $M^{-1}cm^{-1}$)**	**Φ_f (%)**
Probe **A**	Ethanol	498	508	7.89	56
	Buffer (pH 7.4)	501	506	7.48	18
	Buffer (pH 4.0)	495	507	7.62	5.4
Probe **B**	Ethanol	515	529	10.03	8.0
	Buffer (pH 7.4)	511	523	7.86	1.3
	Buffer (pH 4.0)	502	514	9.74	0.14
Probe **C**	Ethanol	565	652	3.15	8.6
	Buffer (pH 7.4)	550	665	5.06	0.32
	Buffer (pH 4.0)	532	654	5.12	0.036

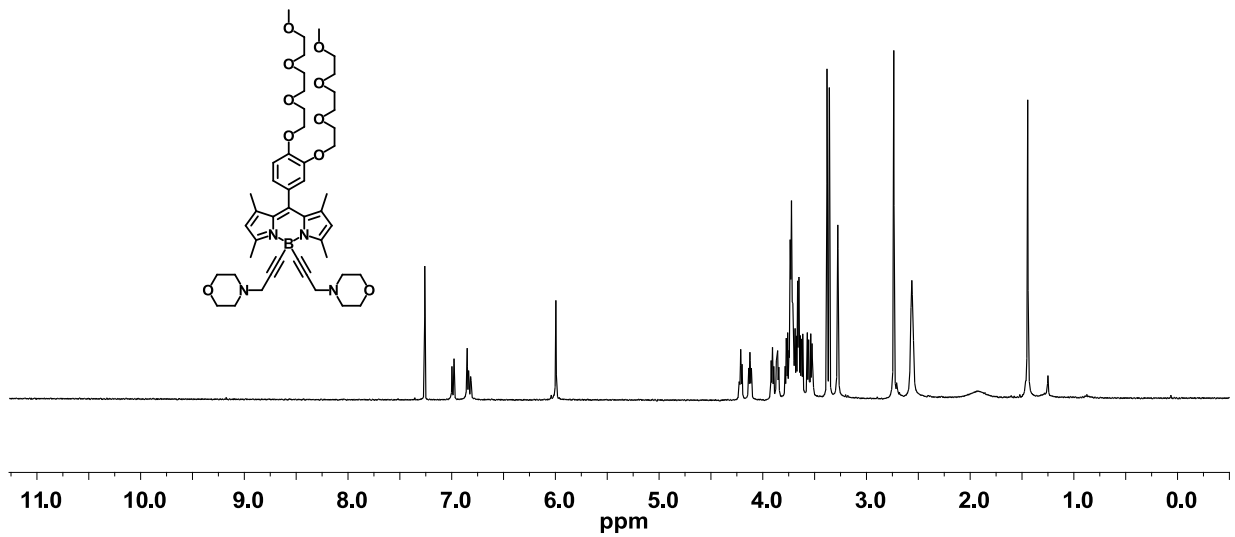

Figure S1. ^{1}H NMR spectrum of fluorescent probe **A** in $CDCl_3$ solution

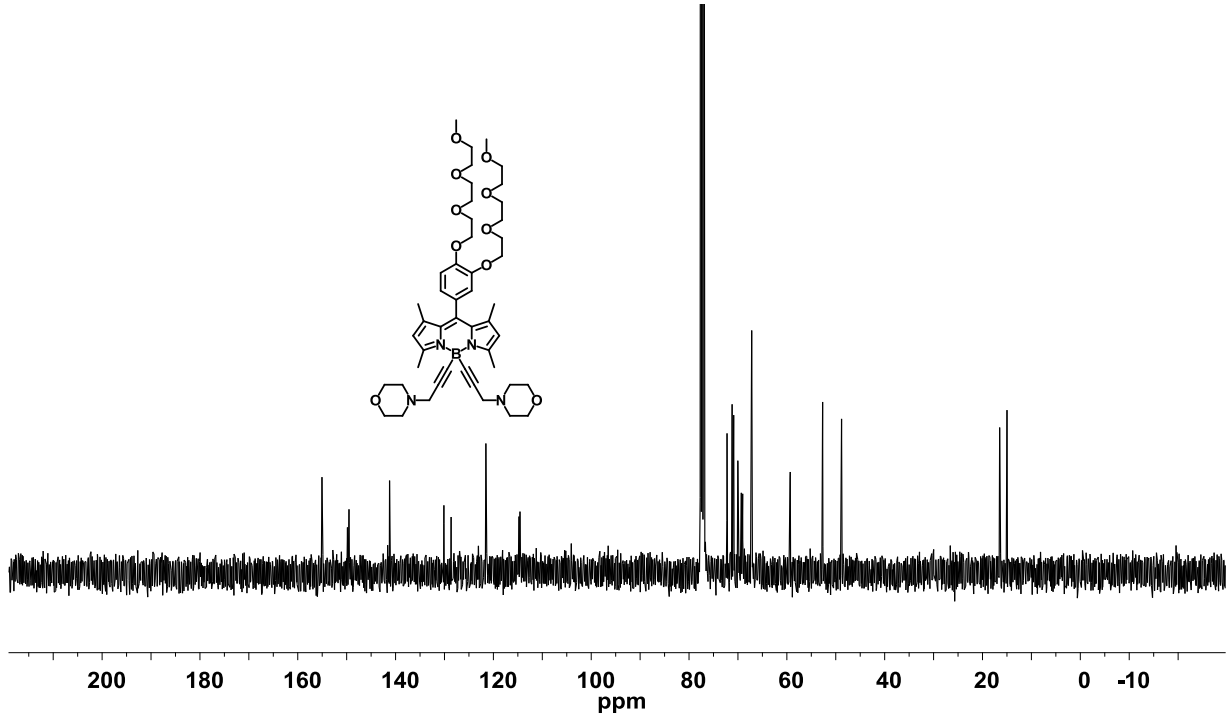

Figure S2. ^{13}C NMR spectrum of fluorescent probe **A** in $CDCl_3$ solution

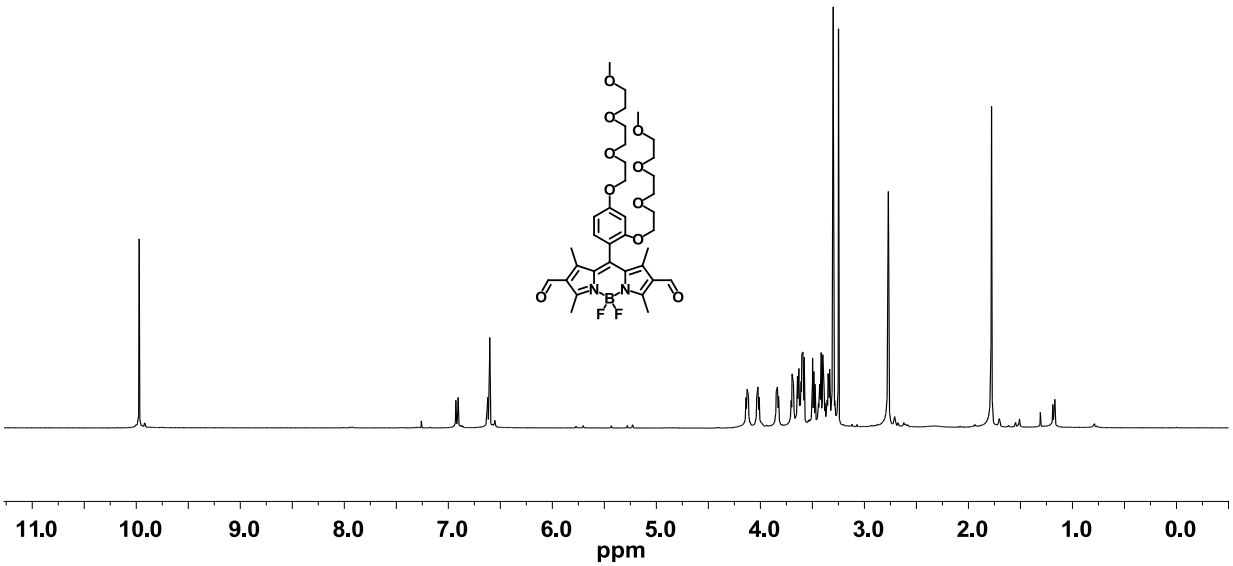

Figure S3. ^{1}H NMR spectrum of BODIPY dye **4** in $CDCl_3$ solution

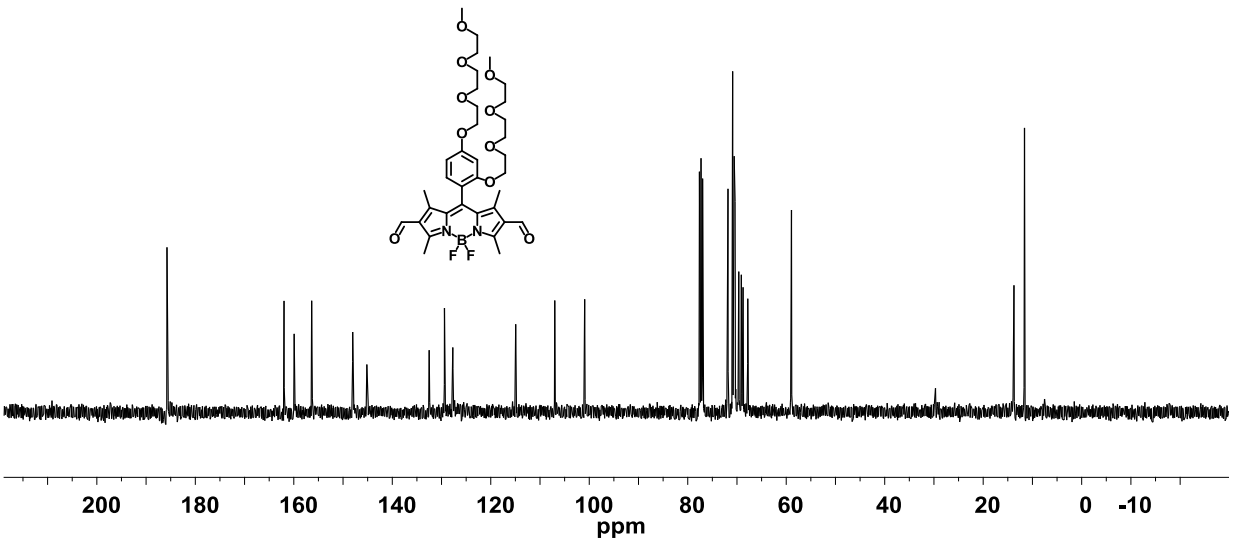

Figure S4. ^{13}C NMR spectrum of BODIPY dye **4** in $CDCl_3$ solution

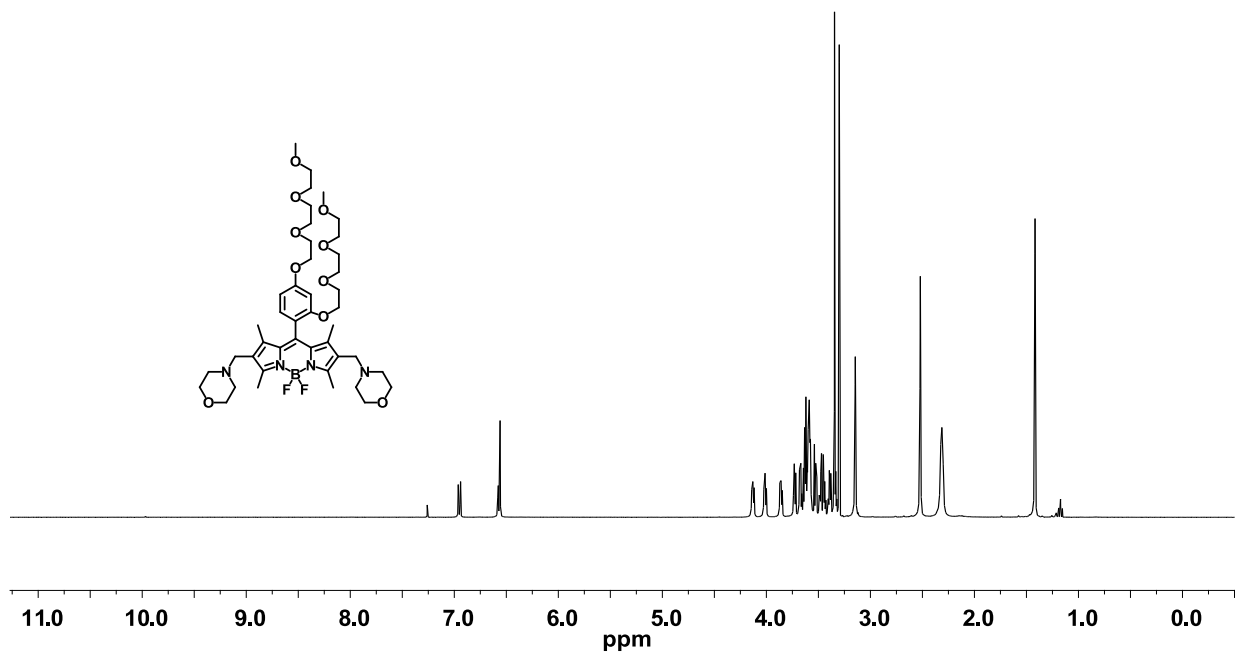

Figure S5. ^{1}H NMR spectrum of fluorescent probe **B** in $CDCl_3$ solution

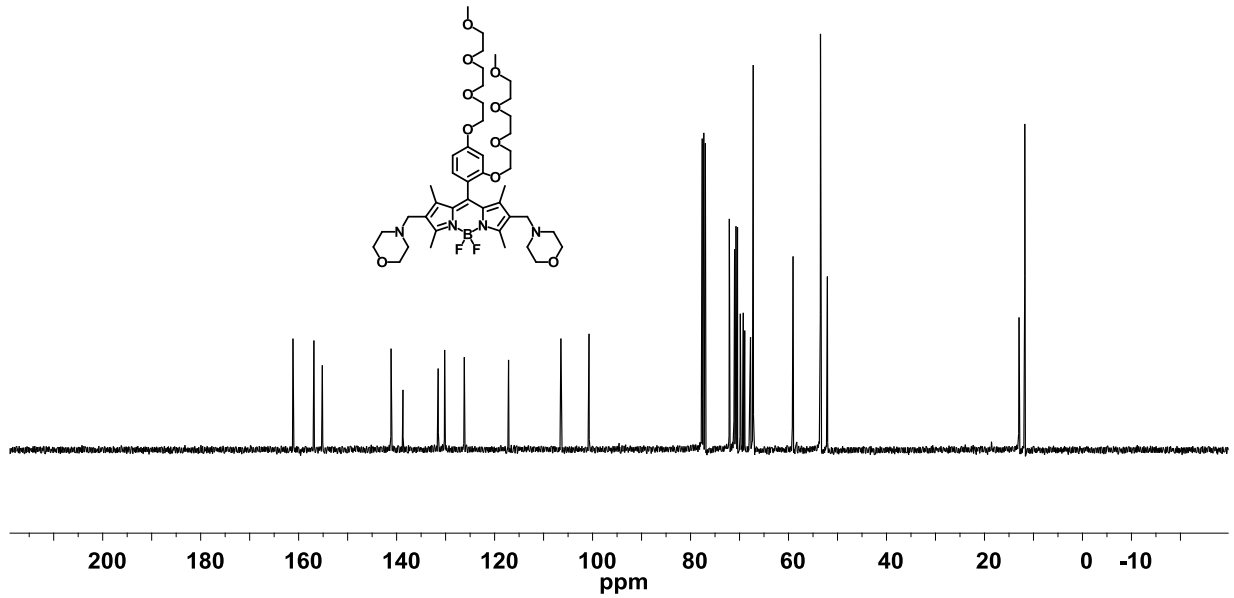

Figure S6. ^{13}C NMR spectrum of fluorescent probe **B** in $CDCl_3$ solution

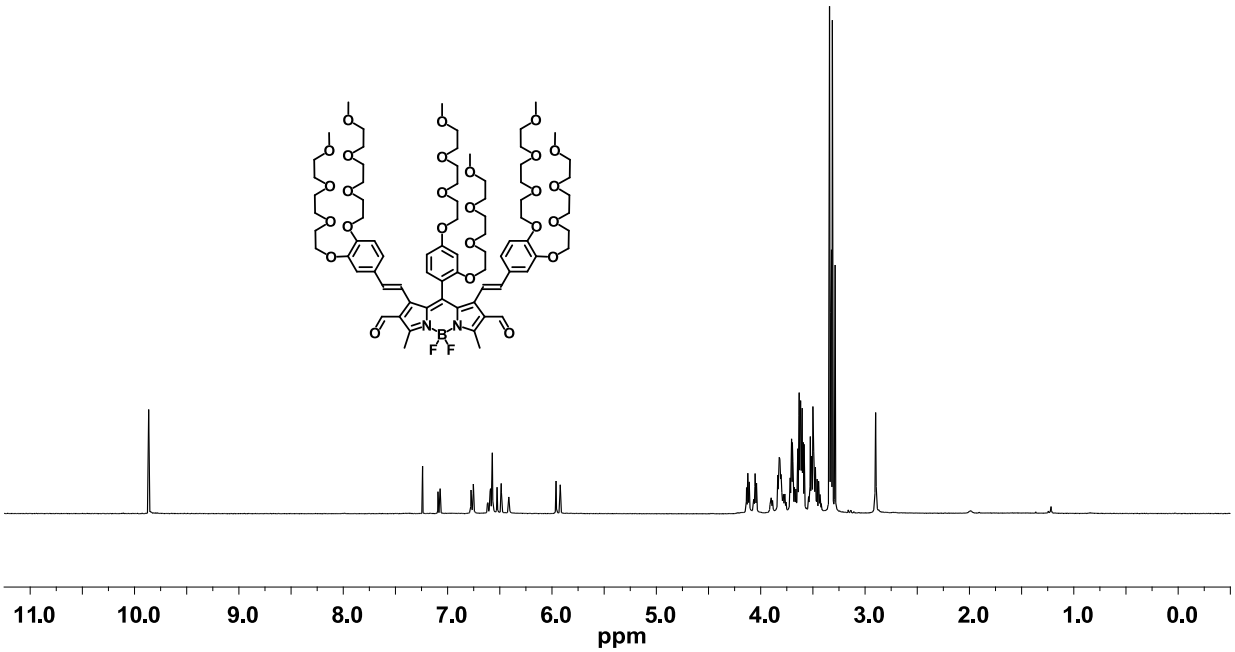

Figure S7. ^{1}H NMR spectrum of BODIPY dye **6** in $CDCl_3$ solution.

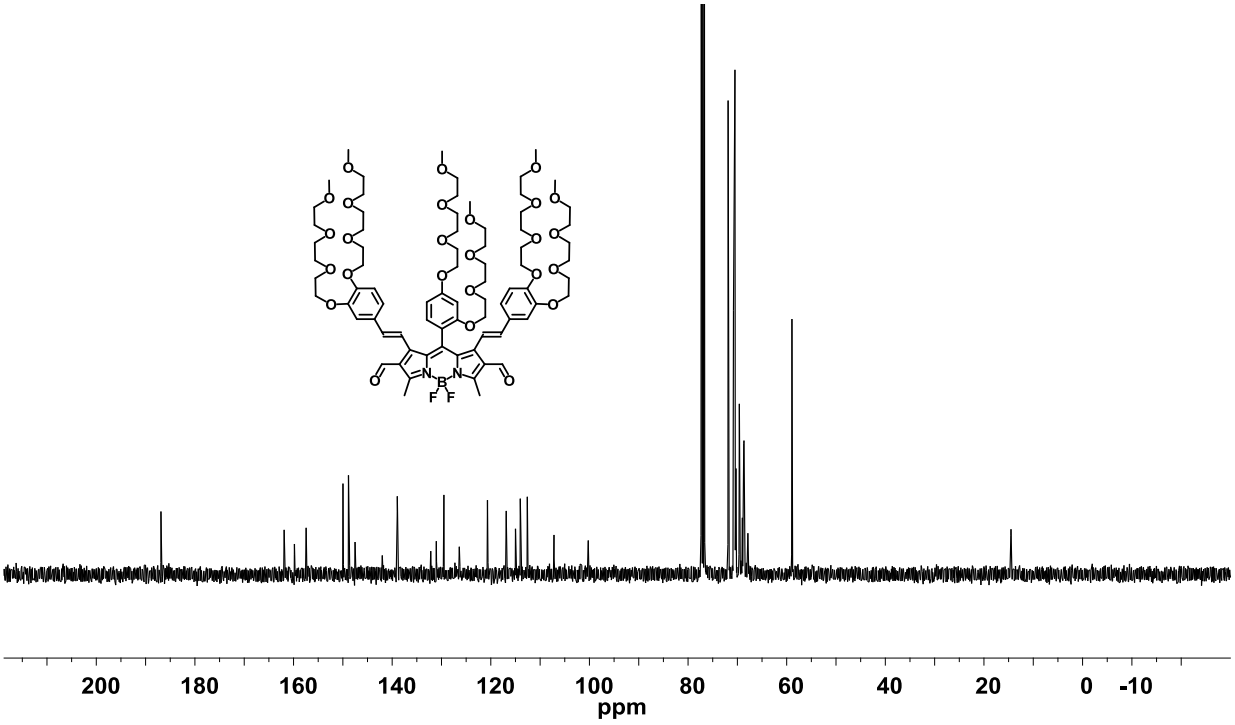

Figure S8. ^{13}C NMR spectrum of BODIPY dye **6** in $CDCl_3$ solution.

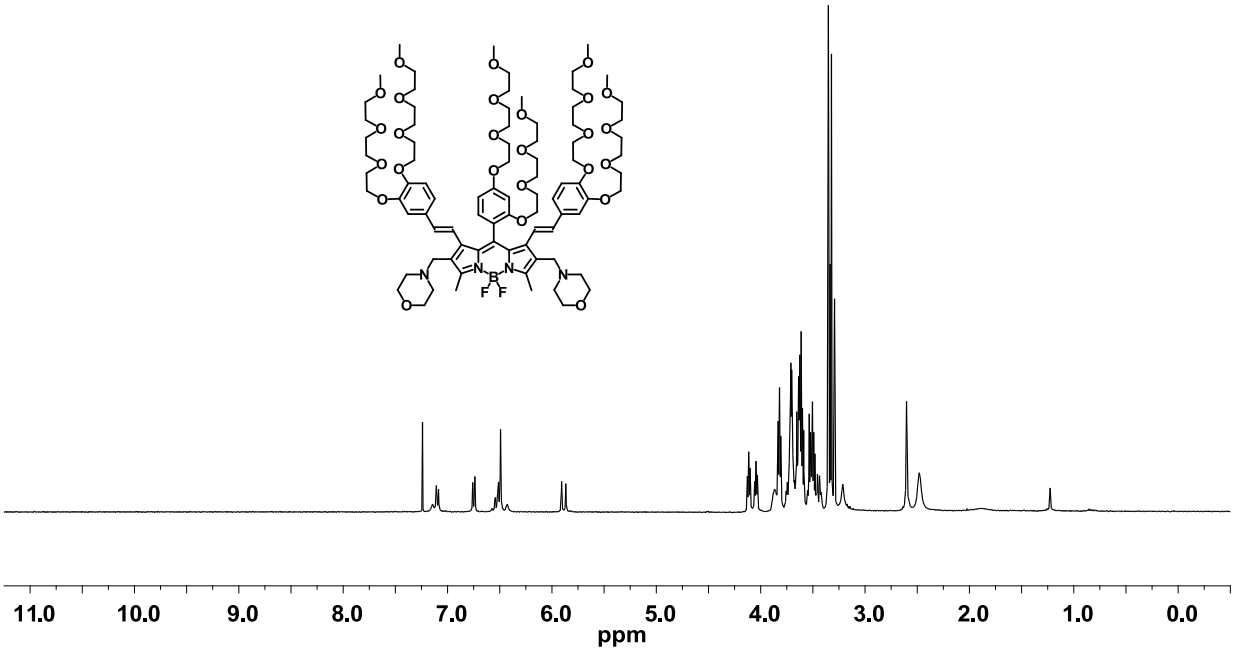

Figure S9. ^{1}H NMR spectrum of fluorescent probe **C** in $CDCl_3$ solution.

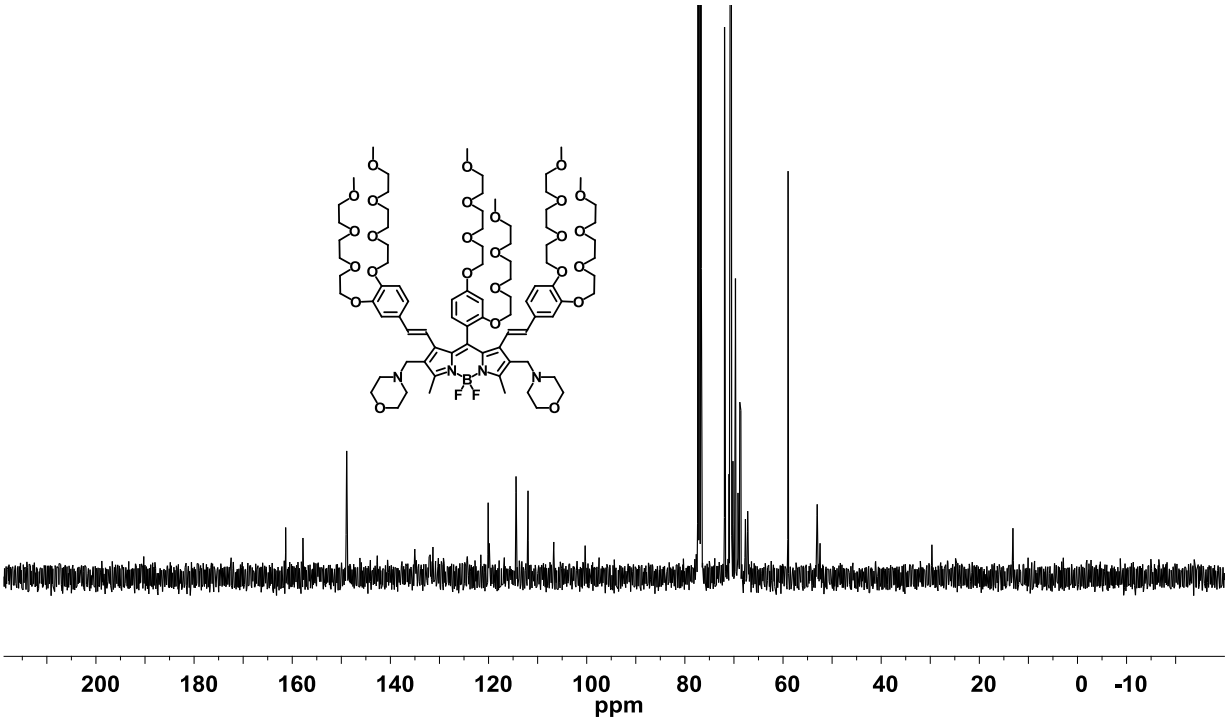

Figure S10. ^{13}C NMR spectrum of fluorescent probe **C** in $CDCl_3$ solution.

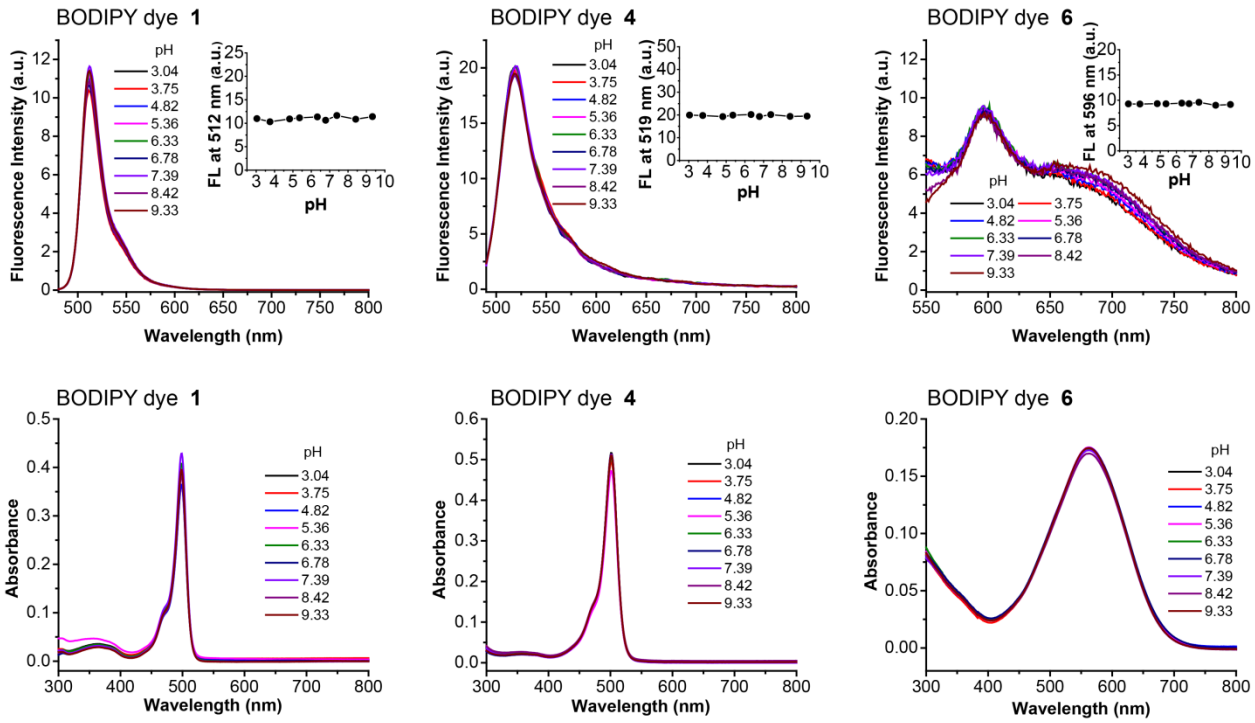

Figure S11. Absorption and emission spectra of BODIPY dyes **1**, **4** and **6** (5 μM) in buffer solution at different pH values. The inset graphs in upper row are the corresponding changes fluorescence intensity at peak wavelengths at different pH conditions.

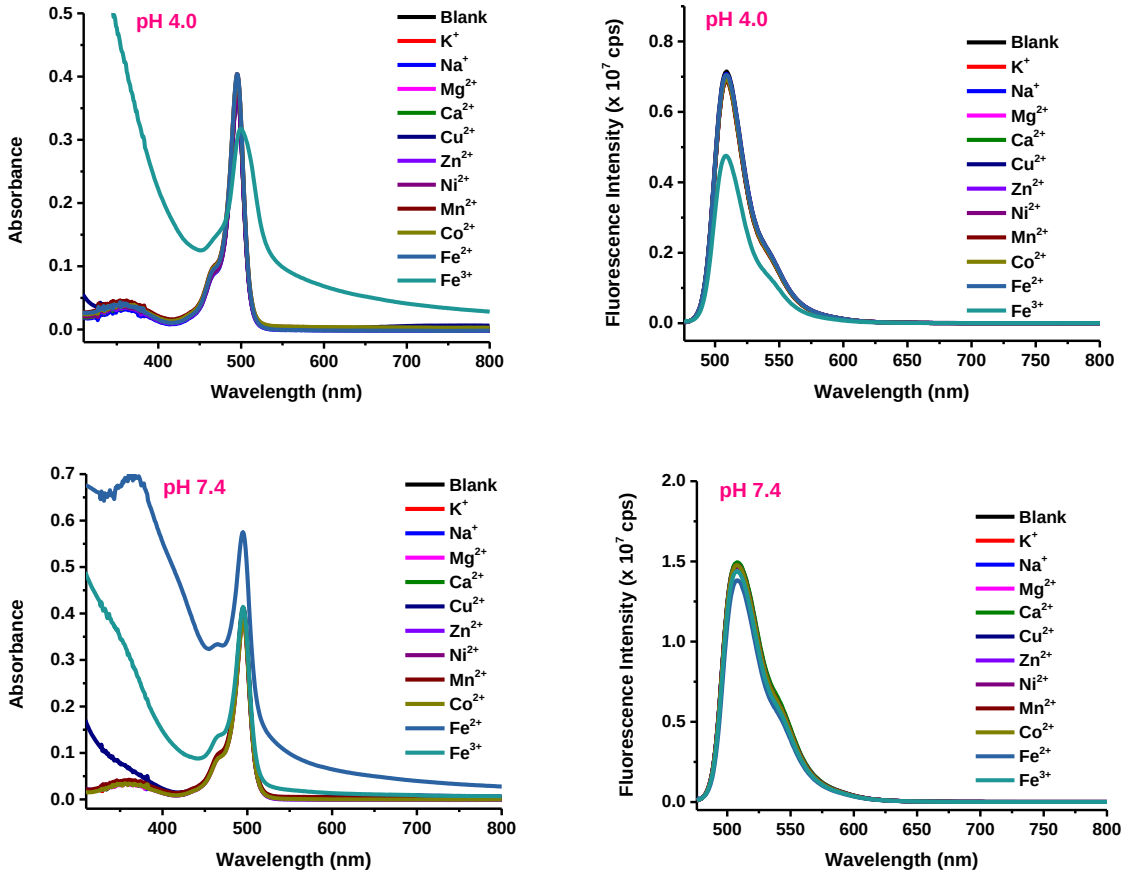

Figure S12. Absorption and emission spectra of fluorescent probe **A** (5 μM) in the presence of various metal cations (200 μM) in buffer solution at pH 4.0 (upper row) and pH 7.4 (lower row).

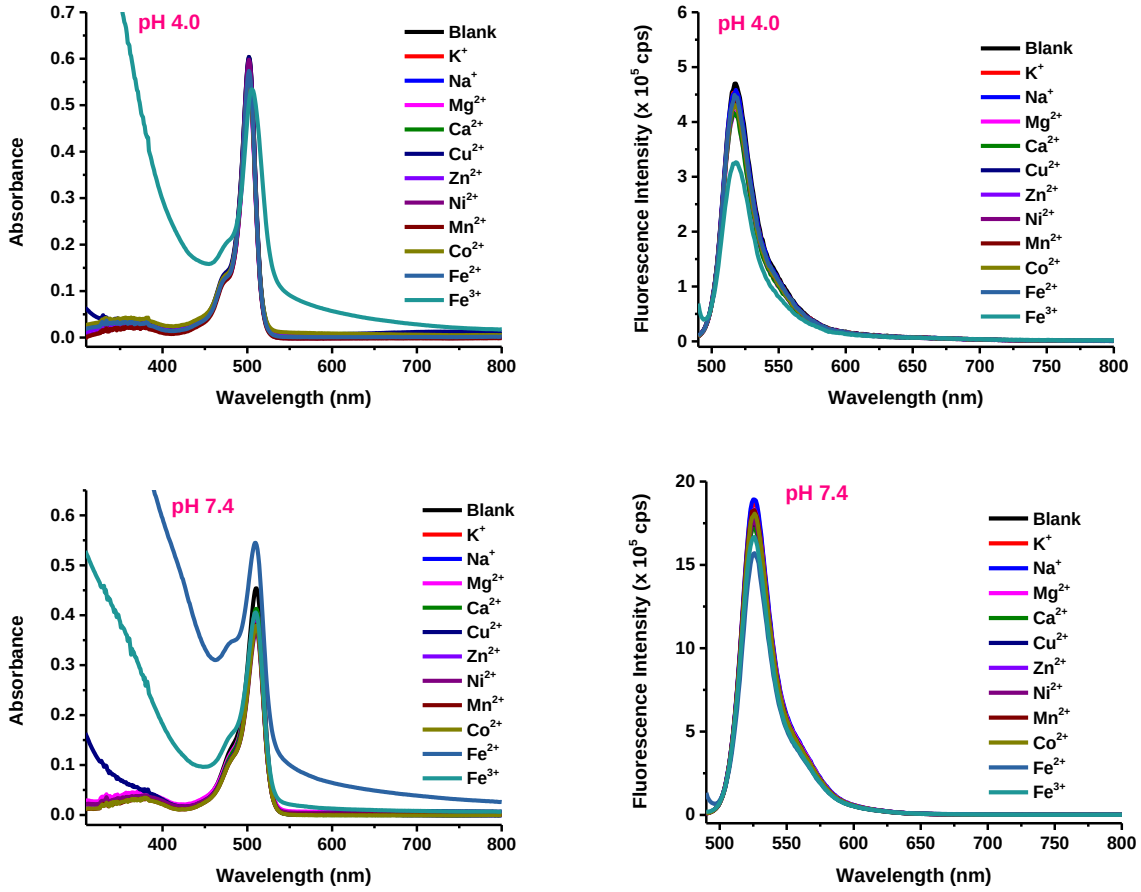

Figure S13. Absorption and emission spectra of fluorescent probe **B** (5 μM) in the presence of various metal cations (200 μM) in buffer solution at pH 4.0 (upper row) and pH 7.4 (lower row).

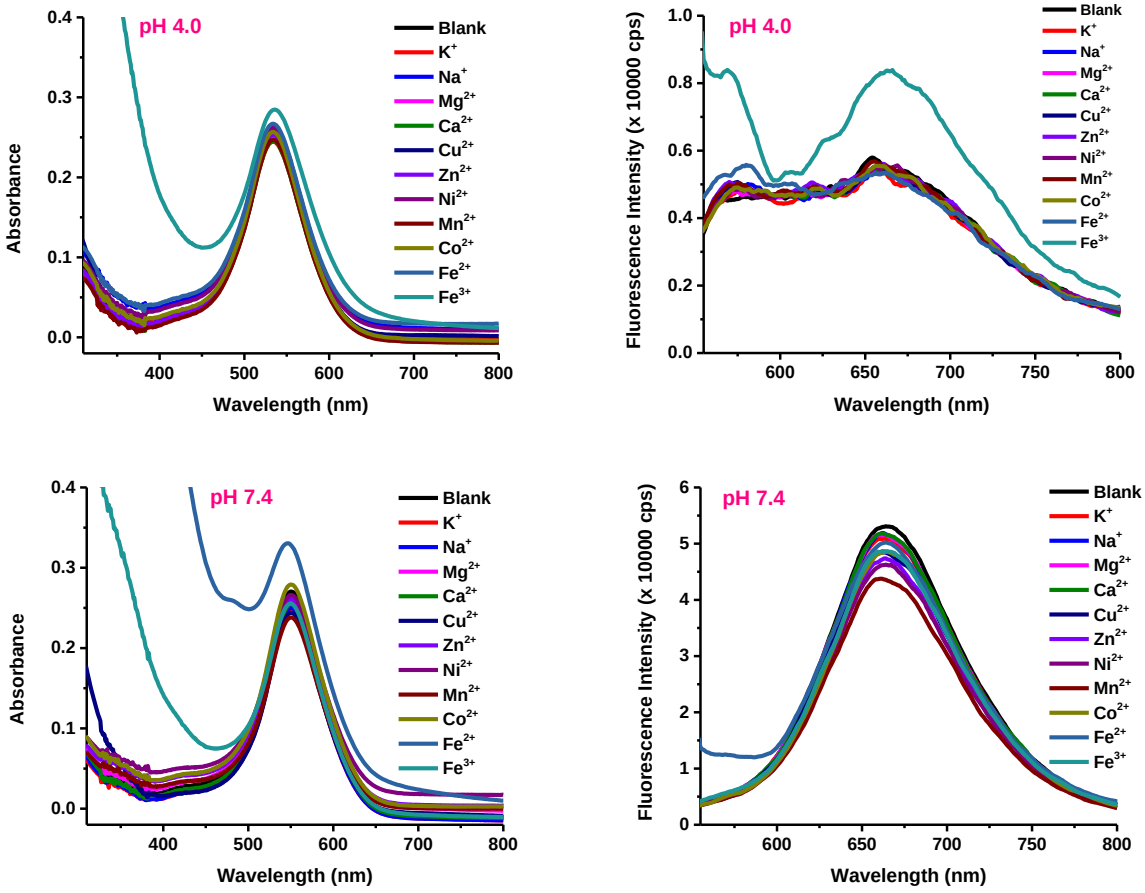

Figure S14. Absorption and emission spectra of fluorescent probe **C** (5 μM) in the presence of various metal cations (200 μM) in buffer solution at pH 4.0 (upper row) and pH 7.4 (lower row).

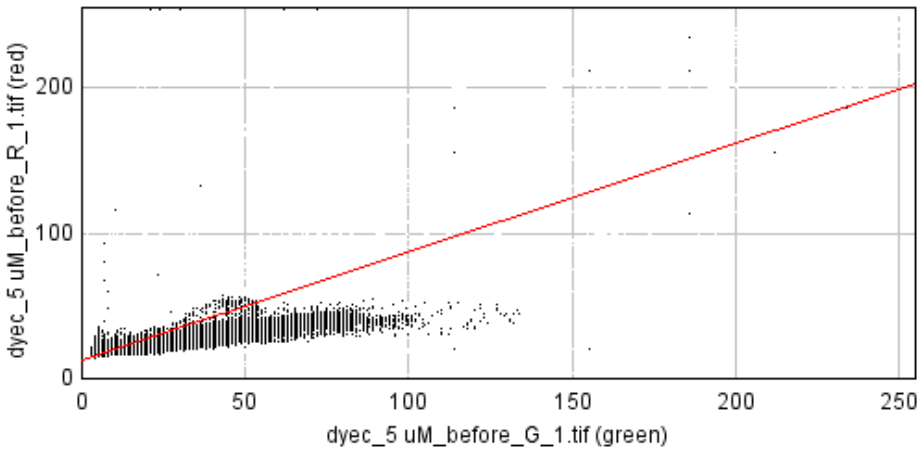

Figure S15. Red and green channel cytofluorogram of fluorescence images of cells incubated with 5 μM fluorescent probe **C** and 1 μM LysoSensor Green DND-189 for verifying their co-localization.

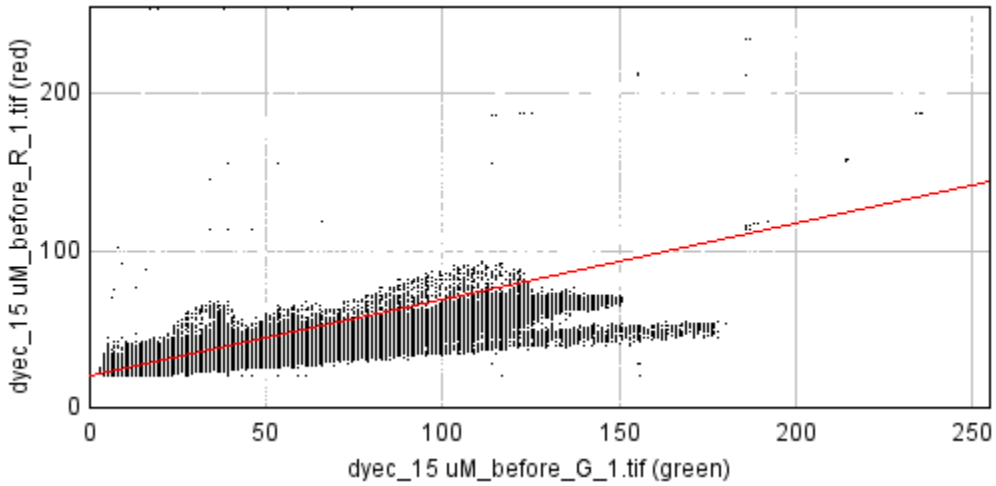

Figure S16. Red and green channel cytofluorogram of fluorescence images of cells incubated with 15 μM fluorescent probe **C** and 1 μM LysoSensor Green DND-189 for verifying their co-localization.

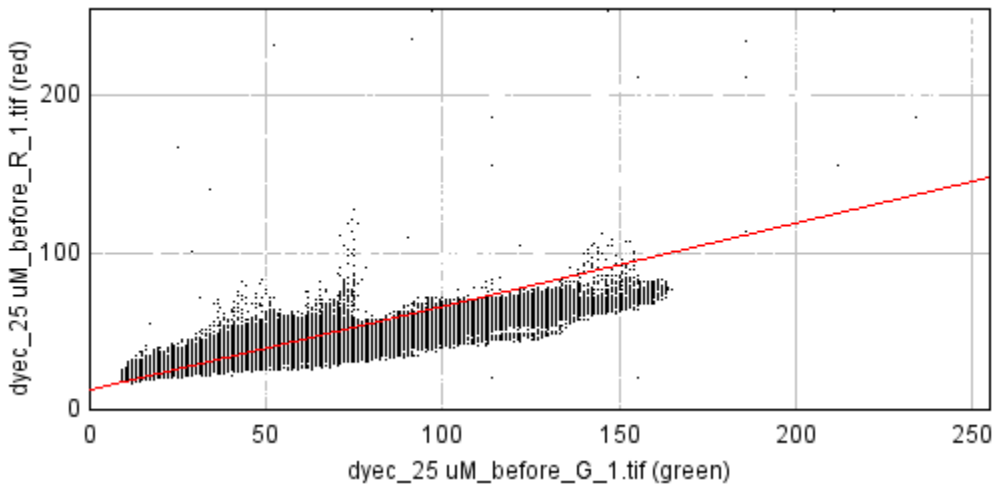

Figure S17. Red and green channel cytofluorogram of fluorescence images of cell incubated with 25 μM fluorescent probe **C** and 1 μM LysoSensor Green DND-189 for verifying their co-localization.

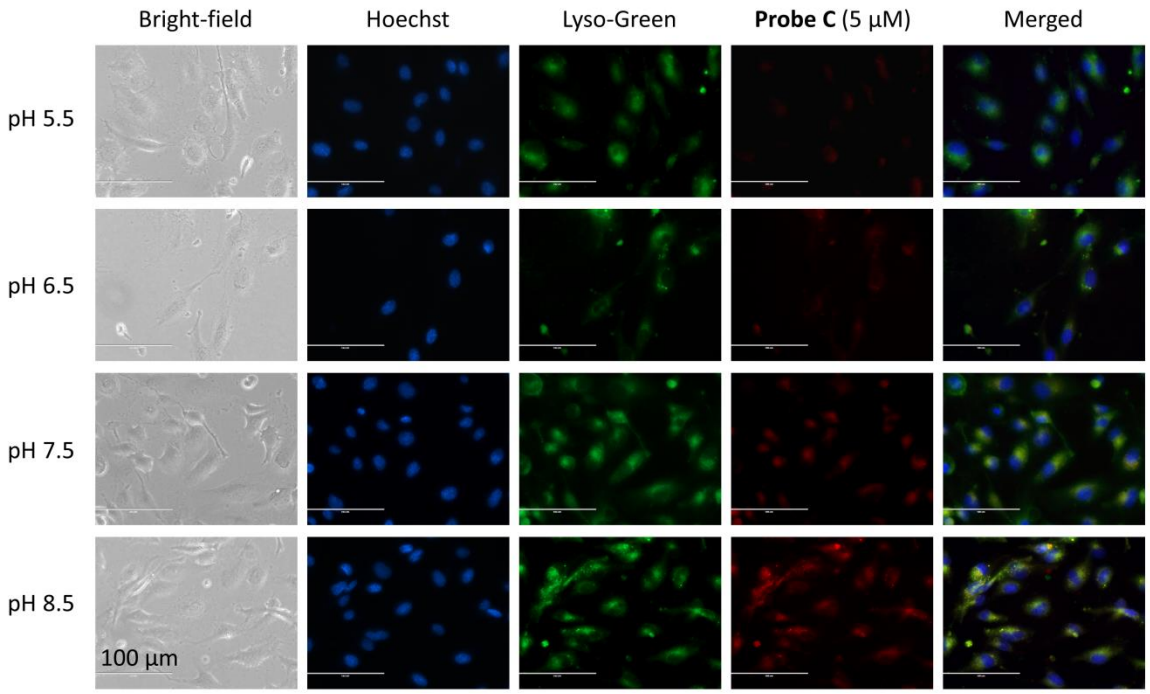

Figure S18. Fluorescence images of HUVEC-C cells incubated with 5 μM fluorescent probe **C** at different intracellular pH values. Intracellular pH were tuned by using nigericin (5 $\mu g \cdot mL^{-1}$) in 2 mL potassium rich PBS at different pH values (5.5, 6.5, 7.5, or 8.5). HUVEC-C cells were incubated with fluorescent probe **C** for 2 h and imaged for colocalization with 1 μM LysoSensor Green and (1 $\mu g \cdot mL^{-1}$) Hoechst 33342 stains. Images were acquired using the inverted fluorescence microscope (AMF-4306, EVOSfl, AMG) at 40× magnification.

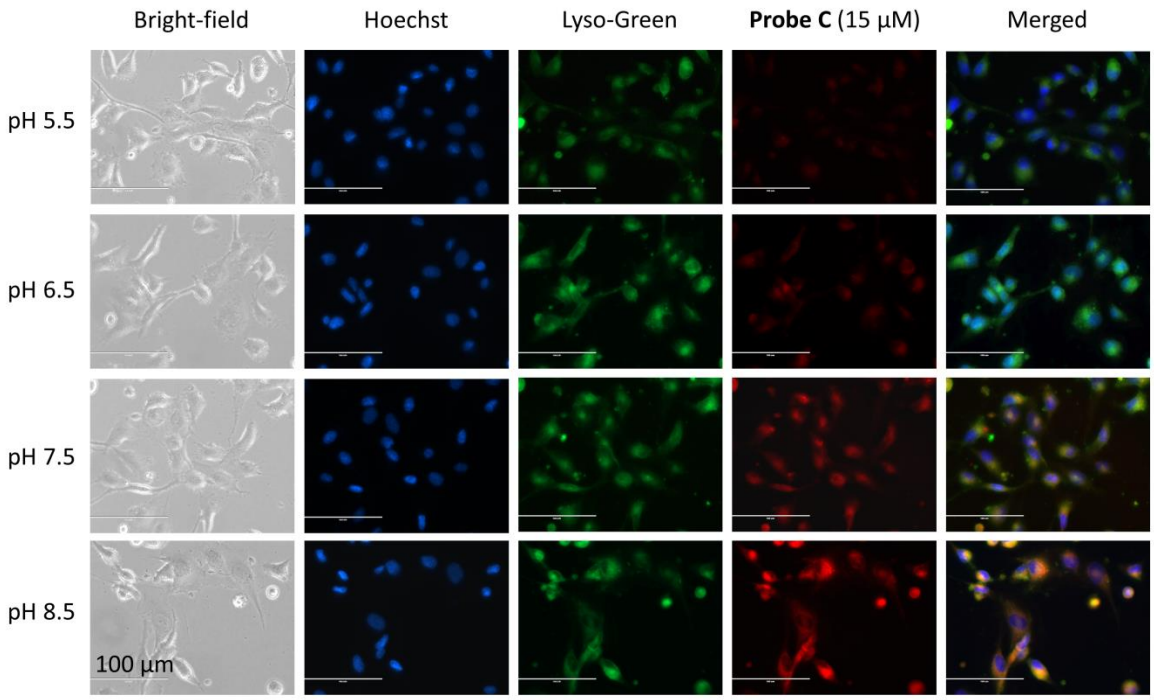

Figure S19. Fluorescence images of HUVEC-C cells incubated with 15 μM fluorescent probe **C** at different intracellular pH values. Intracellular pH were tuned by using nigericin (5 $\mu g \cdot mL^{-1}$) in 2 mL potassium rich PBS at different pH values (5.5, 6.5, 7.5, or 8.5). HUVEC-C cells were incubated with fluorescent probe **C** for 2 h and imaged for colocalization with 1 μM LysoSensor Green and (1 $\mu g \cdot mL^{-1}$) Hoechst 33342 stains. Images were acquired using the inverted fluorescence microscope (AMF-4306, EVOSfl, AMG) at 40× magnification.

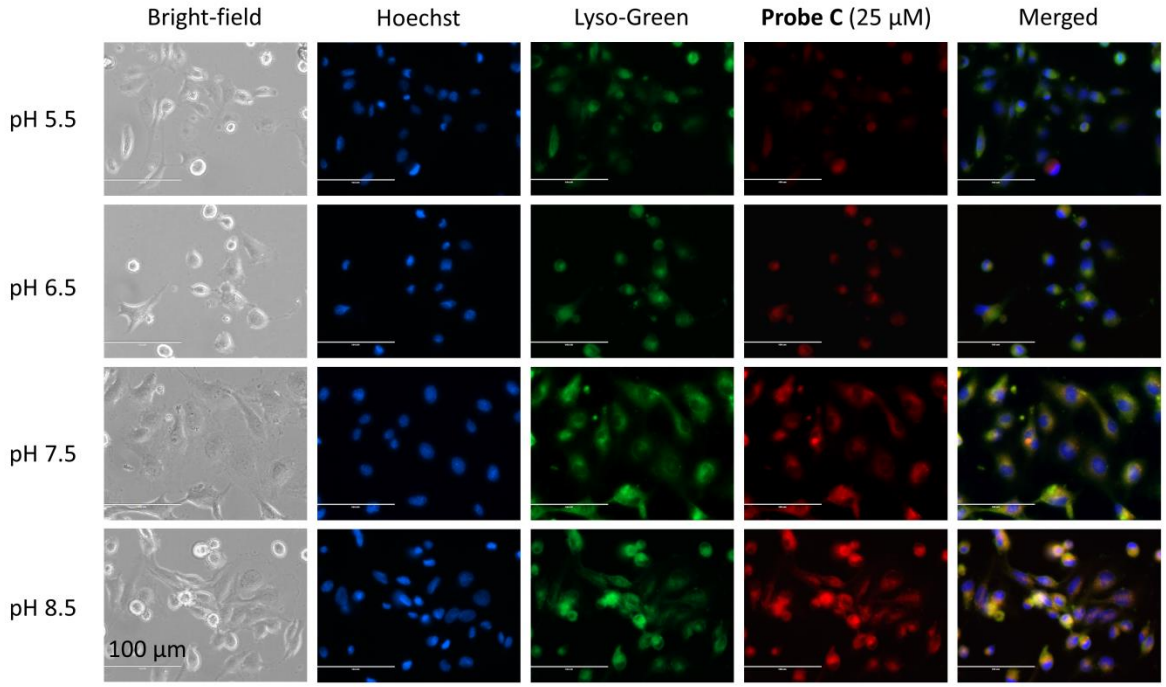

Figure S20. Fluorescence images of HUVEC-C cells incubated with 25 μM fluorescent probe **C** at different intracellular pH values. Intracellular pH were tuned by using nigericin (5 $\mu g \cdot mL^{-1}$) in 2 mL potassium rich PBS at different pH values (5.5, 6.5, 7.5, or 8.5). HUVEC-C cells were incubated with probe **C** for 2 h and imaged for colocalization with 1 μM LysoSensor Green and (1 $\mu g \cdot mL^{-1}$) Hoechst 33342 stains. Images were acquired using the inverted fluorescence microscope (AMF-4306, EVOSfl, AMG) at 40× magnification.

Reference:

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3. Du, H.; Fuh, R. C. A.; Li, J. Z.; Corkan, L. A.; Lindsey, J. S., Photochemcad: A Computer-aided Design and Research Tool in Photochemistry. *Photochemistry and Photobiology* **1998,** *68*, 141-142.
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*Full author list for the reference 15 in the main paper:

Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A.; Peralta, Jr., J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken,V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J.