Supporting Information

pH-Sensitive Fluorescent Dyes: Are They Really pH-Sensitive in Cells?

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Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA) and used as received. 35-mm glass bottom dishes and 8-well chambers were purchased from MatTek Corporation (Ashland, MA).

Instruments

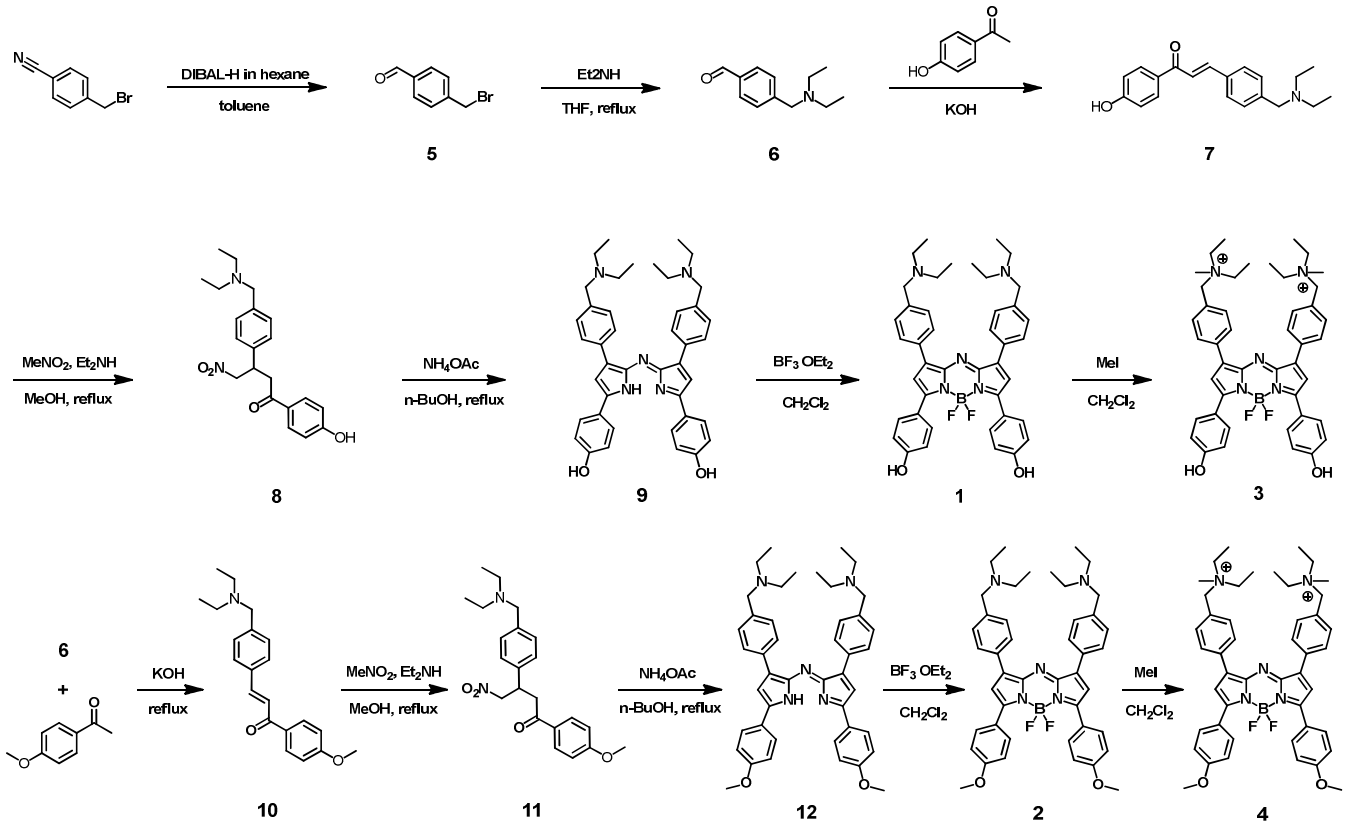
NMRs were taken with a Bruker (Billerica MA) 300 NMR spectrometer. Mass spectra were obtained with a Waters LC-MS system (Waters, Milford, MA) that included an Acquity UPLC system coupled to a Waters Q-Tof Premier high resolution mass spectrometer. HPLC was performed on a Agilent (Santa Clara, CA) 1200 HPLC system with a 1260 DAD VL detector. An Agilent Eclipse XDB-C18 column (5 μm, 4.6×150 mm) was used in analytical HPLC. A Beckman (Brea, CA) Ultrasphere^{TM} C18 column (5 μm, 10×250 mm) was used in preparative HPLC. Fluorescence was measured on an F-7000 spectrofluorometer (Hitachi, Japan) with a PMT voltage of 950V and slit width 5 nm. UV absorbance was measured on a Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Laser scanning confocal microscopy (LSCM) was performed on an Olympus Fluoview FV10i microscope (Olympus, Center Valley, PA). Images were processed using Olympus FV10-ASW 3.0a software. Differential Centrifugation was performed on an Eppendorf Centrifuge 5430R (Hauppauge, NY). Cells were lysed using a Cole-Parmer ultrasonic processor (Vernon Hills, IL).

Cell culture

Human melanoma MDA-MB-435 cells were (ATCC, Manassas, VA) were cultured in DMEM medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) with 1% penicillin-streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. MDA-MB-435 cells were plated 16 h before the start of the experiment in 8-well chamber slides at a density of 5×10^3 cell/cm^2 overnight.

Synthesis

Scheme S1. Synthesis of dyes **1-4**



4-Bromo-tolualdehyde (5): 6.00 g (30.6 mmol) of α-bromo-*p*-tolunitrile was dissolved in 60 mL of toluene and cooled to 0 °C. 40 mL (43.2 mmol) of 1.08 M DIBAL-H in hexane was added dropwise under N2. The solution was stirred for another hour at 0 °C. 80 mL of chloroform was then added followed by ca. 200 mL of 10% HCl, and the solution was stirred at room temperature for 1 h. The organic layer was separated, washed with distilled water, dried over anhydrous Na_2SO_4, and filtered. The solvent was almost completely removed from the filtrate under reduced pressure, and the residue was cooled, filtered, washed with cold hexane, and dried at 50 °C under vacuum to afford the product as white crystals (5.67 g, 95%). ^{1}H NMR (CDCl3) δ (ppm): 10.01 (s, 1H), 7.85 (d, *J* = 8.1 Hz, 2H), 7.56 (d, *J* = 8.2 Hz, 2H), 4.51 (s, 2H). ^{13}C NMR ($CDCl_3$) δ (ppm): 191.5, 144.3, 136.1, 130.2, 129.7, 32.0.

4-Diethylaminomethyl-benzaldehyde (6): To a stirred solution of **5** (3.85 g, 19 mmol) in THF (80 mL) was added diethylamine (5.92 mL, 57 mmol) and the resulting mixture was heated at reflux for 8 h. The solvent was removed under reduced pressure and the resulting oil was partitioned between Et_2O (200 mL) and water (200 mL). The organic layer was extracted 1M HCl (aq) (200 mL), the aqueous layer was neutralized with 2M NaOH (aq) and extracted with Et_2O (2x200 mL). The organic layer was washed with saturated NaCl (aq) (200 mL), dried over Na_2SO_4 and the solvent removed under reduced pressure to give colorless oil (3.51 g, 94%). ^{1}H NMR ($CDCl_3$) δ (ppm): 9.99 (s, 1H), 7.82 (d, *J* = 8.1 Hz, 2H), 7.53 (d, *J* = 8.2 Hz, 2H), 3.63 (s, 2H), 2.58 (q, *J* = 7.1 Hz, 4H), 1.07 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 192.0, 148.1, 135.5, 129.8, 129.4, 57.3, 47.0, 11.5.

3-(4-diethylaminomethyl-phenyl)-1-(4-hydroxyphenyl)-propenone (7): 6 (2 g, 11 mmol) and 1-(4-hydroxyphenyl)ethanone (1.69g, 12 mmol) were dissolved in EtOH (16 mL), and KOH (1 mmol) in H_2O (4

mL) was added. The solution was refluxed for 48 h and the crude was purified by flash column chromatography (40% hexanes in EtOAc to EtOAc). The product is red-brown oil (2.73 g, 79%). ^{1}H NMR ($CDCl_3$) δ (ppm): 8.05 (d, J = 8.9 Hz, 2H), 7.80 (d, J = 15.6, 1H), 7.55 (d, *J* = 8.1 Hz, 2H), 7.53 (d, *J* = 15.6 Hz, 1H), 7.37 (d, J = 8.1 Hz, 2H), 6.99 (d, *J* = 8.9 Hz, 2H), 4.68 (s, 1H), 3.68 (s, 2H), 2.60 (q, *J* = 7.1 Hz, 4H), 1.08 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 188.9, 162.1, 143.9, 143.5, 133.7, 131.3, 130.8, 129.4, 128.3, 121.2, 115.9, 56.6, 46.4, 11.1. HRMS Calcd for $C_{19}H_{22}NO_2$ $[M+H]^+$: 296.1645. Found: 296.1026.

3-(4-diethylaminomethyl-phenyl)-1-(4-hydroxyphenyl)-4-nitrobutanone (8): A solution of **7** (1.56 g, 5 mmol) in MeOH (50 mL) was treated with diethylamine (5.2 mL, 50 mmol) and nitromethane (2.7 mL, 50 mmol) and heated under reflux for 48 h. The solution was cooled, partitioned between CH_3Cl (100 mL) and water (100 mL) and extracted with CH_3Cl (100 mL). The combined organics were extracted with 1M HCl (200 mL), and the aqueous layer was then neutralized with 2M NaOH and extracted with CH_3Cl (2X100 mL). The combined organics were washed with water (100 mL) and brine (100 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the resulting oil was purified by column chromatography (0.465 g, 25%). ^{1}H NMR ($CDCl_3$) *δ* (ppm): 7.90 (d, *J* = 8.8 Hz, 2H), 7.39 (d, *J* = 8.2 Hz, 2H), 7.25 (d, *J* = 8.2 Hz, 2H), 6.84 (d, *J* = 8.8 Hz, 2H), 4.82 (dd, *J* = 12.4, 6.4 Hz, 1H), 4.67 (dd, *J* = 12.4, 8.1 Hz, 1H), 4.14-4.24 (m, 1H), 3.76 (s, 2H), 3.30-3.45 (m, 2H), 2.64 (q, *J* = 7.1 Hz, 4H), 1.12 (t, *J* = 7.1 Hz, 6H). 13C NMR ($CDCl_3$) *ä*: 196.6, 170.0, 167.8, 143.7, 141.6, 134.4, 133.5, 131.3, 117.9, 83.7, 61.1, 56.6, 45.3, 46.4, 11.1. HRMS Calcd for $C_{20}H_{25}N_2O_4$ $[M+H]^+$: 357.1809. Found: 357.2613.

(9): A 100 mL round-bottomed flask was charged with **8** (0.43 g, 1.16 mmol), ammonium acetate (3.0 g, 40 mol), and n-butanol (40 mL) and heated under reflux for 48 h. The reaction was cooled to room temperature, the solvent concentrated to 10 mL and filtered, and the isolated solid washed with ethanol (2X 10 mL) to yield the product **9** as a blue-black solid (0.212 g, 56%). ^{1}H NMR (CD_3OD) *δ* (ppm): 8.10 (d, *J* = 8.1 Hz, 4H), 7.90 (d, *J* = 8.7 Hz, 4H), 7.55 (d, *J* = 8.4 Hz, 4H), 7.50 (s, 2H), 7.00 (d, *J* = 8.4 Hz, 4H), 4.39 (s, 4H), 3.25 (q, *J* = 5.7 Hz, 8H), 1.38 (t, *J* = 7.2 Hz, 12H). ^{13}C NMR (CD_3OD) *δ* (ppm): 161.8, 154.2, 149.4, 142.4, 134.2, 129.2, 128.4, 128.3, 127.8, 125.5, 114.9, 114.7, 55.9, 46.9, 10.8. HRMS Calcd for $C_{42}H_{46}N_5O_2$ $[M+H]^+$: 652.3652. Found: 652.3169.

(1): 9 (150 mg, 0.2 mmol) was dissolved in dry CH_2Cl_2 (50 mL), treated with diisopropylethylamine (0.4 mL, 2.4 mmol) and boron trifluoride diethyl etherate (0.4 mL, 3.6 mmol), and stirred at room temperature under N_2 for 24 h. The mixture was washed with water (2X 50 mL), and organic layer was dried over Na_2SO_4 and evaporated to dryness. Purification by flash column chromatography eluting with 5% MeOH in CH_2Cl_2 gave the product **1** as a metallic brown solid (56 mg, 35%). ^{1}H NMR (CD_3OD) *δ* (ppm): 8.05 (d, *J* = 8.1 Hz, 4H), 7.78 (d, *J* = 8.7 Hz, 4H), 7.46 (d, *J* = 8.4 Hz, 4H), 6.90 (d, *J* = 8.4 Hz, 4H), 3.68 (s, 4H), 2.72 (q, *J* = 5.7 Hz, 8H), 1.21 (t, *J* = 7.2 Hz, 12H). ^{13}C NMR (CD_3OD) *δ* (ppm): 159.4, 154.2, 145.6, 144.1, 141.8, 131.7, 130.9, 130.8, 129.6, 128.6, 118.7, 63.4, 48.9, 12.9. HRMS Calcd for $C_{42}H_{45}BF_2N_5O_2$ $[M+H]^+$: 700.3634. Found: 700.2681.

(3): 1 (10 mg, 0.014 mmol) was dissolved in dry CH_2Cl_2 (2 mL), treated with diisopropylethylamine (10 µL, 0.06 mmol) and methyl iodide (10 µL, 0.16 mmol), and stirred at room temperature under N2 for 24 h. The solvent was removed under reduced pressure and the residue was purified by preparative HPLC. The product is a metallic brown solid (5 mg, 48%). HRMS Calcd for $C_{45}H_{51}BF_2N_5O_4$ $[M+HCOO]^+$: 774.3997. Found: 774.4839.

3-(4-Diethylaminomethyl-phenyl)-1-(4-methoxyphenyl)-propenone (10): Same procedure as **7.** ^{1}H NMR ($CDCl_3$) δ (ppm): 8.02 (d, J = 8.9 Hz, 2H), 7.76 (d, J = 15.6, 1H), 7.59 (d, *J* = 8.1 Hz, 2H), 7.54 (d, *J* = 15.6 Hz, 1H), 7.37 (d, J = 8.1 Hz, 2H), 6.96 (d, *J* = 8.9 Hz, 2H), 3.87 (s, 3H), 3.58 (s, 2H), 2.54 (q, *J* = 7.1 Hz, 4H), 1.04 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ (ppm): 188.8, 163.4, 143.9, 143.0, 133.6, 131.2, 130.8, 129.4, 128.3, 121.3, 113.8, 57.4, 55.5, 46.9, 11.8. HRMS Calcd for $C_{20}H_{24}NO_2$ $[M+H]^+$: 310.1802. Found: 310.1237.

3-(4-Diethylaminomethyl-phenyl)-1-(4-methoxyphenyl)-4-nitrobutanone (11): Same procedure as **8.** ^{1}H NMR ($CDCl_3$) *δ* (ppm): 7.91 (d, *J* = 8.8 Hz, 2H), 7.28 (d, *J* = 8.2 Hz, 2H), 7.22 (d, *J* = 8.2 Hz, 2H), 6.89 (d, *J* = 8.8 Hz, 2H), 4.84 (dd, *J* = 12.4, 6.4 Hz, 1H), 4.68 (dd, *J* = 12.4, 8.1 Hz, 1H), 4.17-4.24 (m, 1H), 3.86 (s, 3H), 3.53 (s, 2H), 3.30-3.45 (m, 2H), 2.54 (q, *J* = 7.1 Hz, 4H), 1.03 (t, *J* = 7.1 Hz, 6H). 13C NMR ($CDCl_3$) *ä*: 195.5, 163.8, 139.4, 137.7, 130.4, 129.5, 127.3, 113.9, 79.7, 57.0, 55.5, 41.2, 39.2, 11.6. HRMS Calcd for $C_{21}H_{27}N_2O_4$ $[M+H]^+$: 371.1966. Found: 371.1169.

(12): Same procedure as **9**. ^{1}H NMR (CD_3OD) *δ* (ppm): 8.10 (d, *J* = 8.1 Hz, 4H), 7.90 (d, *J* = 8.7 Hz, 4H), 7.55 (d, *J* = 8.4 Hz, 4H), 7.50 (s, 2H), 7.00 (d, *J* = 8.4 Hz, 4H), 4.39 (s, 4H), 3.83 (s, 6H), 3.25 (q, *J* = 5.7 Hz, 8H), 1.38 (t, *J* = 7.2 Hz, 12H). ^{13}C NMR (CD_3OD) *δ* (ppm): 161.8, 154.2, 149.4, 142.4, 134.2, 129.2, 128.4, 128.3, 127.8, 125.5, 114.9, 114.7, 57.9, 55.9, 46.9, 10.8. HRMS Calcd for $C_{44}H_{50}N_5O_2$ $[M+H]^+$: 680.3965. Found: 680.4235.

(2): Same procedure as **1**. The product is a metallic brown solid (35 mg, 42%). ^{1}H NMR (CD_3OD) *δ* (ppm): 8.05 (d, *J* = 8.1 Hz, 4H), 7.78 (d, *J* = 8.7 Hz, 4H), 7.46 (d, *J* = 8.4 Hz, 4H), 6.90 (d, *J* = 8.4 Hz, 4H), 3.83 (s, 6H), 3.68 (s, 4H), 2.72 (q, *J* = 5.7 Hz, 8H), 1.21 (t, *J* = 7.2 Hz, 12H). ^{13}C NMR (CD_3OD) *δ* (ppm): 159.4, 154.2, 145.6, 144.1, 141.8, 131.7, 130.9, 130.8, 129.6, 128.6, 118.7, 63.4, 55.8, 48.9, 12.9. HRMS Calcd for $C_{44}H_{49}BF_2N_5O_2$ $[M+H]^+$: 728.3947. Found: 728.4579.

(4): Same procedure as **3**. The product is a metallic brown solid (8 mg, 80%). HRMS Calcd for $C_{47}H_{55}BF_2N_5O_4$ $[M+HCOO]^+$: 802.4310. Found: 802.4995.

HPLC methods:
Eluents: 0.1%TFA in H2O (A) and 0.1%TFA in acetonitrile (B).
Analytical: 0-30min 20-80% B.
Preparative: 0-5min 5% B; 5-35min 5-65% B.
Under these conditions, the compounds **1-3** should be all in protonated forms. Elution at higher pH with formic acid or ammonium acetate was either not successful or resulted in poor resolution.

Table S1. lecithin-cholesterol liposome sizes in PBS at different pH

pH	Avg	SD	PI
6.36	130.9	30.5	0.309
6.85	125.6	32.3	0.343
7.38	129.6	36.9	0.338
7.85	126.9	31.9	0.300
8.18	130.6	33.0	0.331
8.65	122.0	31.2	0.299
9.46	130.1	32.7	0.362
9.79	120.7	28.8	0.257
10.27	119.7	31.8	0.257

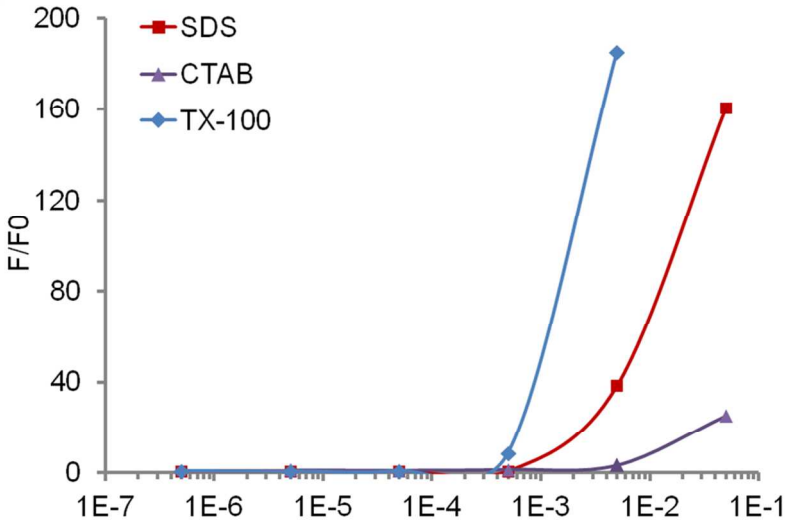


Figure S1. Fold of **1** fluorescence (Ex695/Em740) increase (F/F0) in different concentrations of surfactants (SDS, CTAB, TX-100) in pH7.40 PBS

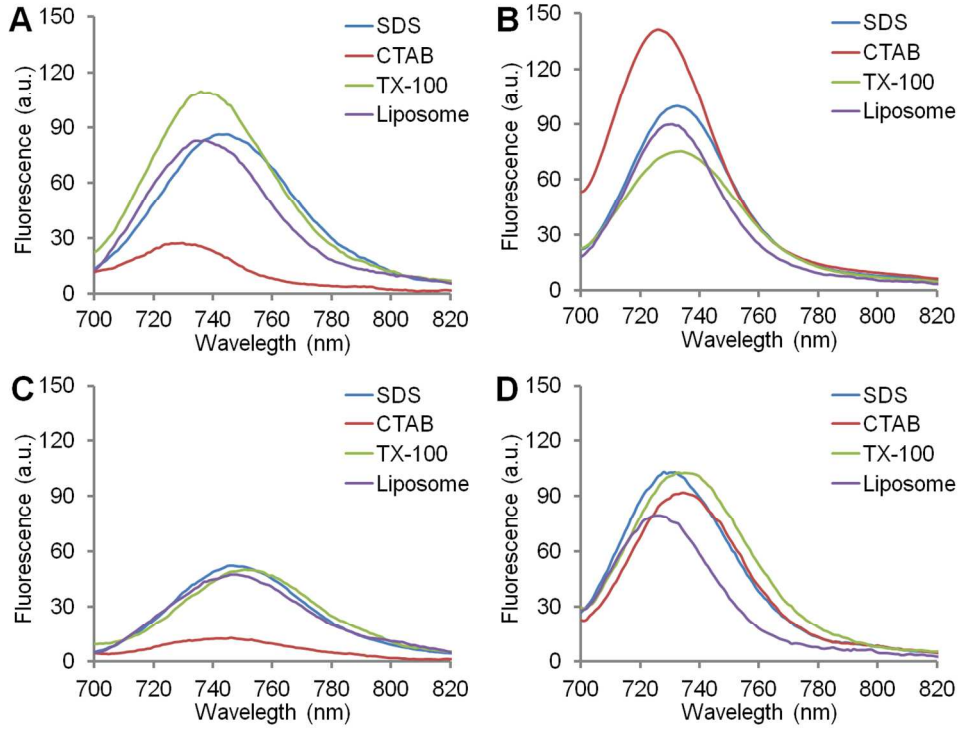


Figure S2. Fluorescence spectra (Ex = 680 nm) of dyes 1-4 (A: 1, B: 2, C: 3, D: 4) in micelle and liposome solutions (PBS pH7.40). The fluorescence is normalized that FL = 100 under acidic conditions for each dye respectively.

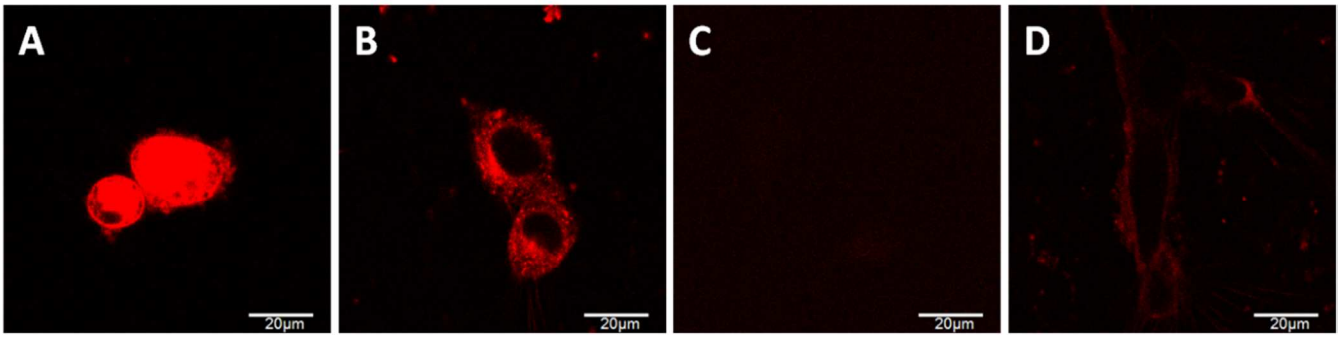


Figure S3. Confocal images of MDA-MB-435 cells after incubated with **1-4** (A, **1**; B, **2**; C, **3**; D, **4**) for 30 min in the absence of FBS. Cells were washed and incubated in serum-free media for 1 h before the dyes were loaded.

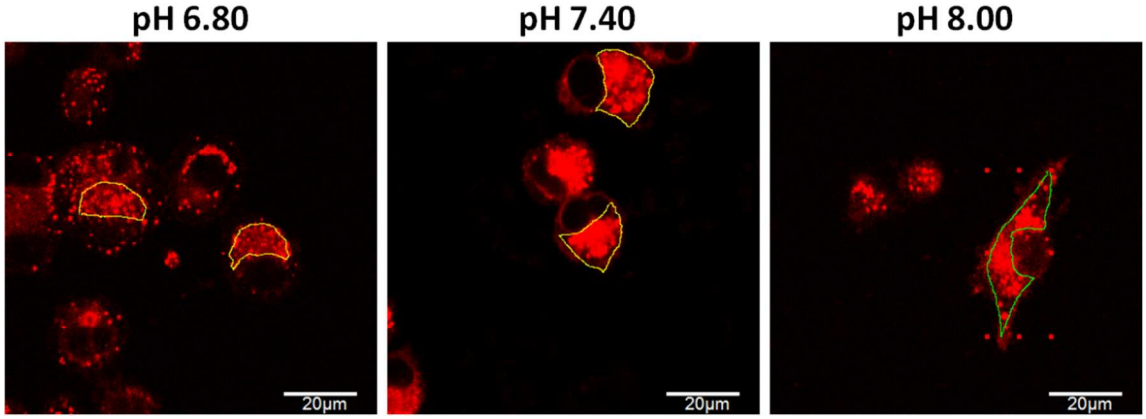


Figure S4. The ROIs for calculating the average fluorescence intensity at different intracellular pH.