Electronic Supplementary Information

Near-infrared Fluorescent Probes Based on Piperazine-Functionalized BODIPY Dyes for Sensitive Detection of Lysosomal pH

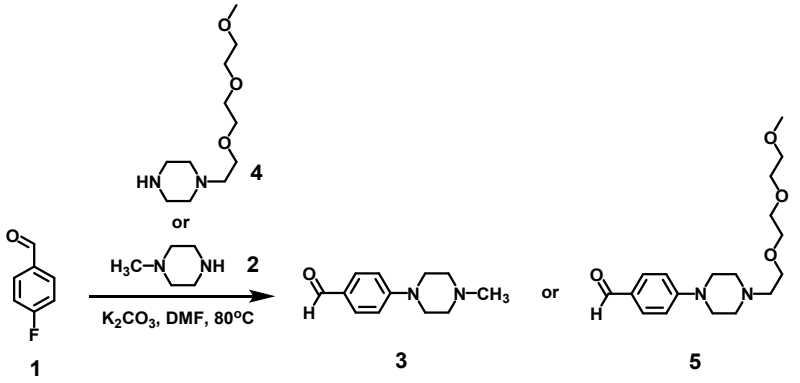
Jingtuo Zhang,[a] Mu Yang,[a] Cong Li,[a] Nethaniah Dorh,[a] Fei Xie,[a] Fen-Tair Luo,[b] Ashutosh Tiwari*[a] and Haiying Liu*[a]*

[a] Department of Chemistry, Michigan Technological University, Houghton, MI 49931
[b] Institute of Chemistry, Academia Sinica, Taipei, Taiwan 11529, Republic of China

Content: page

Experimental section:

Synthesis:



Compound **4** was prepared according to the reported literature/patent[1]

4-(4-Methylpiperazin-1-yl)benzaldehyde (**3)**:

4-Fluorobenzaldehyde (1 g, 8.0 mmol), 1-methylpiperazine (2.4 g, 24.0 mmol) and K_2CO_3 (3.3 g, 24 mmol) were added into 18 mL DMF in a 50 mL round-bottom flask. The mixture was stirred for 14 hours at 80 °C, washed with water and extracted by 100 mL CH_2Cl_2. The organic layer was collected, dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated in vacuum and the crude product was purified by column chromatograph with eluent (CH_2Cl_2/EtOH, 9/0.8, v/v) to get compound **3** as yellow solid (1.41 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.76 (s, 1H), 7.73 (d, J = 8 Hz, 2H), 6.90 (d, J = 8 Hz, 2H), 3.40 (t, J = 4.4 Hz, 4H), 2.54 (t, J = 4.4 Hz, 4H), 2.34 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.6, 155.2, 132.1, 127.4, 113.8, 54.9, 47.3, 46.3. HRMS (EI+) Calculated for $C_{12}H_{16}N_2O$ $[M]^+$ 204.1263, found 204.1262.

4-(4-(2-(2-(2-Methoxyethoxy)ethoxy)ethyl)piperazin-1-yl)benzaldehyde (**5)**:

4-Fluorobenzaldehyde (0.4 g, 3.2 mmol), compound **2** (1.2 g, 4.8 mmol) and K_2CO_3 (0.66 g, 4.8 mmol) were added into 7 mL DMF in a 25 mL round-bottom flask. The mixture was stirred for 12 hours at 80 °C, washed with water and extracted by 100 mL CH_2Cl_2. The organic layer was collected, dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated in vacuum

and the crude product was purified by column chromatograph with eluent (CH_2Cl_2/EtOH, 9/0.6, v/v) to get compound **5** as yellow oil (0.60 g, 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.76 (s, 1H), 7.73 (d, *J* = 8 Hz, 2H), 6.89 (d, *J* = 8 Hz, 2H), 3.77 – 3.30 (m, 17H), 2.67 (brs, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.3, 154.9, 131.7, 126.9, 113.3, 71.9, 70.3, 68.8, 59.0, 57.6, 53.1, 46.9. HRMS (EI+) Calculated for $C_{18}H_{28}N_2O_4$ $[M]^+$ 336.2049, found 336.2045

Optical measurements:

50 mM citrate-phosphate buffer was used for pH dependency and photostability measurements of the fluorescent probes. Diluted citrate-phosphate buffer (10 mM, pH 7.4) was used for preparing the mixed solvent with ethanol for the study of solvent effects of the fluorescent probes. In order to avoid the interference of metal-phosphate and metal-citrate binding interactions (forming precipitate of divalent cation phosphate and forming complex of the metal-citrate), 10 mM KHP buffer (pH 4.5) and 10 mM HEPES buffer (pH 7.4) were used for selectivity measurements of fluorescent probes **A**, **B** and **C**.

The UV-Vis spectra of fluorescent probes **A**, **B** and **C** for pH dependency, selectivity, photostability and solvent effect measurements were collected in the range of 300 – 850 nm with increments of 1 nm. Their corresponding fluorescence spectra were collected at the excitation wavelength of 620 nm with increments of 1 nm. The excitation and emission slit widths were set up to 3 nm and 5 nm, respectively. The concentration of the dye in each sample is 5 μM. The fluorescence quantum yields of BODIPY dyes **7** and **9** were calculated by using fluorescein ($\Phi_{f=}$ 0.95 in 0.1 N NaOH aq. with excitation at 490 nm)[2] as standard. Sulforhodamine 101 dye (Φ_f = 0.95 with excitation wavelength at 577 nm in ethanol)[3] was used as a reference standard to determine the fluorescence quantum yields of fluorescent probes **A**, **B**, **C** as well as their protonated derivatives in dichloromethane, ethanol, DMSO and aqueous solutions. Both samples and references were prepared fresh under identical conditions. The quantum yields were calculated using the following equation:

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

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 p$K_{\mathbf{a}}$ by fluorometric titration:

The constants K_a of fluorescent probes **A**, **B** and **C** were determined in aqueous buffered 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 is expressed by the equation as below:[4]

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

$F_{\max}$ and $F_{\min}$ stand for the fluorescence intensities at maximal and minimal H^+ concentration, 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 that were recoded as a function of H^+ concentration with K_a and n as free adjustable parameters yields the estimated apparent constant of K_a.

Reference:

1. B.-Y. Chung, I.-S. Lee, B.-J. Park, Y.-K. Kim, S.-J. Kim and S.-H. Yoon, LG Chem Investment Ltd., S. Korea . 2002, p. 37.
2. Y. Gabe, Y. Urano, K. Kikuchi, H. Kojima and T. Nagano, *Journal of the American Chemical Society*, 2004, **126**, 3357-3367.
3. R. A. Velapoldi and H. H. Tonnesen, *Journal of Fluorescence*, 2004, **14**, 465-472.
4. E. Cielen, A. Tahri, K. V. Heyen, G. J. Hoornaert, F. C. De Schryver and N. Boens, *Journal of the Chemical Society-Perkin Transactions 2*, 1998, 1573-1580.

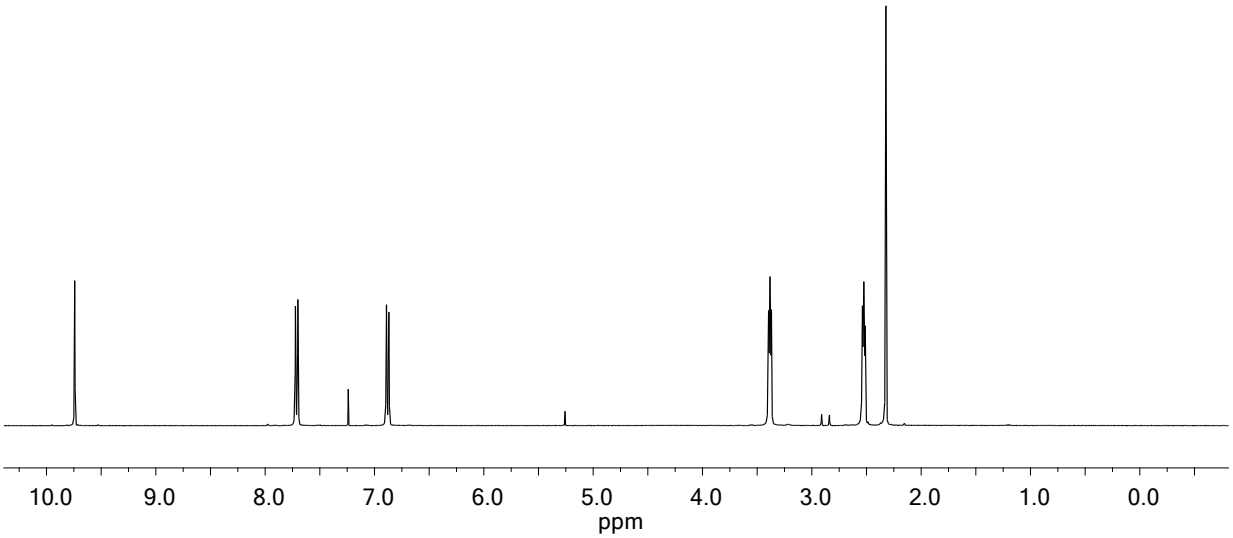


Figure S1. ^{1}H NMR spectrum of compound **4** in $CDCl_3$ solution.

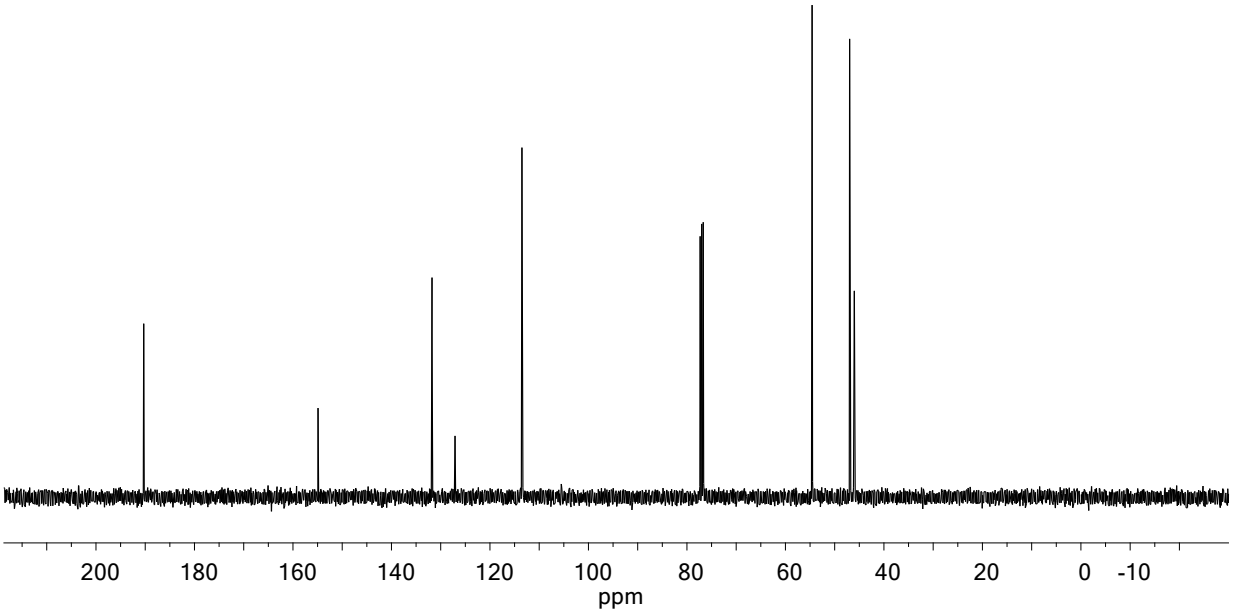


Figure S2. ^{13}C NMR spectrum of compound **4** in $CDCl_3$ solution.

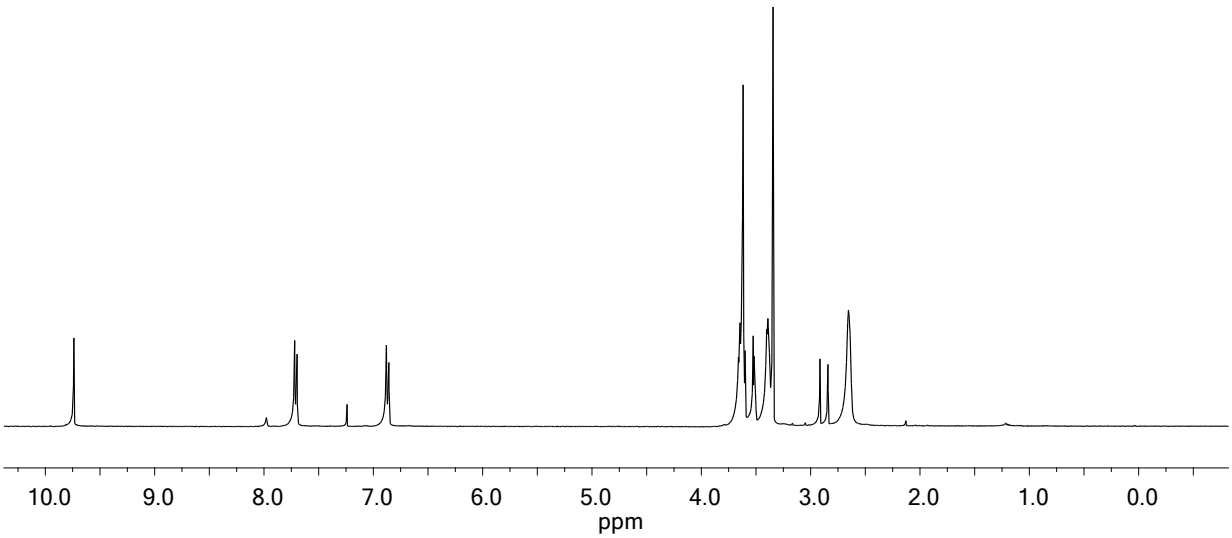


Figure S3. ^{1}H NMR spectrum of compound **5** in $CDCl_3$ solution.

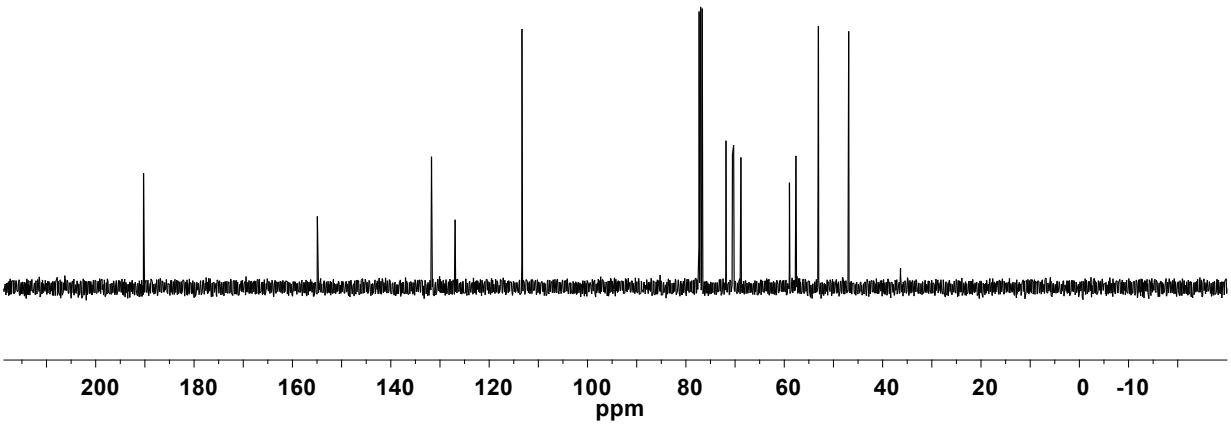


Figure S4. ^{13}C NMR spectrum of compound **5** in $CDCl_3$ solution.

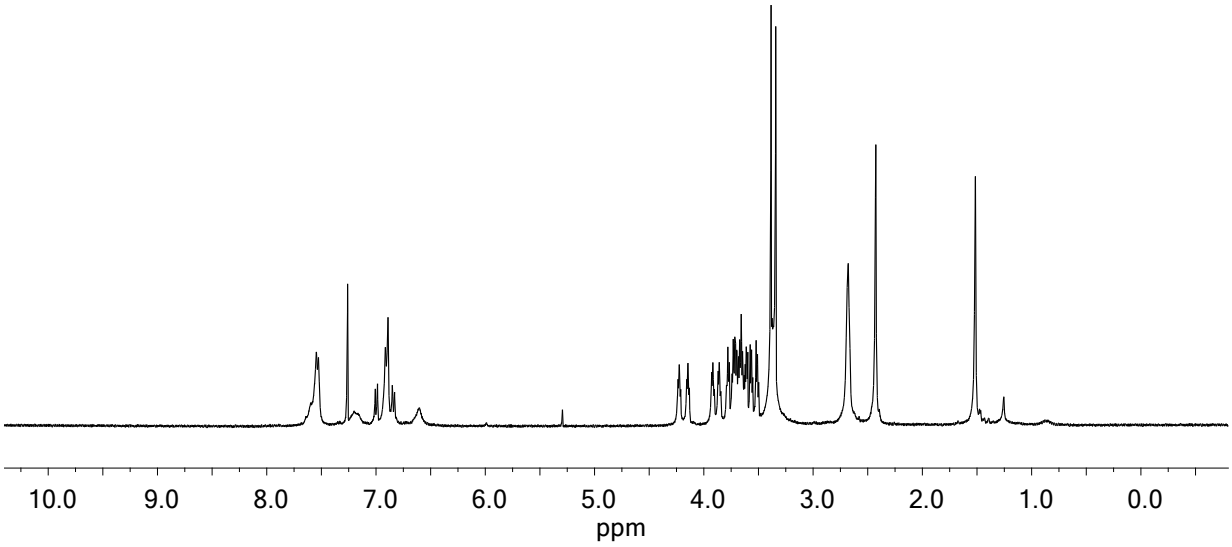


Figure S5. ^{1}H NMR spectrum of fluorescent probe **A** in $CDCl_3$ solution.

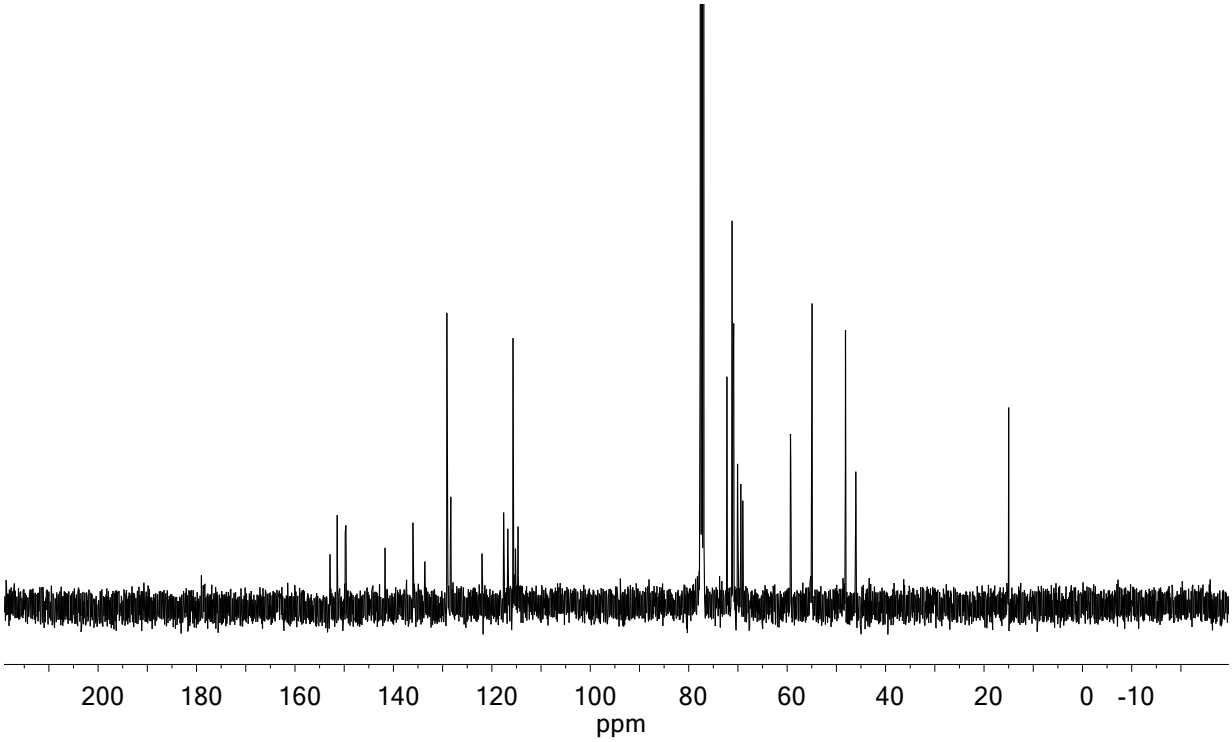


Figure S6. ^{13}C NMR spectrum of fluorescent probe **A** in $CDCl_3$ solution.

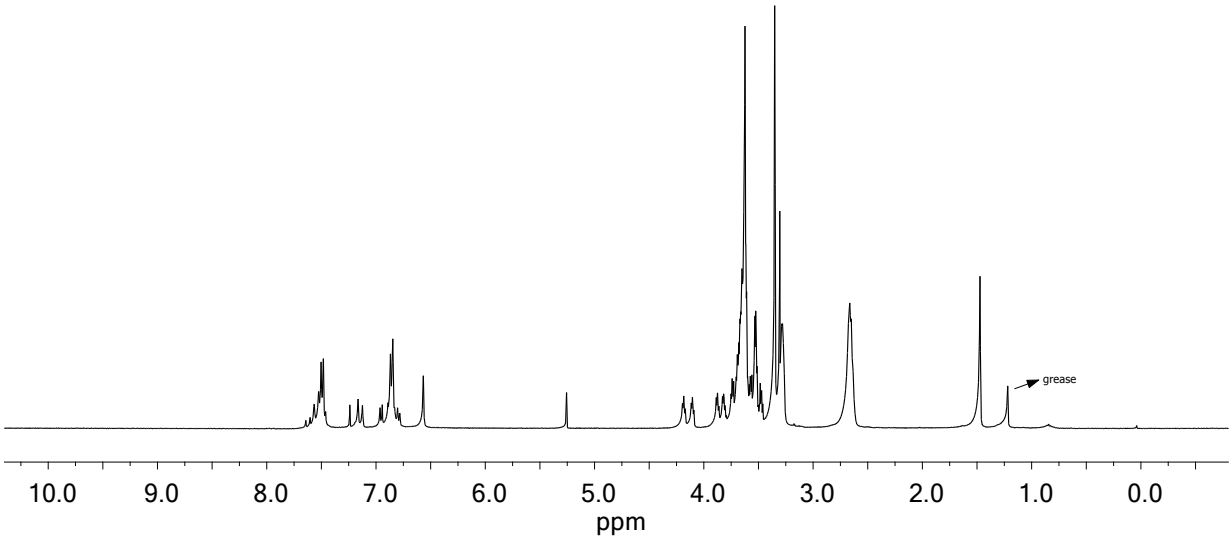


Figure S7. ^{1}H NMR spectrum of fluorescent probe **B** in $CDCl_3$ solution.

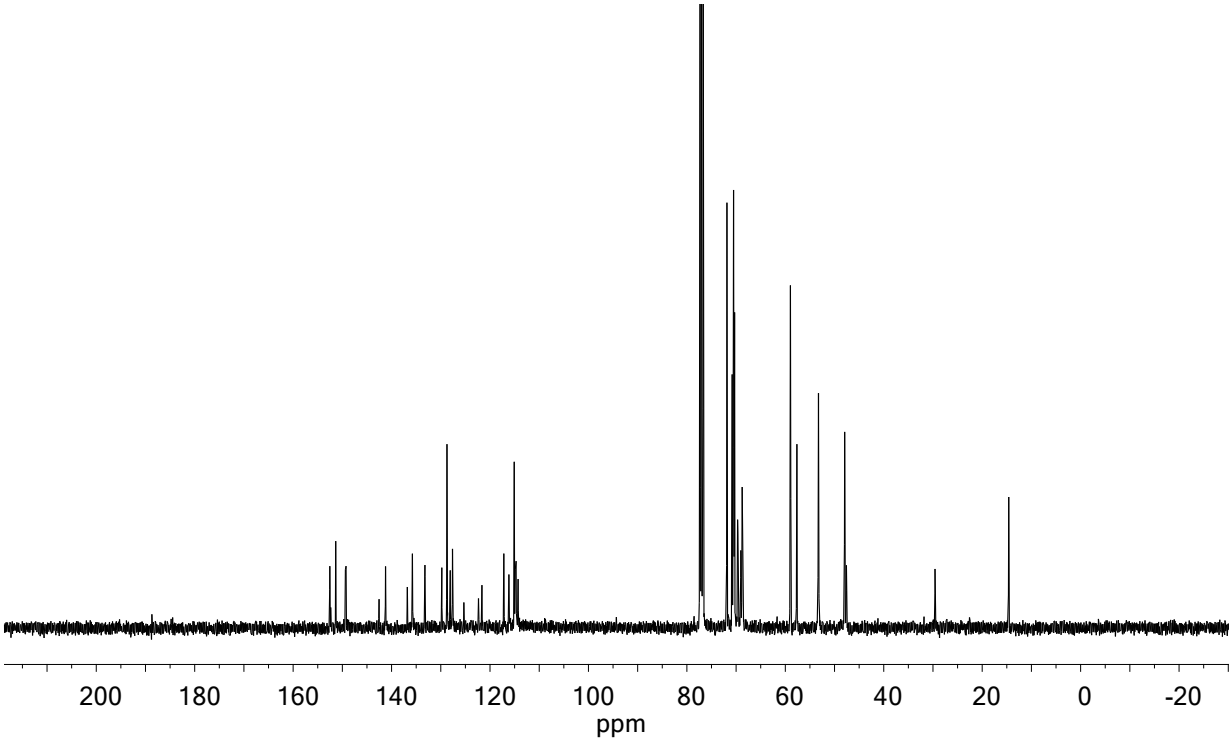


Figure S8. ^{13}C NMR spectrum of fluorescent probe **B** in $CDCl_3$ solution.

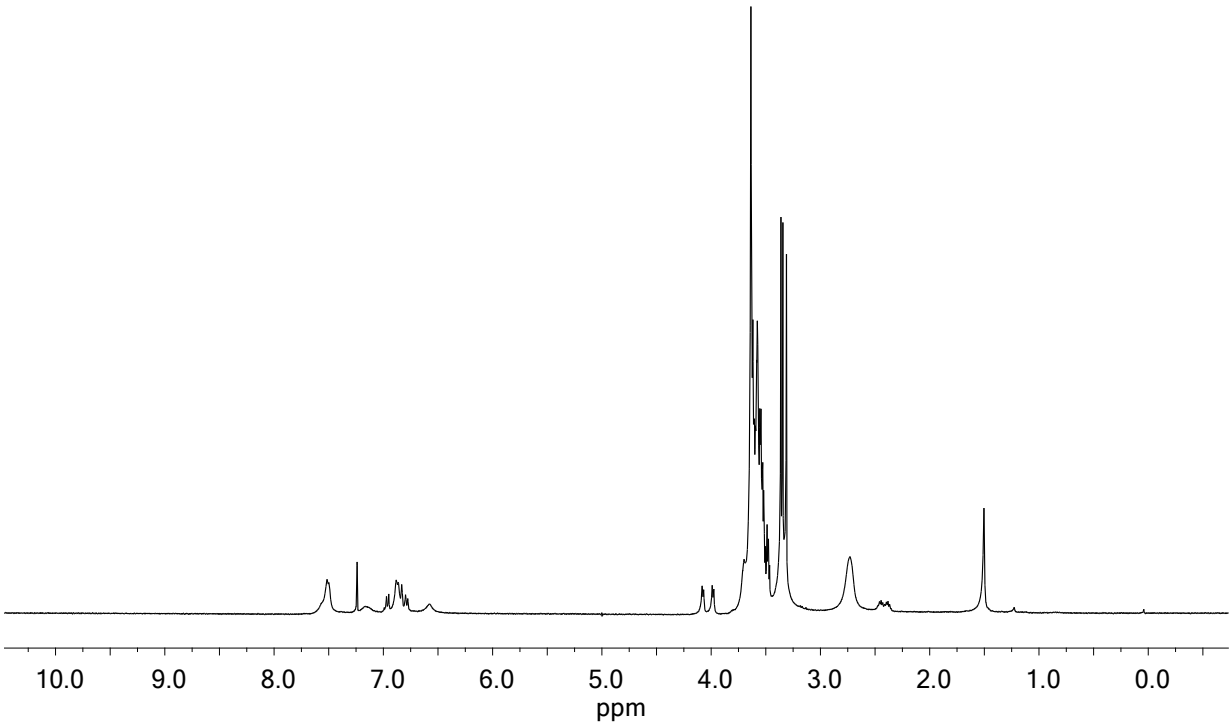


Figure S9. ^{1}H NMR spectrum of fluorescent probe **C** in $CDCl_3$ solution.

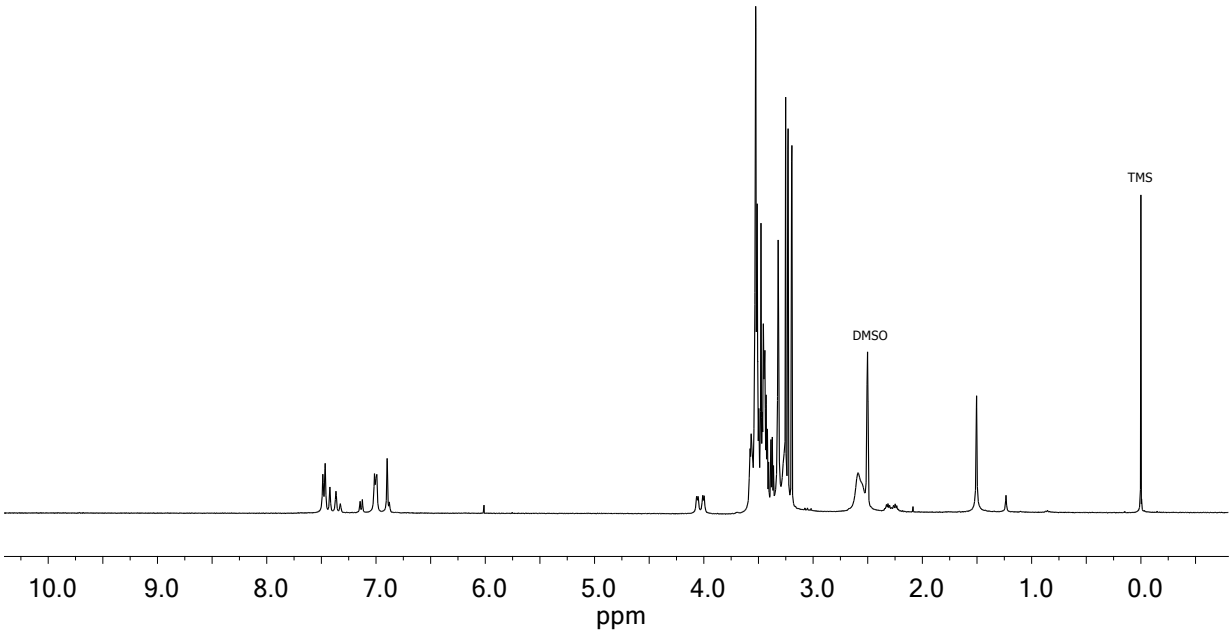


Figure S10. ^{1}H NMR spectrum of fluorescent probe **C** in DMSO-d_6 solution.

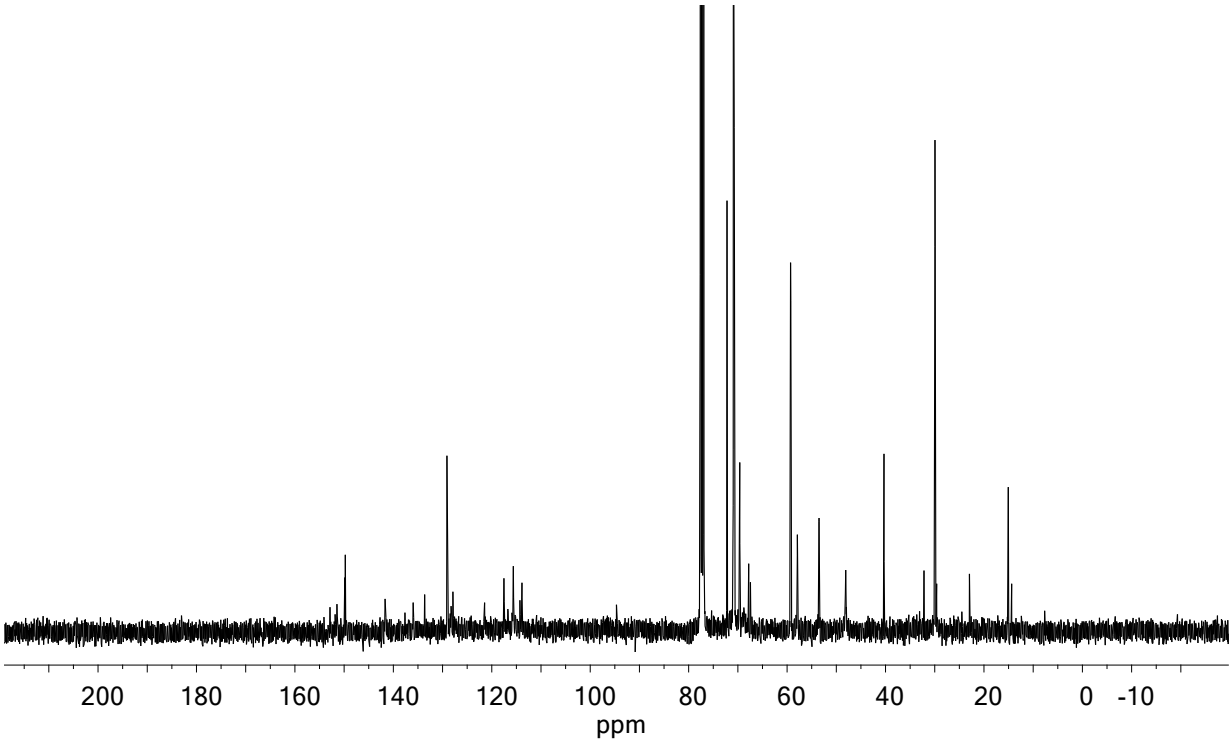


Figure S11. ^{13}C NMR spectrum of fluorescent probe **C** in $CDCl_3$ solution.

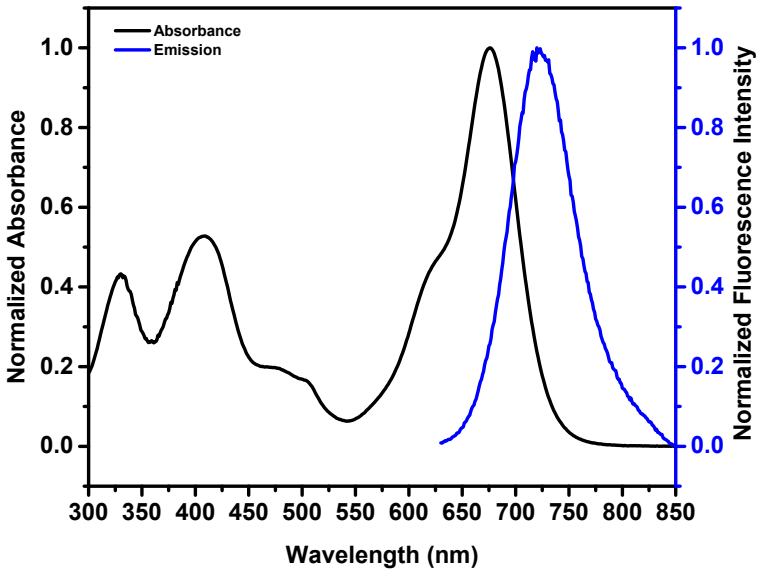


Figure S12. Normalized absorption and emission spectra of fluorescent probe **A** in dichloromethane solution.

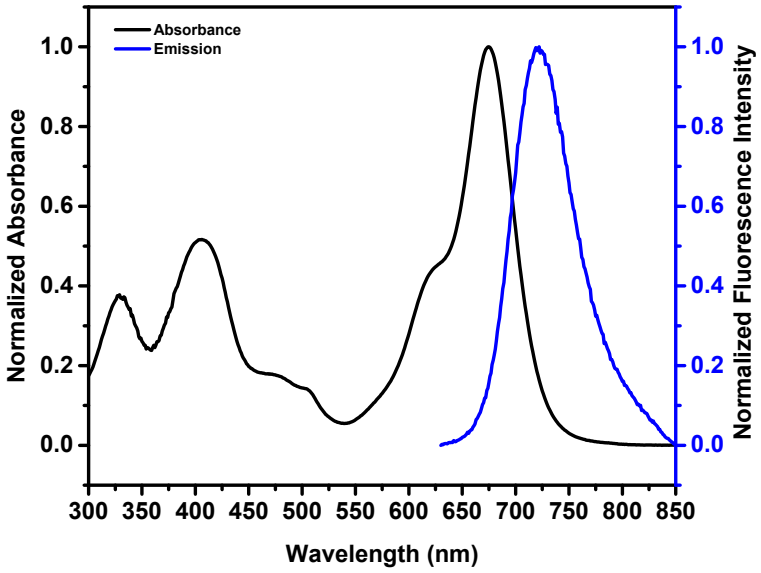


Figure S13. Normalized absorption and emission spectra of fluorescent probe **A** in DMSO solution.

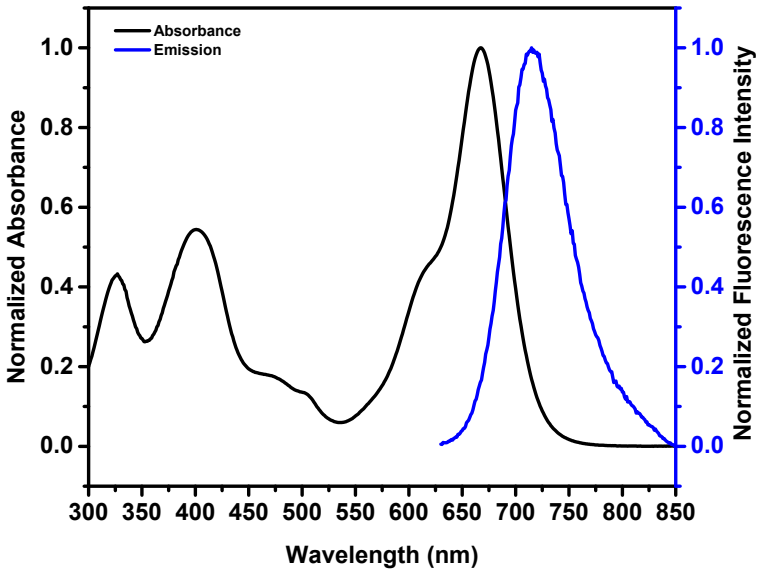


Figure S14. Normalized absorption and emission spectra of fluorescent probe **A** in ethanol solution.

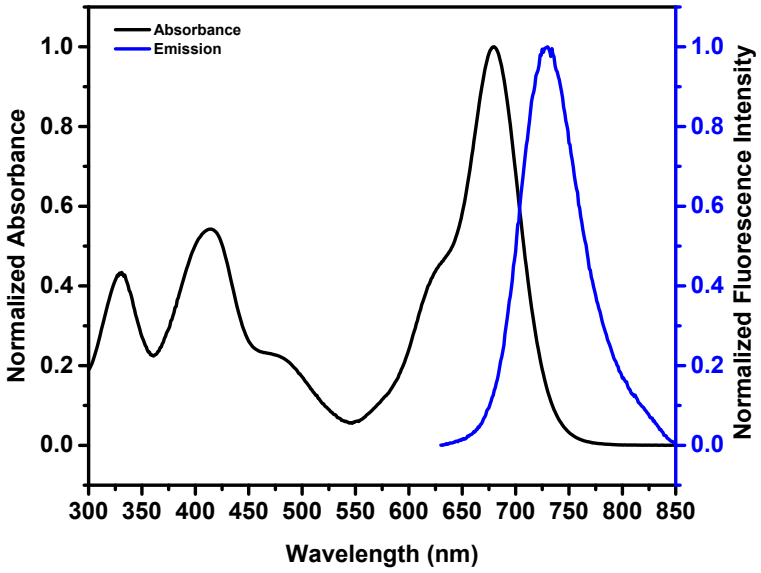


Figure S15. Normalized absorption and emission spectra of fluorescent probe **B** in dichloromethane solution.

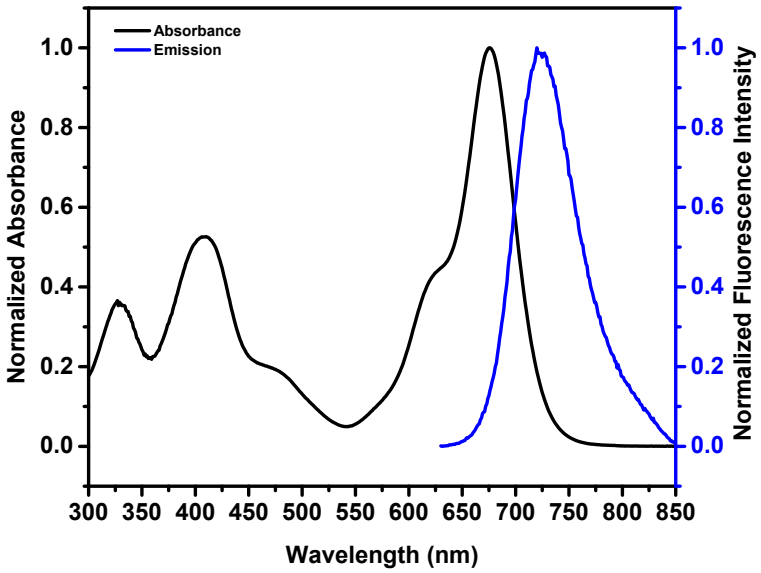


Figure S16. Normalized absorption and emission spectra of fluorescent probe **B** in DMSO solution.

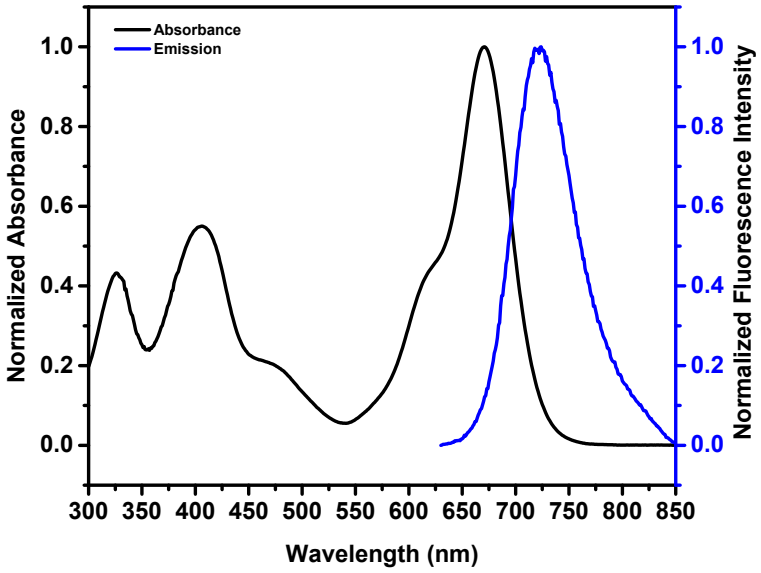


Figure S17. Normalized absorption and emission spectra of fluorescent probe **B** in ethanol solution.

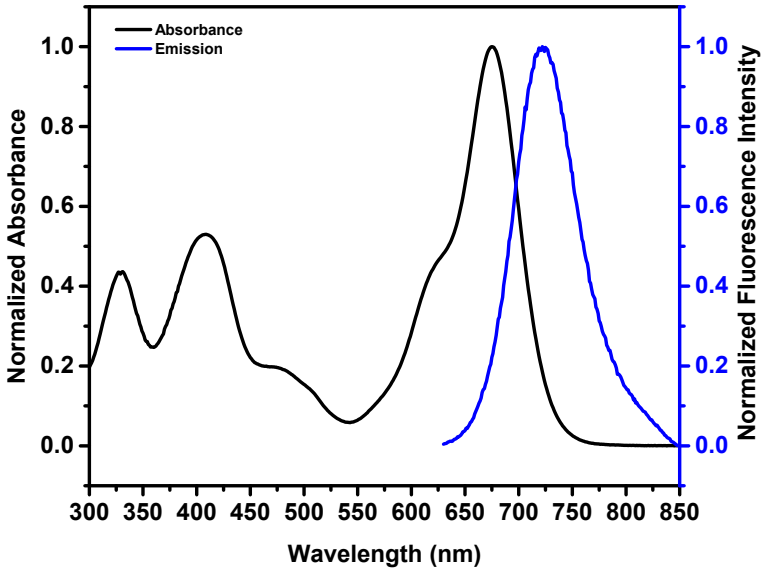


Figure S18. Normalized absorption and emission spectra of fluorescent probe **C** in dichloromethane solution.

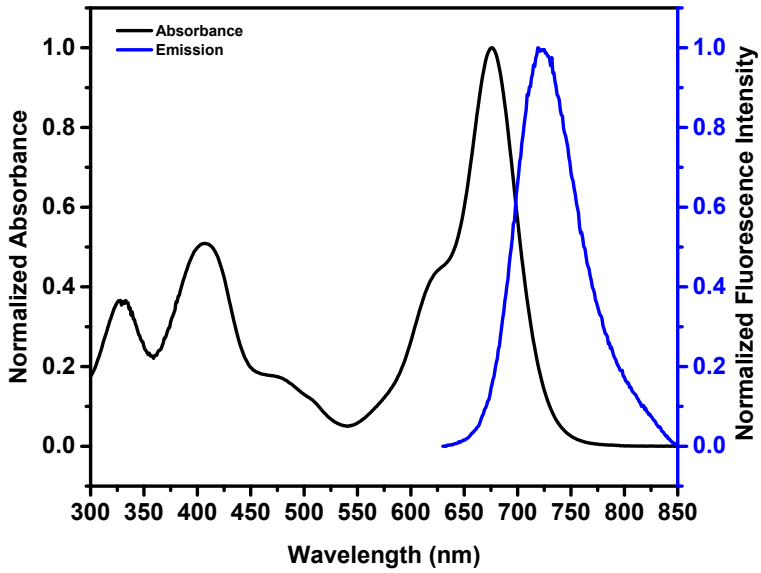


Figure S19. Normalized absorption and emission spectra of fluorescent probe **C** in DMSO solution.

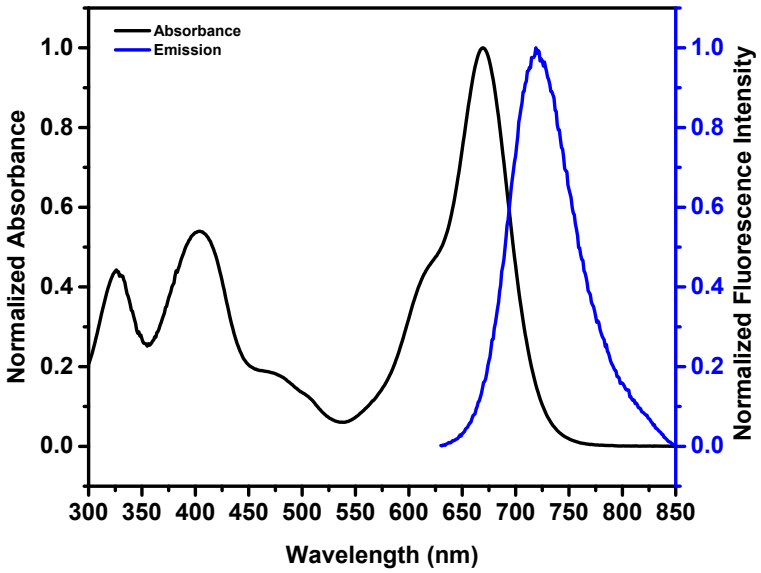


Figure S20. Normalized absorption and emission spectra of fluorescent probe **C** in ethanol solution.

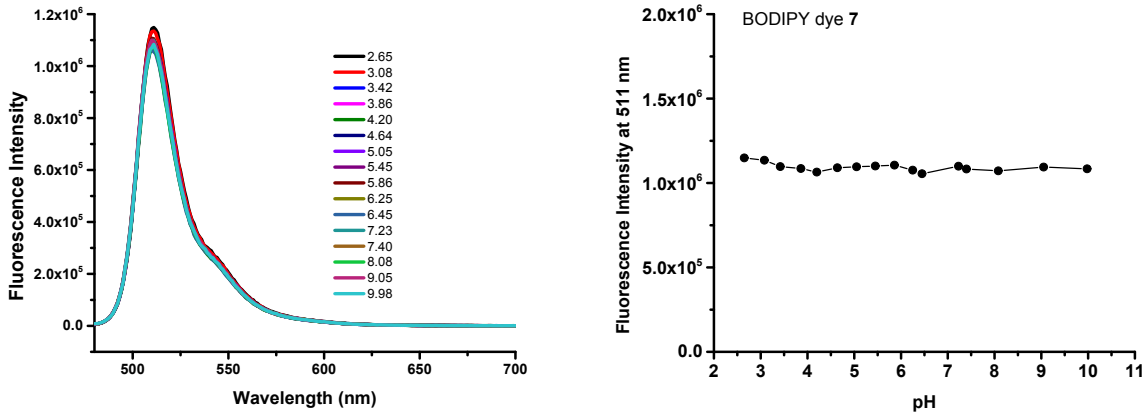


Figure S21. Left: fluorescence spectra of BODIPY dye **7** (5 μM) under different pH conditions. Right: fluorescence intensity responses of BODIPY dye **7** (5 μM) to different pH values.

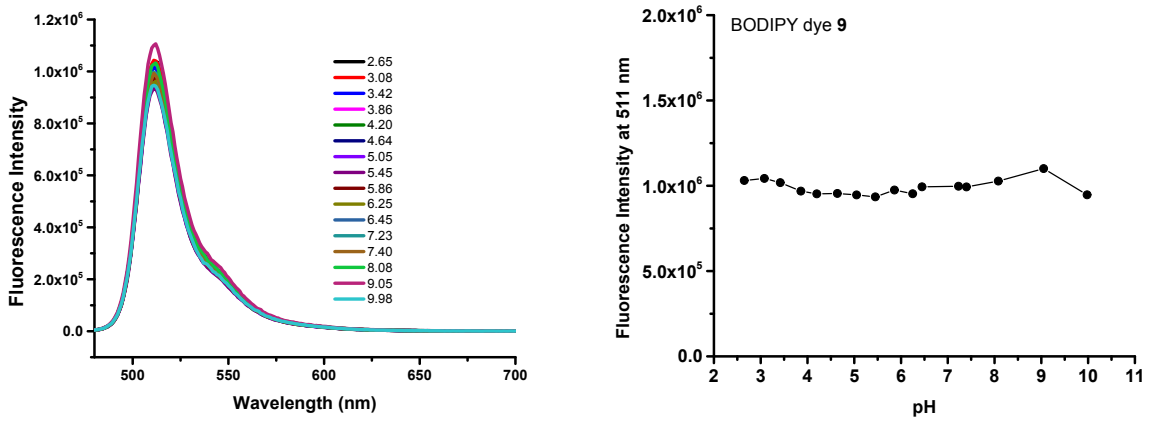


Figure S22. Left: fluorescence spectra of BODIPY dye **9** (5 μM) under different pH conditions. Right: fluorescence intensity responses of BODIPY dye **9** (5 μM) to pH.

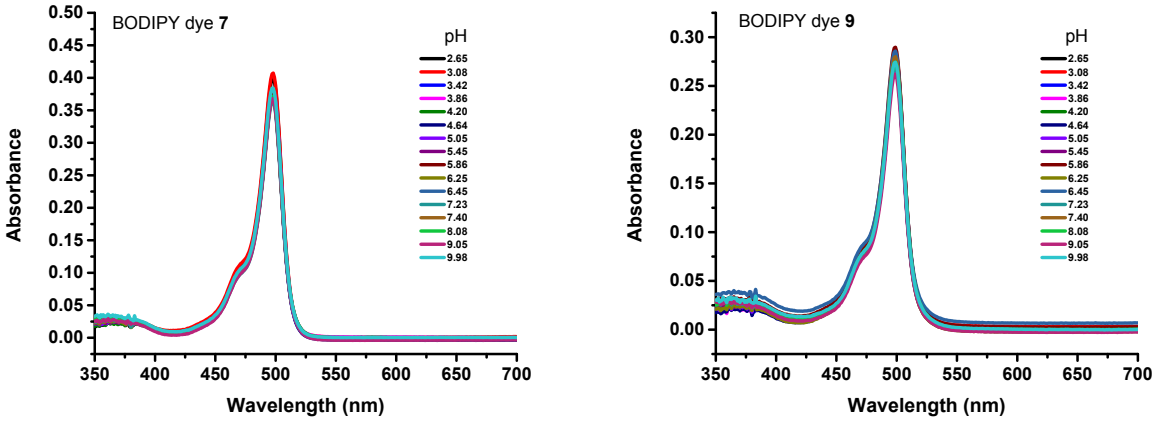


Figure S23. Absorption spectra of BODIPY dyes **7** (left) and **9** (right) at different pH conditions in buffer solutions.

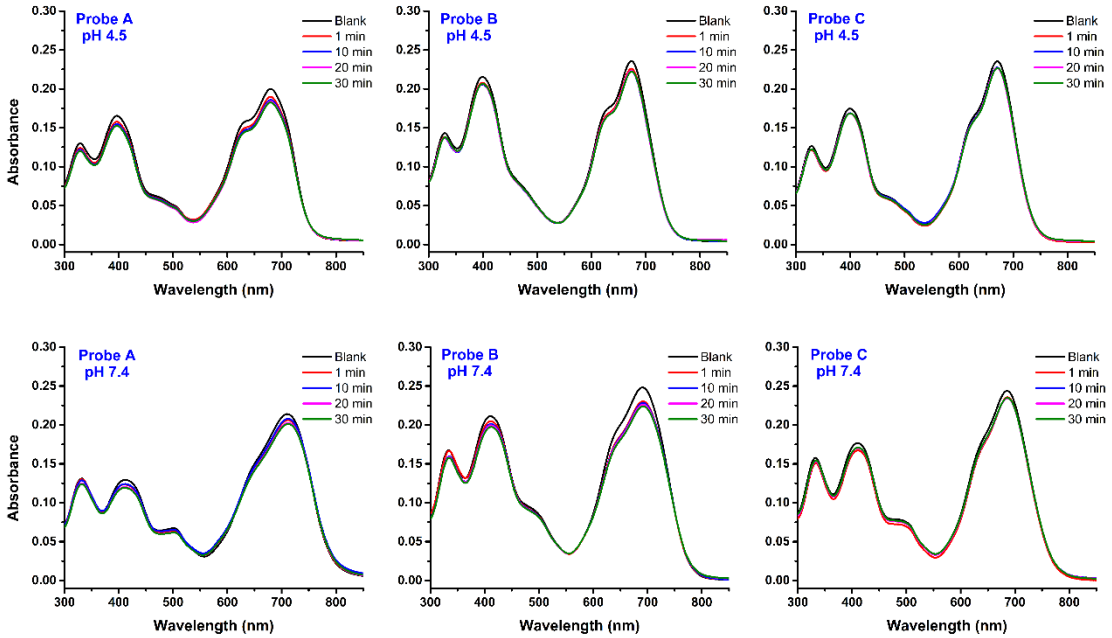


Figure S24. Absorption spectra of 5 μM probes **A**, **B** and **C** in absence (Blank) and presence of 20 μM cysteine in buffer solutions (pH 4.5: upper row, pH 7.4: lower row) as function of time.

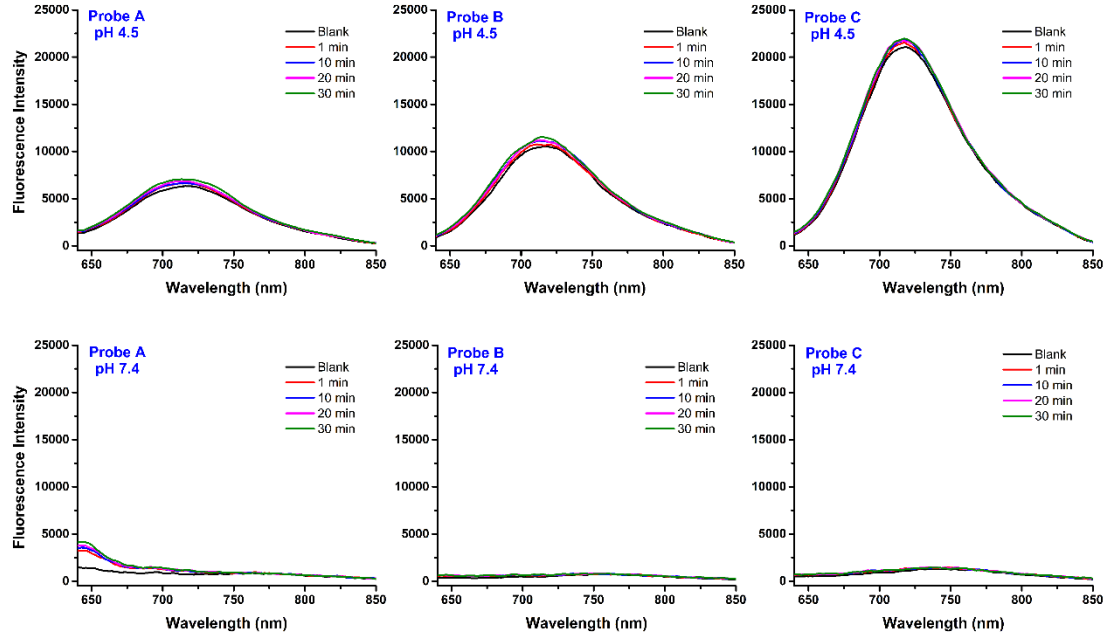


Figure S25. Fluorescence spectra of 5 μM probes **A**, **B** and **C** in absence (Blank) and presence of 20 μM cysteine in buffer solutions (pH 4.5: upper row, pH 7.4: lower row) as function of time.

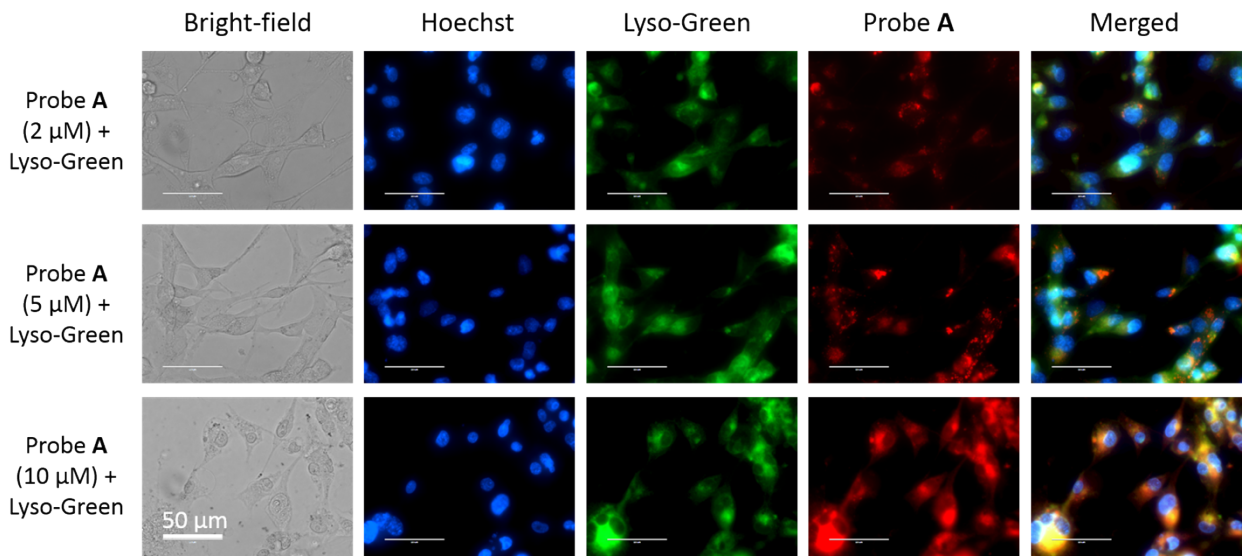


Figure S26. Fluorescence images of MDA-MB-231 cells incubated with different concentrations of probe **A**. After 2 h serum starvation, cells were incubated with 2 µM, 5 µM and 10 µM of fluorescent probe **A** for 2 h and imaged for co-localization with lysosomal stain LysoSensor Green DND-189 (1 µM). 1 µg/mL of Hoechst was used to stain the nucleus. All images were acquired at 60× magnification using inverted fluorescence microscope (AMF-4306, $EVOS_{fl}$, AMG).

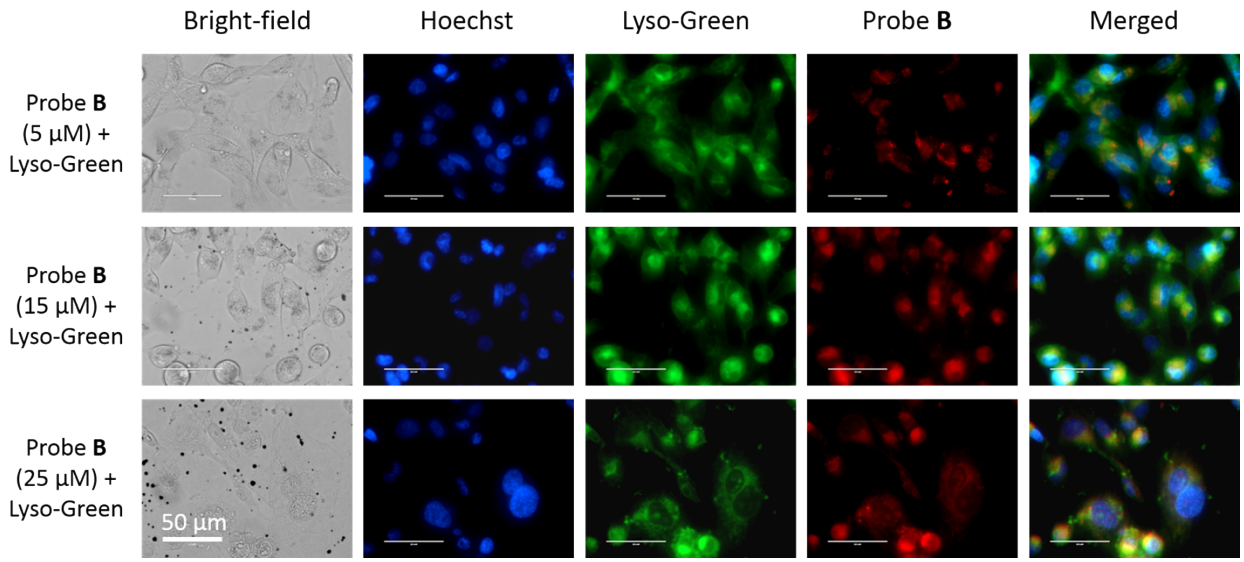


Figure S27. Fluorescence images of MDA-MB-231 cells incubated with different concentrations of probe **B**. After 2 h serum starvation, cells were incubated with 5 µM, 15 µM and 25 µM of fluorescent probe **B** for 2 h and imaged for co-localization with lysosomal stain LysoSensor Green DND-189 (1 µM). 1 µg/mL of Hoechst was used to stain the nucleus. All images were acquired at 60× magnification using inverted fluorescence microscope (AMF-4306, $EVOS_{fl}$, AMG).

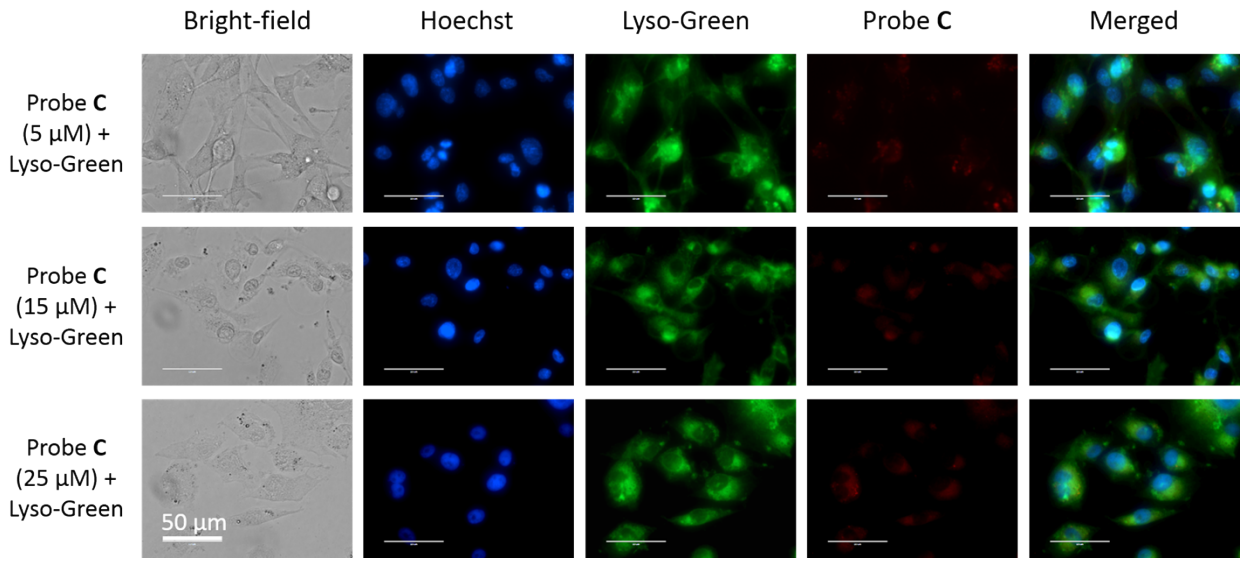


Figure S28. Fluorescence images of MDA-MB-231 cells incubated with different concentrations of probe **C**. After 2 h serum starvation, cells were incubated with 5 µM, 15 µM and 25 µM of fluorescent probe **C** for 2 h and imaged for co-localization with lysosomal stain LysoSensor Green DND-189 (1 µM). 1 µg/mL of Hoechst was used to stain the nucleus. All images were acquired at 60× magnification using inverted fluorescence microscope (AMF-4306, $EVOS_{fl}$, AMG).

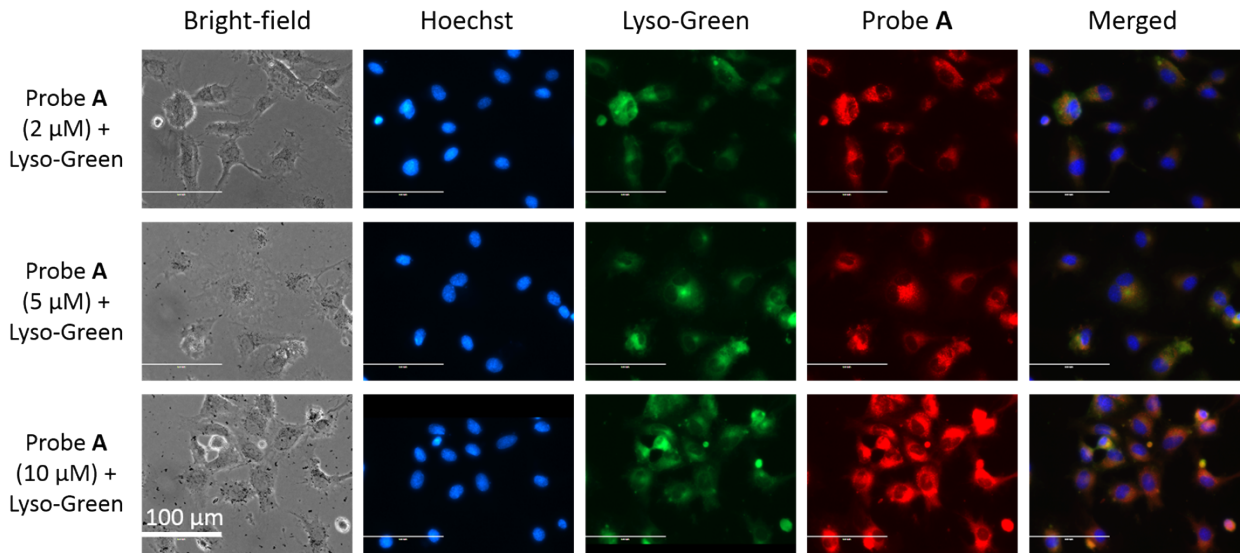


Figure S29. Fluorescence images of HUVEC-C cells incubated with different concentrations of fluorescent probe **A**. After 2 h serum starvation, HUVEC-C cells were incubated with 2 µM, 5 µM and 10 µM probe **A** for 2 h and imaged for co-localization with (1 µM) LysoSensor Green DND-189 and (1 µg/mL) Hoechst 33342 stains. Images were acquired at 40× magnification using the inverted fluorescence microscope (AMF-4306, $EVOS_{fl}$, AMG).

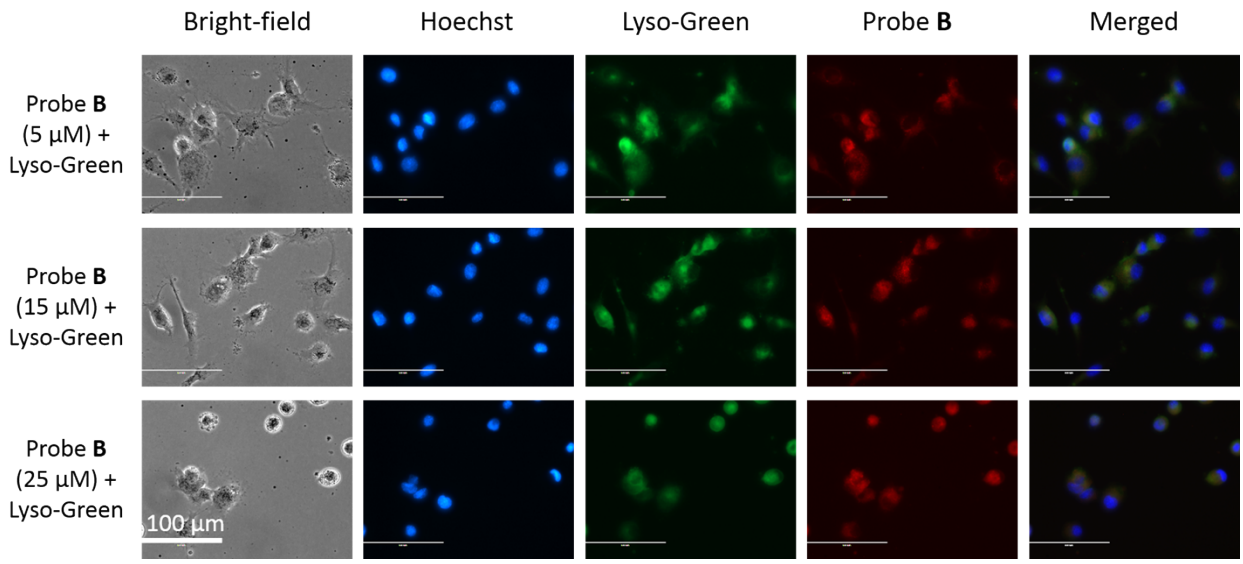


Figure S30. Fluorescence images of HUVEC-C cells incubated with different concentrations of fluorescent probe **B**. After 2 h serum starvation, HUVEC-C cells were incubated with 5 μM, 15 μM or 25 μM of probe **B** for 2 h and imaged for co-localization with 1 μM LysoSensor Green DND-189 and 1 μg/mL Hoechst 33342 stains. Images were acquired at 40× magnification using the inverted fluorescence microscope (AMF-4306, $EVOS_{fl}$, AMG).

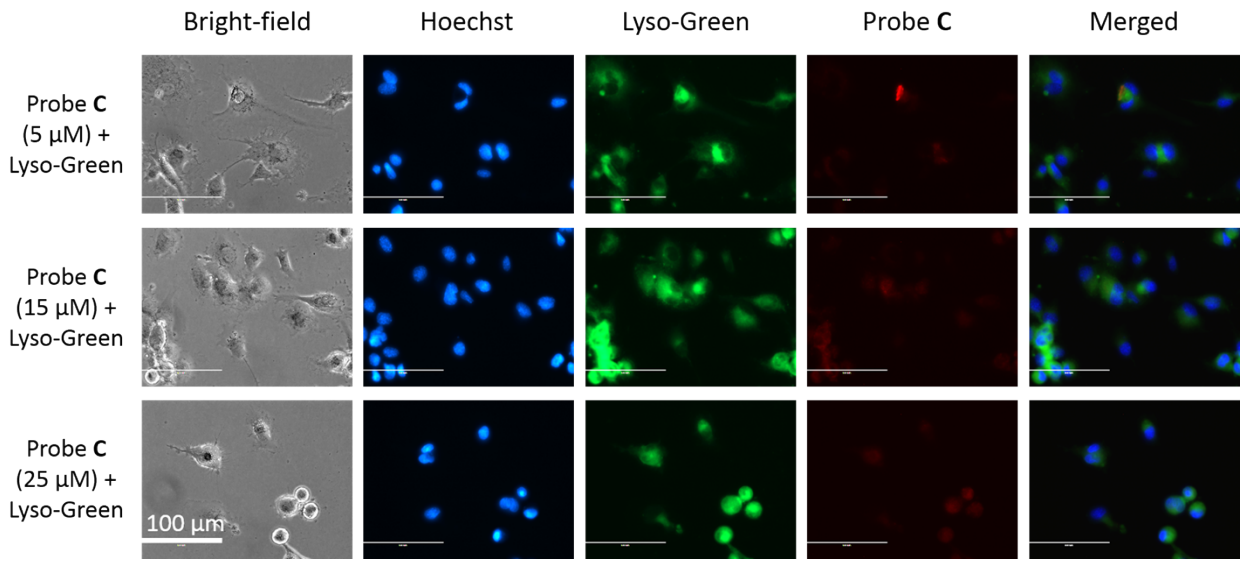


Figure S31: Fluorescence images of HUVEC-C cells incubated with different concentrations of fluorescent probe **C**. After 2 h serum starvation, HUVEC-C cells were incubated with 5 µM, 15 µM and 25 µM of probe **C** for 2 h and imaged for co-localization with 1 µM LysoSensor Green DND-189 and 1 µg/mL Hoechst 33342 stains. Images were acquired at 40× magnification using the inverted fluorescence microscope (AMF-4306, $EVOS_{fl}$, AMG).

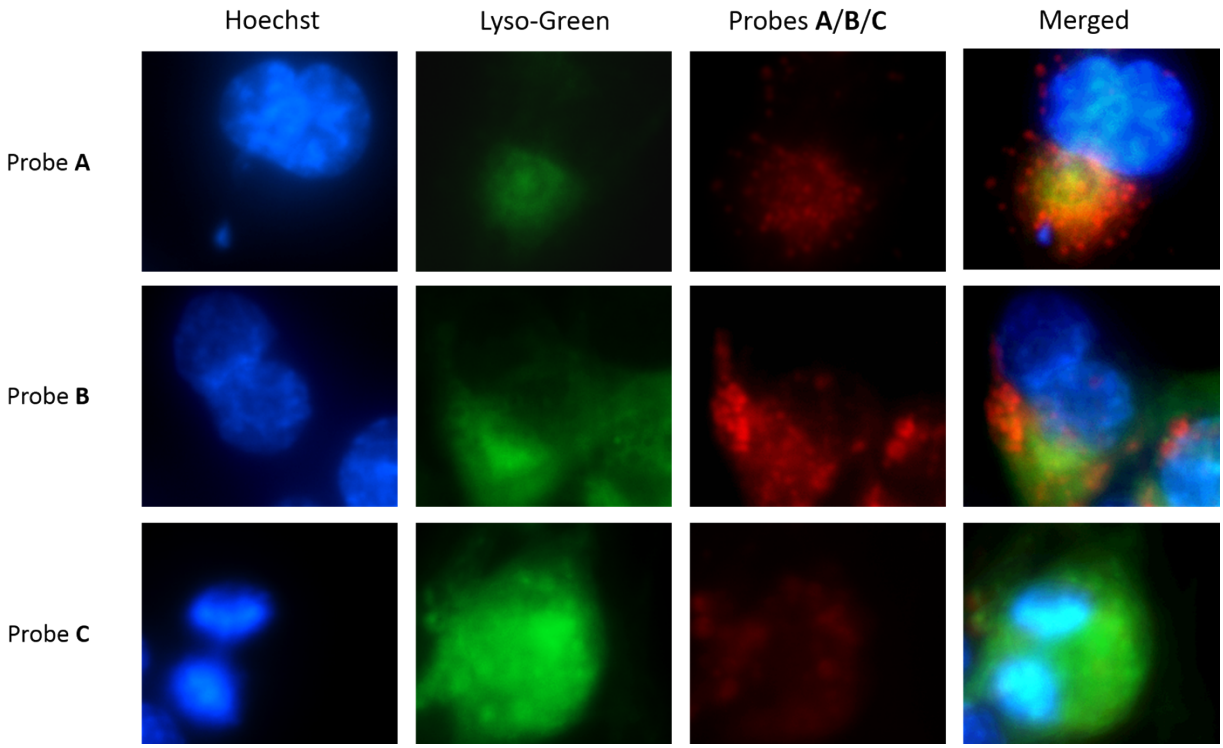


Figure S32. Enlarged fluorescence images of MDA-MB-231 cells with fluorescent probes **A**, **B** and **C** taken at 60× magnification showing co-localization of probes with LysoSensor Green DND-189 in lysosomes. The probe **A** showed much stronger signal than probes **B** and **C**. The lysosomes were mostly perinuclear in cells as they were serum starved for 2 hours before staining.

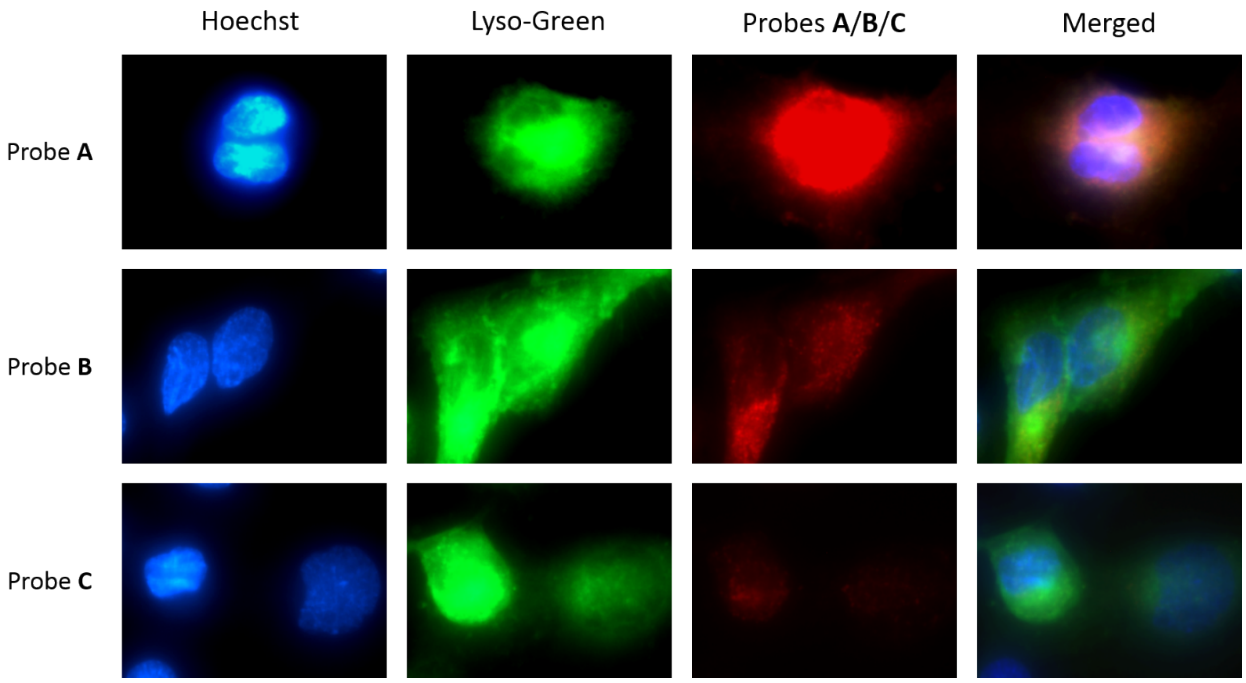


Figure S33. Enlarged fluorescence images of HUVEC-C cells with fluorescent probes **A**, **B** and **C** taken at 60× magnification showing co-localization of probes with LysoSensor Green DND-189 in lysosomes. The probe **A** showed much stronger signal compared to probes **B** and **C**. Probe **B** showed punctate staining under merged image while probe **C** showed very faint staining. The lysosomes were mostly perinuclear in cells as they were serum starved for 2 hours before staining.

Pearson's co-localization coefficients of fluorescent probes **A**, **B** and **C** with LysoSensor Green DND-189

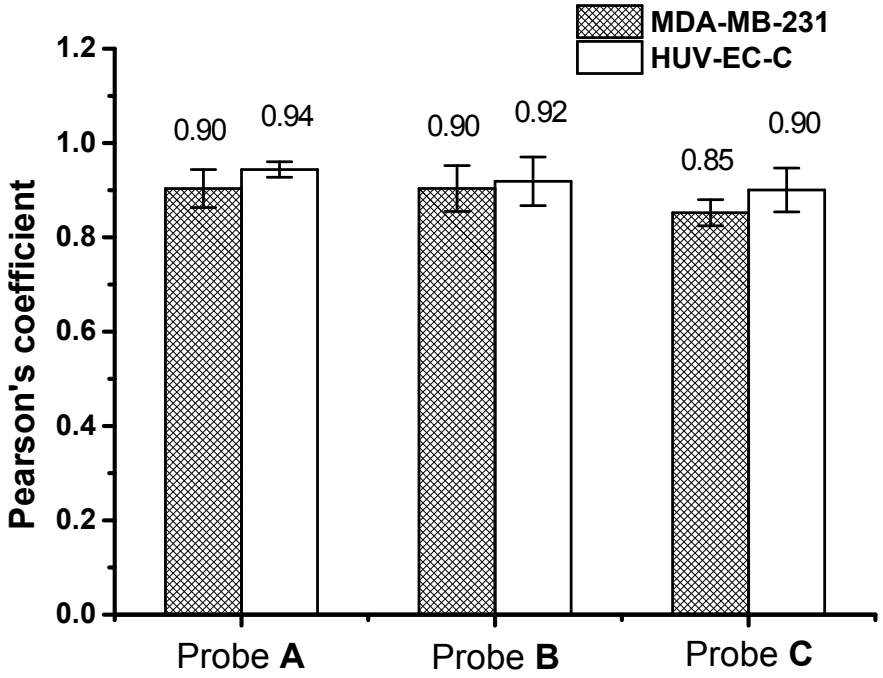


Figure S34. The co-localization of fluorescent probes **A**, **B** and **C** with LysoSensor Green DND-189 were quantified by Perason's coefficient (mean ± SD, n=6).

Methods: Pearson's coefficient was quantified using JACoP plug in from imageJ. (Bolte and Cordelieres, 2006, Journal of microscopy, 224, 213-232)

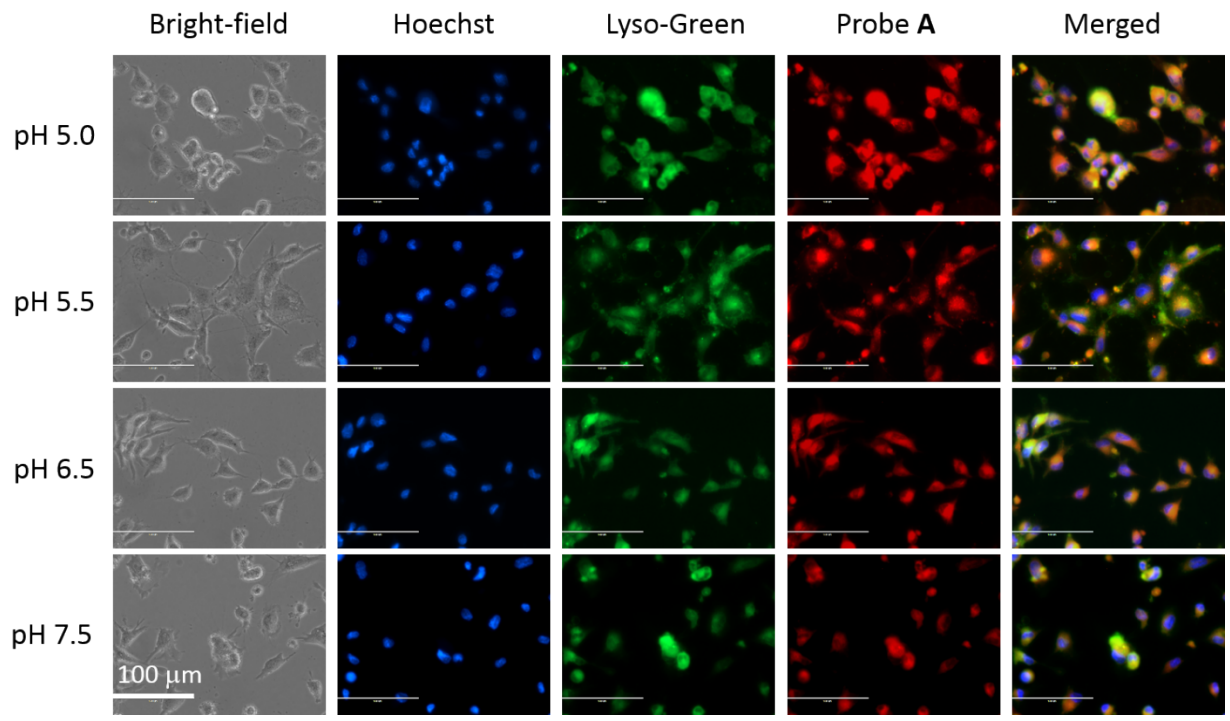


Figure S35. Fluorescence images of HUVEC-C cells incubated with 5 µM probe **A** at different pH values. 1 µM LysoSensor Green DND-189 and 1 µg/mL Hoechst 33342 were used as co-stains. Images were acquired at 40× magnification using the inverted fluorescence microscope (AMF-4306, $EVOS_{fl}$, AMG).

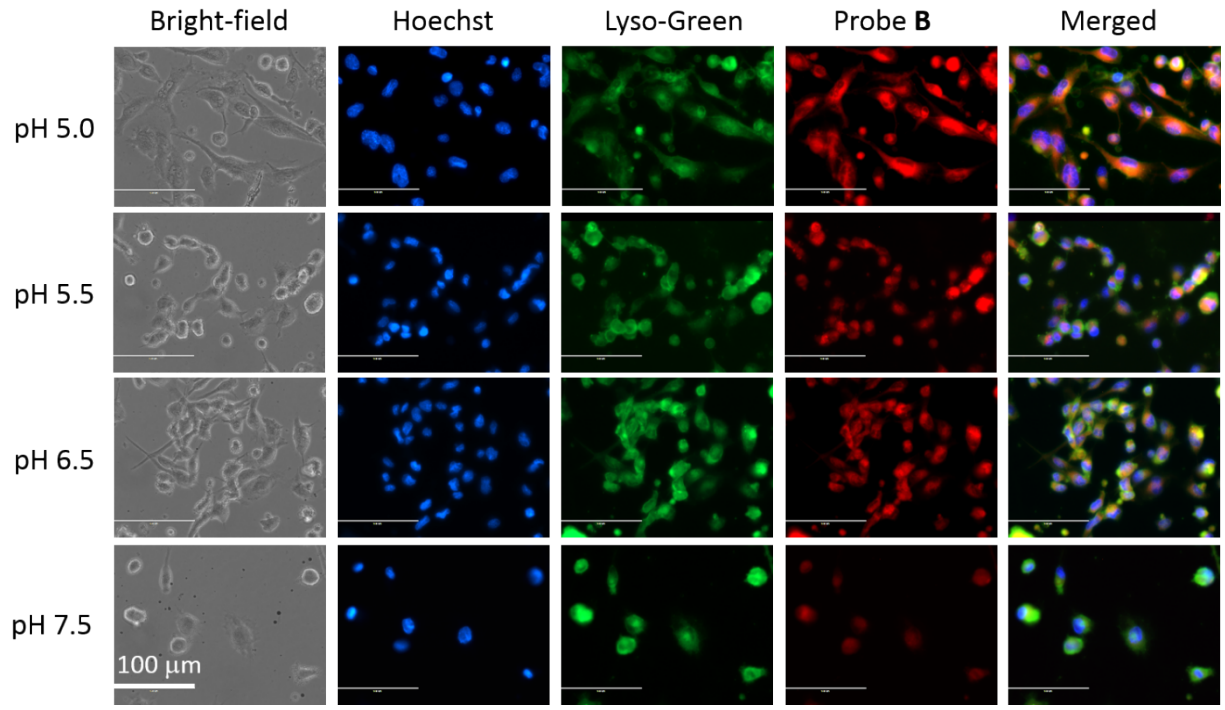
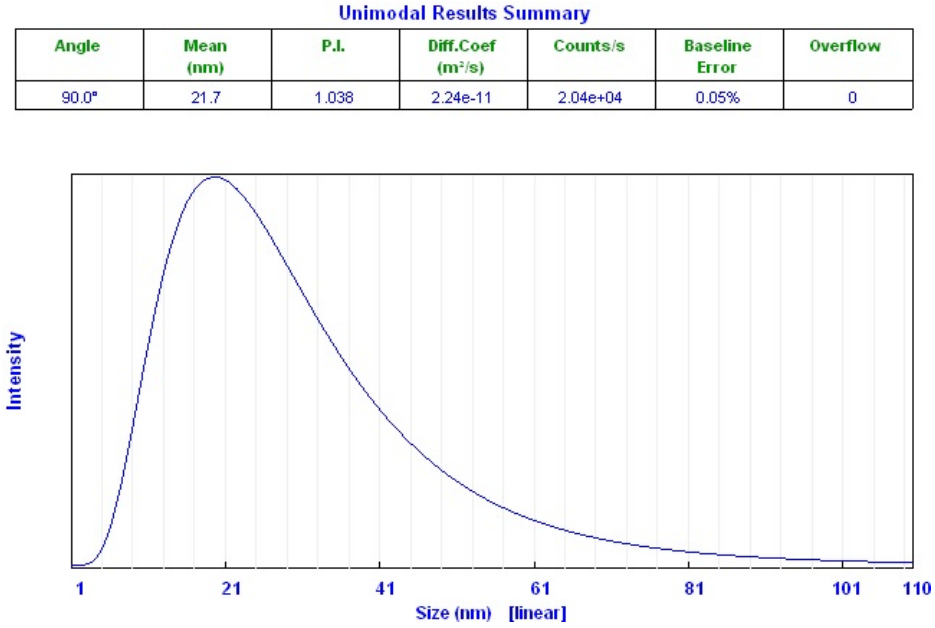


Figure S36. Fluorescence images of HUVEC-C cells incubated with 15 µM probe **B** at different pH values. 1 µM LysoSensor Green DND-189 and 1 µg/mL Hoechst 33342 were used as co-stains. Images were acquired at 40× magnification using the inverted fluorescence microscope (AMF-4306, $EVOS_{fl}$, AMG).



Unimodal Results Summary

Angle	Mean (nm)	P.I.	Diff.Coef (m^2/s)	Counts/s	Baseline Error	Overflow
90.0°	21.7	1.038	2.24e-11	2.04e+04	0.05%	0

Figure S37. Dynamic light scattering measurement result of aqueous solution containing 5 µM probe **A** at pH 2.6.

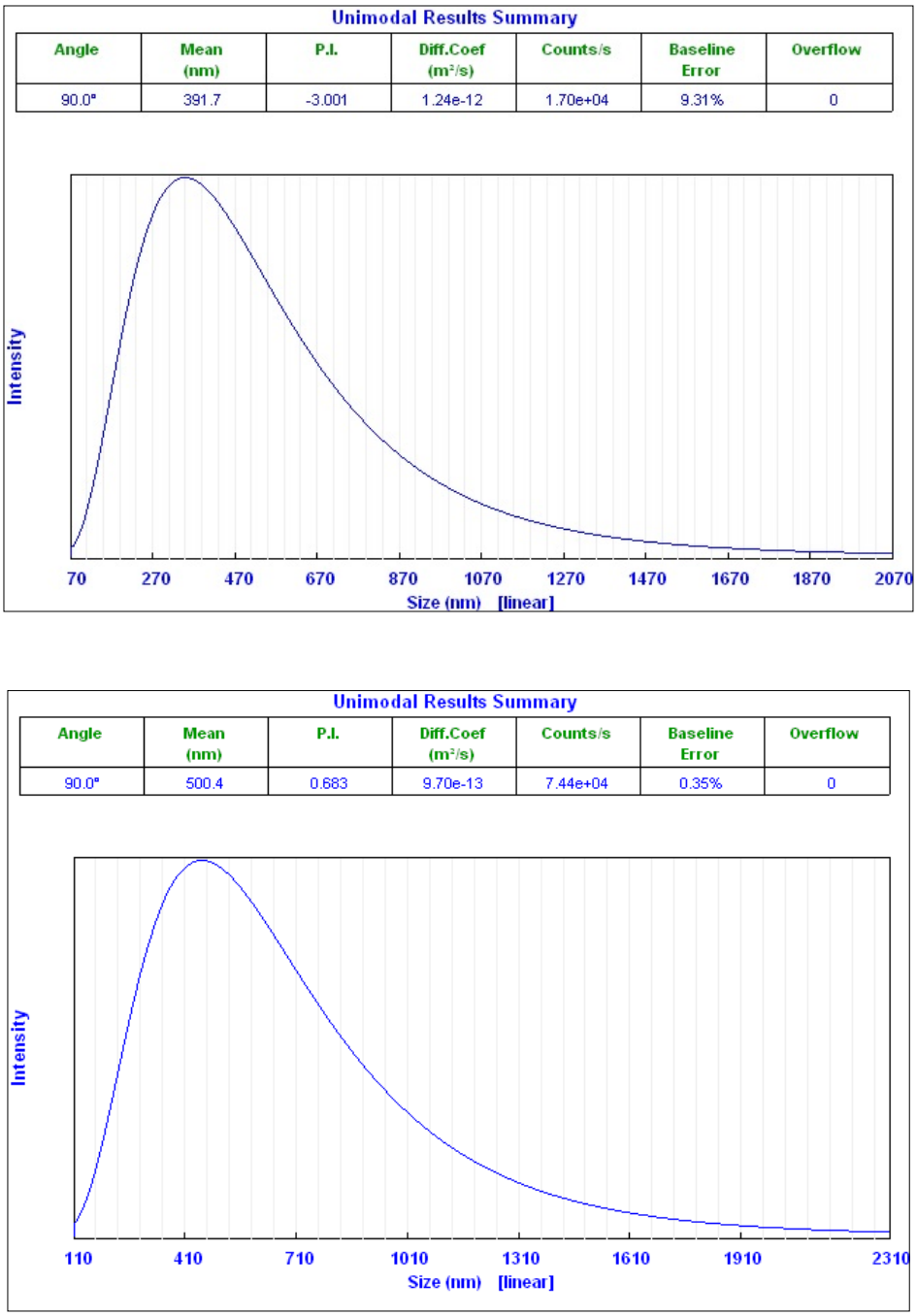


Figure S38. Dynamic light scattering measurement result of aqueous solution containing 5 μM probe **A** at pH 4.5 (top) and pH 7.4 (bottom).

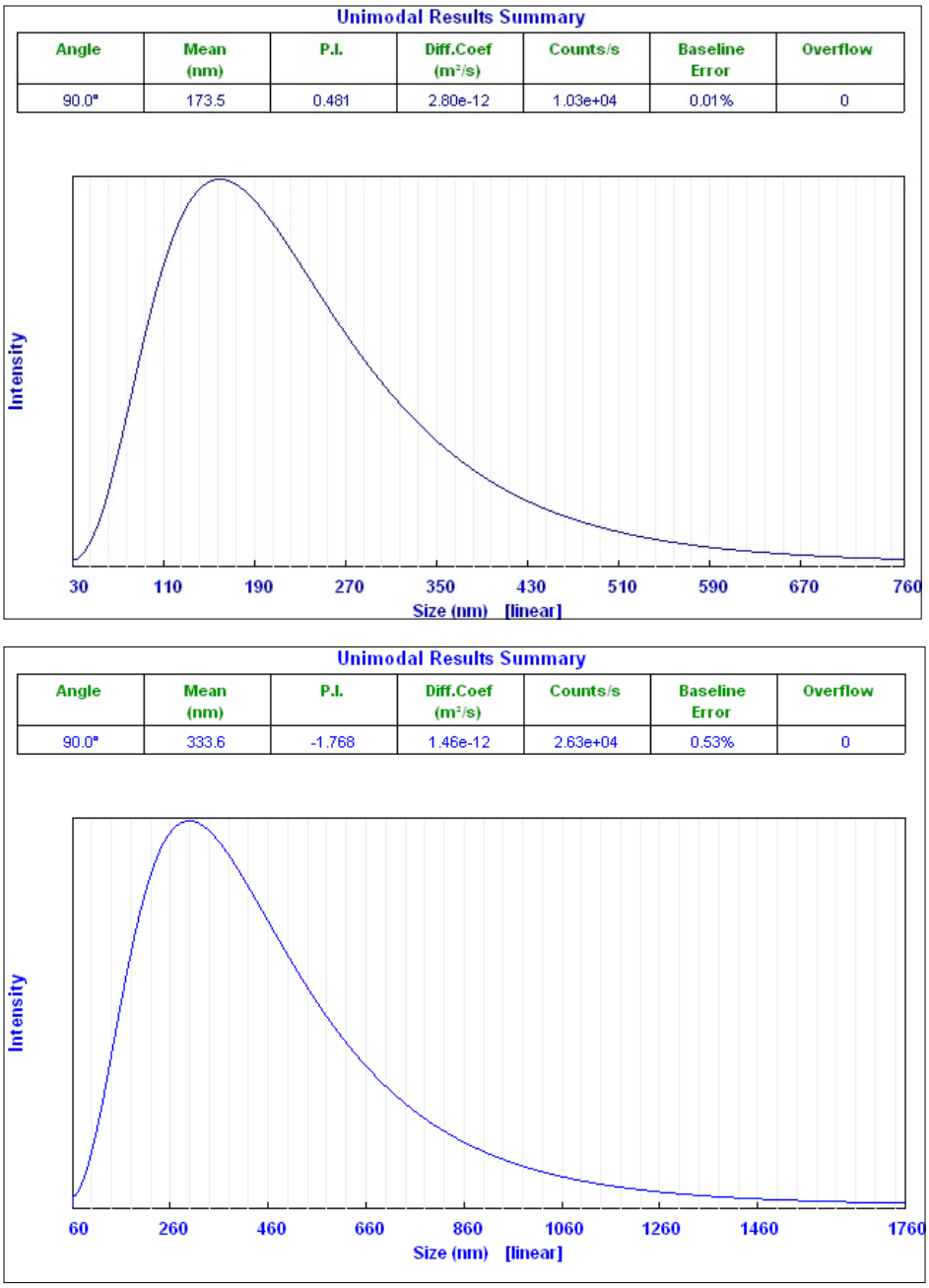


Figure S39. Dynamic light scattering measurement result of aqueous solution containing 5 μM probe **B** at pH 4.5 (top) and pH 7.4 (bottom).

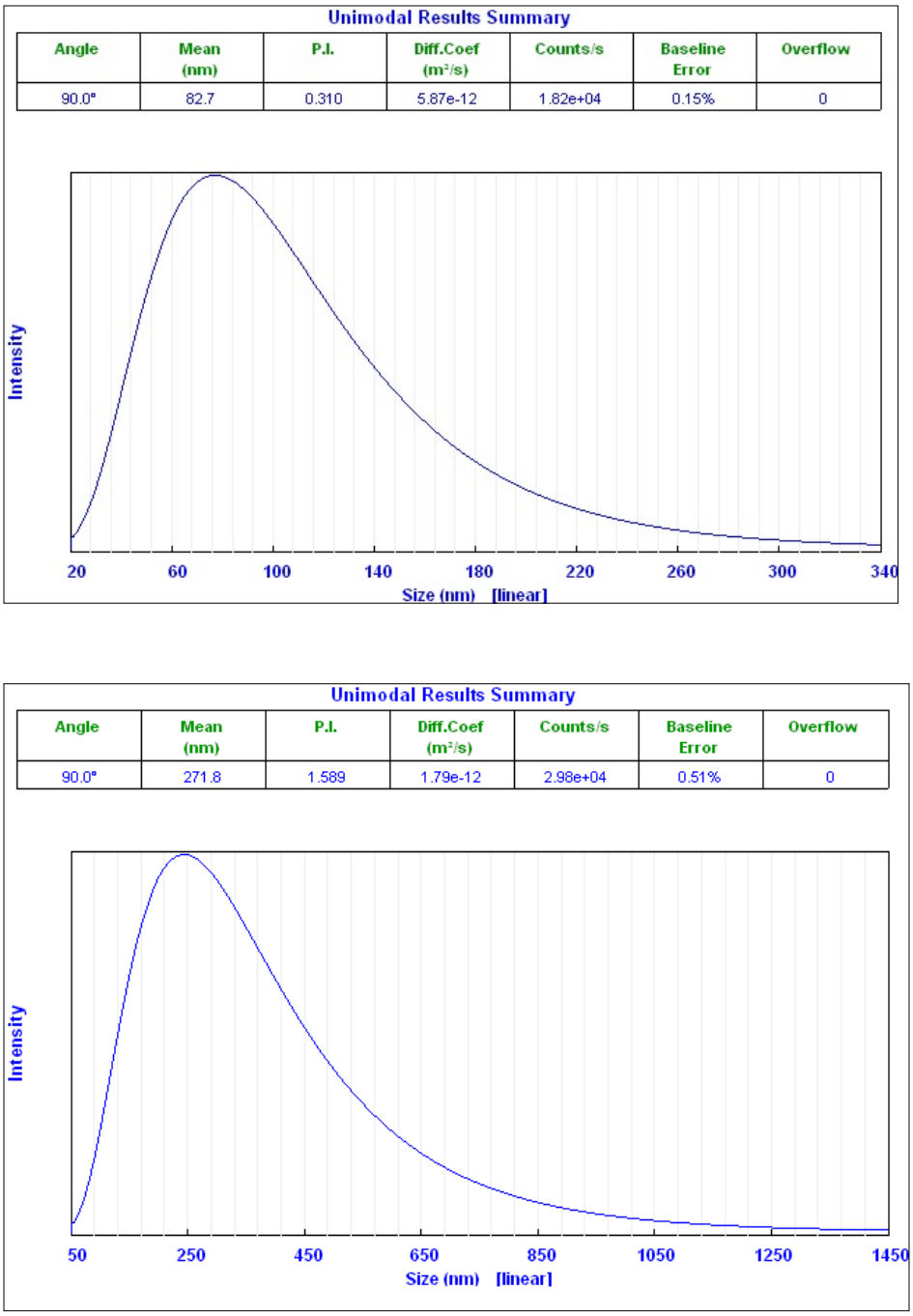


Figure S40. Dynamic light scattering measurement result of aqueous solution containing 5 μM probe **C** at pH 4.5 (top) and pH 7.4 (bottom).