Supporting Information for

Amino-Coumarin Based Fluorescence Ratiometric Sensors for Acidic pH and Their Application for Living Cells Imaging

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Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents 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 recorded on a LXQ Spectrometer (Thermo Scientific) operating on ESI. ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz and 100 MHz respectively. Elemental (C, H, N) analysis were carried out using Flash EA 1112 analyzer. The Crystallographic data were collected on a Saturn 724^{+} CCD X-ray diffractometer by using graphite monochromated Mo *Kα* (λ = 0.71070 Å). Electronic absorption spectra were obtained on a SHIMADZU UV-2450 spectrometer. Fluorescence spectra were measured on a Photon Technology International (PTI) Quantamaster fluorometer with 3 nm excitation and emission slit widths. Cells imaging were performed with an inverted fluorescence microscope (Carl Zeiss, Axio Observer A1). All pH measurements were performed with a pH-3c digital pH-meter (Shanghai ShengCi Device Works, Shanghai, China) with a combined glass-calomel electrode. 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.

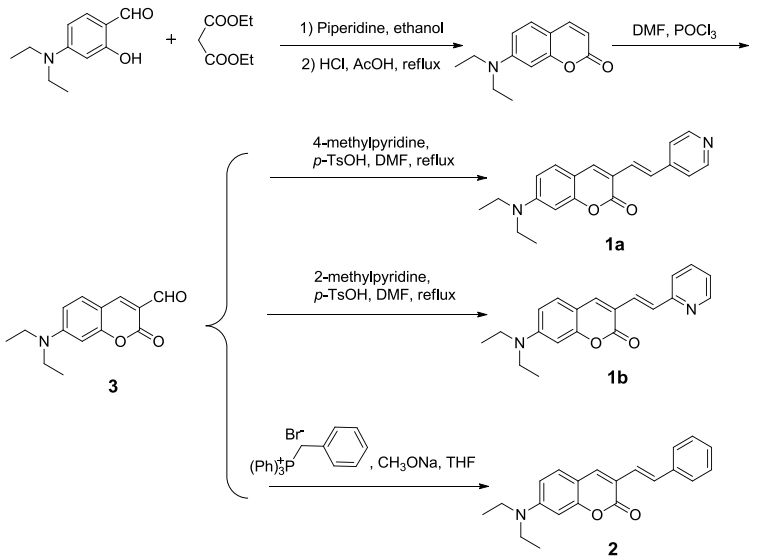

Figure S1. Synthesis of sensor **1a**, **1b** and the compound **2**.

Synthesis of 7-diethylaminocoumarin-3-aldehyde (3):

7-diethylaminocoumarin-3-aldehyde **3** was synthesized according to a reported procedure.[1] mp: 152-154 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.13 (s, 1H), 8.26 (s, 1H), 7.41 (d, J = 8.8 Hz, 1H), 6.64 (dd, J = 2.4 Hz, 8.8 Hz, 1H), 6.49 (d, J = 2.4 Hz, 1H), 3.48 (q, J = 7.2 Hz, 4H), 1.26 (t, J = 7.2 Hz, 6H); MS (*m/z*): 246.1 $[M+H]^{+}$.

Synthesis of compound 1a:

Under nitrogen atmosphere, 7-diethylaminocoumarin-3-aldehyde **3** (1g, 4.08 mmol) and 4-methylpyridine (458 mg, 4.92 mmol) were dissolved in dry DMF (30 mL), and then *p*-toluenesulfonic acid (1.94 g, 11.2 mmol) was added. After refluxed for 4 hours, the reaction mixture was poured into 100 ml of ice cold water and further stirred for 10 minutes. The precipitate was collected by filtration. The crude product was purified by chromatography on silica gel (dichloromethane: petroleum ether: ethanol = 20:40:1 for the first time; ethyl acetate : petroleum ether = 1:2 for the second time) to give the red solid compound **1a** (430 mg, yield 32.9%). mp: 163-164 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.54 (d, J = 6.4 Hz, 2H), 7.71 (s, 1H), 7.44 (d, J = 16.4 Hz, 1H), 7.35 (d, J = 6.4 Hz, 2H), 7.30 (d, J = 8.8 Hz, 1H),7.22 (d, J = 16.4 Hz, 1H), 6.60 (dd, J = 2.4 Hz, 8.8 Hz, 1H), 6.50 (d, J = 2.4 Hz, 1H), 3.43 (q, J = 7.2 Hz, 4H), 1.23 (t, J = 7.2 Hz, 6H); MS (*m/z*):321.4 $[M+H]^{+}$.

Synthesis of compound 1b:

The synthesis procedure is similar as compound **1a**, and the crude product was purified by chromatography on silica gel (ethyl acetate : petroleum ether = 1:3 for the first time; dichloromethane: petroleum ether = 1:2 for the second time) to give the orange solid compound **1b** (360 mg, yield 27.6%). mp: 156-157 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.58 (d, J = 4.8 Hz, 1H), 7.68 (d, J = 16 Hz, 2H), 7.64 (m, 1H), 7.59 (d, J = 16 Hz, 1H), 7.37 (d, J = 7.2 Hz, 1H),7.29 (d, J = 8.8 Hz, 1H), 7.11(m, 1H), 6.58 (dd, J = 2.4 Hz, 8.8 Hz, 1H), 6.49 (d, J = 2.4 Hz, 1H), 3.42 (q, J = 7.2 Hz, 4H),

1.21 (t, *J* = 7.2 Hz, 6H); MS (*m/z*):321.4 $[M+H]^+$.

Synthesis of compound 2:
Compound **3** (100 mg, 0.40 mM) and benzyltriphenylphosphonium bromide (212 mg, 0.49 mM) were dissolved in dry THF (10 mL), and then CH_3ONa (30 mg, 0.55 mM) was added. After the mixture was stirred at room temperature overnight, the reaction mixture was neutralized with hydrochloric acid, and 20 mL water were added, the mixture was further stirred at room temperature for 1 hour, extracted with CH_2Cl_2, the organic layer was collected and dried over Na_2SO_4. After concentration under reduced pressure, the crude product was purified by chromatography on a silica gel column (ethyl acetate : petroleum ether : ethanol = 4:4:1, v/v), affording compound **2** as a yellow solid (92.0 mg, 72%). mp: 188-191 ^oC; ^1H NMR (400 MHz, $CDCl_3$) δ = 7.67 (s, 1H), 7.51 (d, *J* = 7.2Hz, 2H), 7.45 (d, *J* = 16.4Hz, 1H), 7.34 (t, *J* = 7.2Hz, 2H), 7.28 (d, *J* = 8.8 Hz, 1H),7.24 (t, *J* = 7.2 Hz, 1H), 7.09 (d, *J* = 16.4Hz, 1H), 6.58 (dd, *J* = 2.4 Hz, 8.8 Hz, 1H), 6.50 (d, *J* = 2.4 Hz, 1H), 3.41 (q, *J* = 7.2 Hz, 4H), 1.21 (t, *J* = 7.2 Hz, 6H); MS (*m/z*):320.4 $[M+H]^+$.

Preparation of the test solution: The stock solution of sensor **1a** and **1b** (250 μM) were prepared in ethanol. The test solution of the sensors (5 μM) in 0.1 M potassium phosphate buffer (containing 40% ethanol as a co-solvent) was prepared by placing 0.1 mL of the sensor stock solution, 1.9 mL ethanol, and 3 mL of 0.167 M sodium phosphate buffer with various pH value. The resulting solution was shaken well and incubated for 20 min at room temperature before recording the spectra.

Calculation of pK_a Values: the pK_a values of **1a** and **1b** were calculated by regression analysis of the fluorescence data to fit the following equation: [2]

$$pH = pK_a + c[\log(R-R_{min})/(R_{max}-R)] + \log(I_a / I_b)$$

where *R* is the ratio of emission intensity at two wavelengths, R_{max} and R_{min} are the corresponding maximum and minimum respectively, and c is the slope (positive for the basic forms of the dyes and negative for the acidic forms). I_a / I_b is the ratio of the absorption intensity in acid to the absorption intensity in base at the wavelength chosen for the denominator of *R*.

Determination of fluorescence quantum yield: Fluorescence quantum yield was determined using the solutions of Quinine Sulfate (Φ_F = 0.546 in 1N H_2SO_4[3]) as a standard. The quantum yield was calculated using the following equation: [4-6]

$$\Phi_{F(X)} = \Phi_{F(S)} (A_S F_X / A_X F_S) (n_X / n_S)^2$$

Where Φ_F is the fluorescence quantum yield, *A* is the absorbance at the excitation wavelength, *F* is the area under the corrected emission curve, and *n* is the refractive index of the solvents used. Subscripts S and X refer to the standard and to the

unknown, respectively.

Cell culture and fluorescence imaging: Pancreatic cancer cells were seeded in a 24-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum for 24 h. Before the experiments, the cells were washed with PBS buffer, and then the cells were incubated with sensor **1a** (2 μM) for 20 min at 37^{o}C. After that, the dye loaded cells were washed in PBS medium at pH 7.0 or 4.5 with the addition of nigericin (5 μg/mL). Then, the fluorescence images were acquired with a fluorescent microscope (Carl Zeiss, Axio Observer A1). Excitation wavelength of laser was 475 nm, and emissions were centered at 530-550 nm and 575-640 nm.

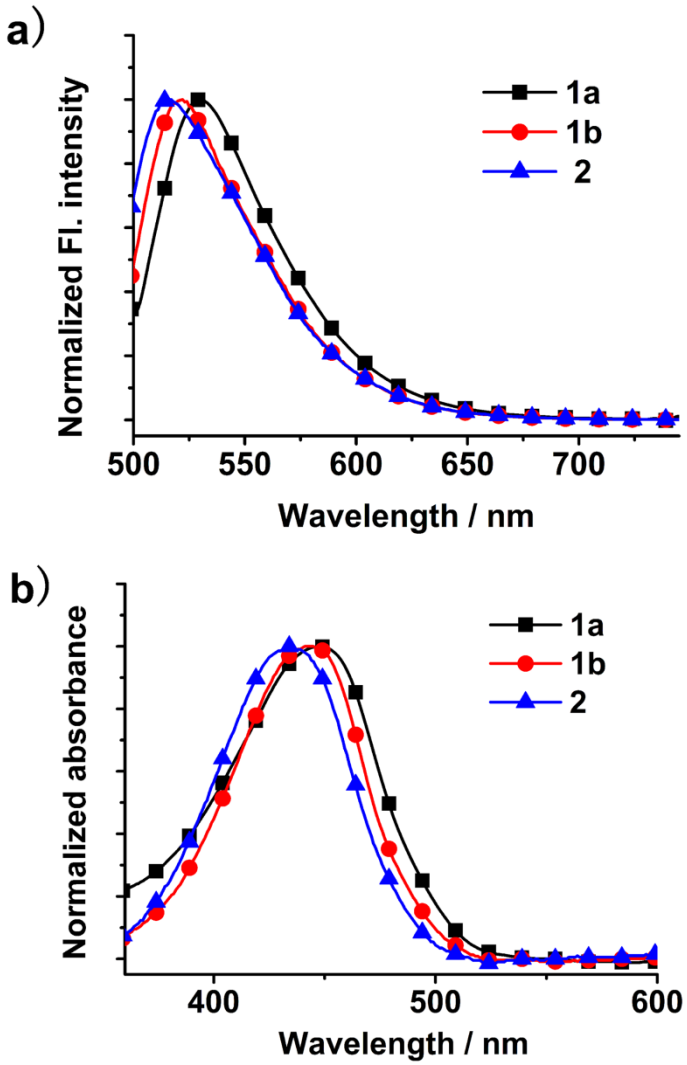

Figure S2. The normalized fluorescence emission spectra (a) and absorption spectra (b) of sensor **1a**, **1b** and compound **2** in 0.1 M potassium phosphate buffer (pH 7.0, containing 40% ethanol as co-solvent) at room temperature.

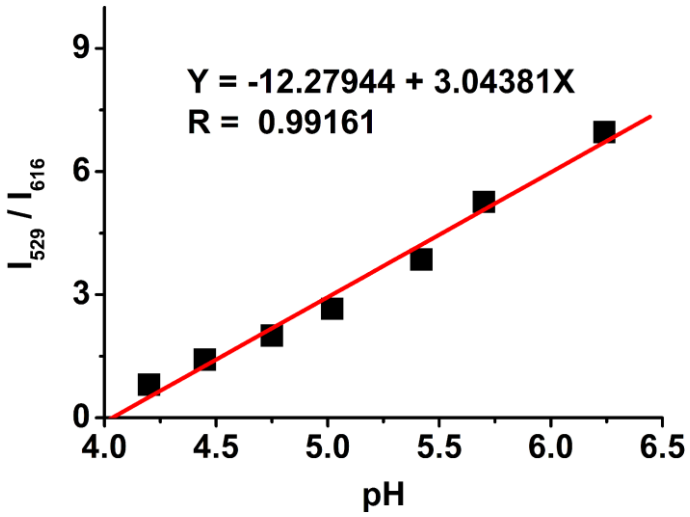

Figure S3. The linear relationship between the ratiometric response (I_{529} /I_{616}) of **1a** and the pH value (4.0-6.5), excitation was performed at 495 nm.

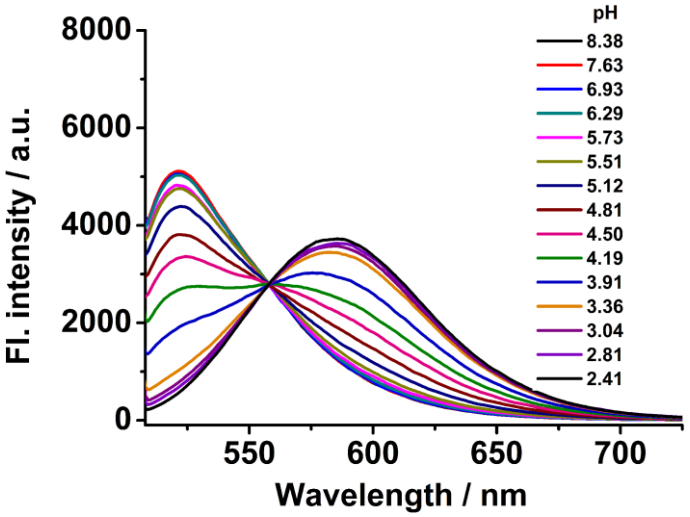

Figure S4. Changes in fluorescence emission spectra of **1b** (5 μM) in 0.1 M potassium phosphate buffer (containing 40% ethanol as co-solvent) with various pH values. Spectra were obtained with excitation at 505 nm.

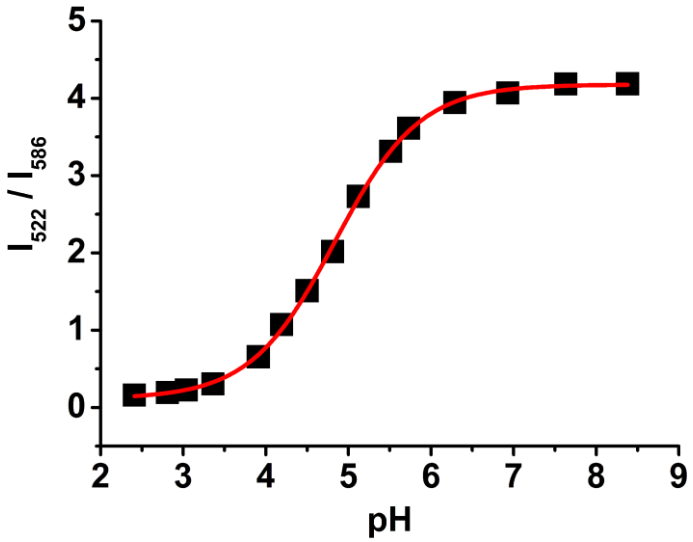

Figure S5. The ratiometric response (I_{522}/ I_{586}) of **1b** (5 μM) to various pH values (from 2.41 to 8.38), excitation was performed at 505 nm.

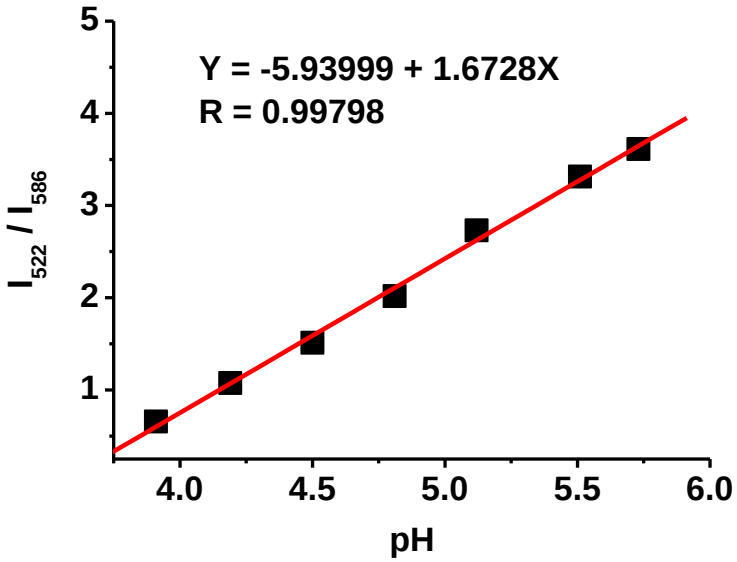

Figure S6. The linear relationship between the ratiometric response (I_{522} /I_{586}) of **1b** and the pH value (4.0-6.5), excitation was performed at 505 nm.

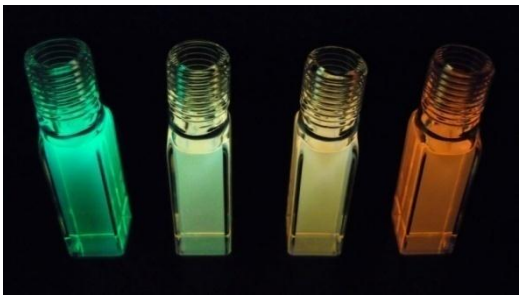

Figure S7. Visual fluorescence color changes of sensor **1b** in the solution with various pH values (from left to right: 7.0, 5.2, 3.9, 2.8), the photo was taken under illumination of a handheld UV lamp.

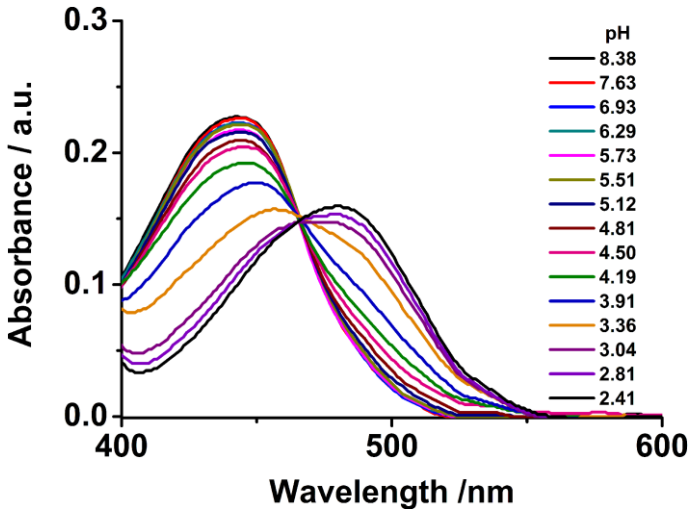

Figure S8. Changes in absorption spectra of **1b** (5 μM) in 0.1 M potassium phosphate buffer (containing 40% ethanol as co-solvent) with various pH values.

Figure S9. Visual color changes of sensor **1b** in the solution with various pH values (from left to right: 7.0, 5.2, 3.9, 2.8).

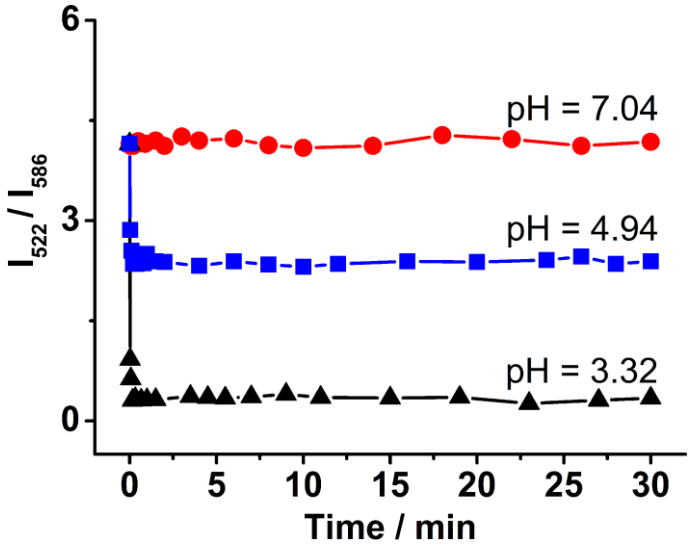

Figure S10. Reaction-time profile of sensor **1b** (5 μM) in the presence of buffer solutions at different pH values. Kinetic studies were performed at room temperature. The excitation wavelength was 505 nm, and the emission ratio changes (I_{522} / I_{586}) were continuously monitored at time intervals.

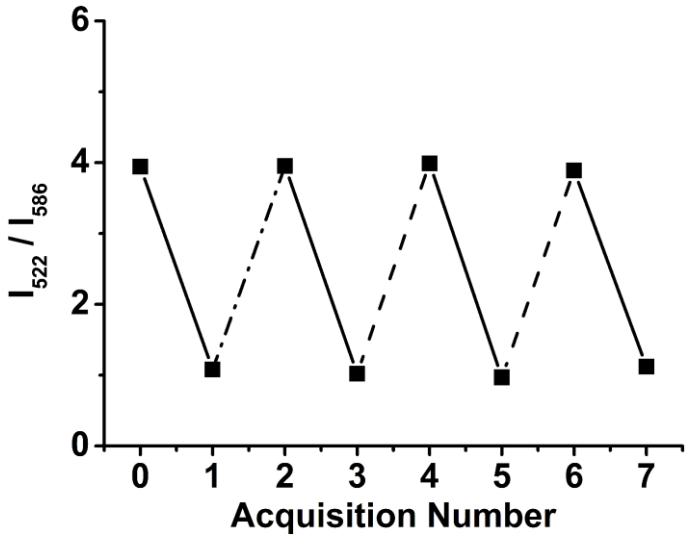

Figure S11. Changes in the emission ratio (I_{522} / I_{586}) of sensor **1b** (5 μM) in 0.1 M potassium phosphate buffer (containing 40% ethanol as co-solvent). The pH value was switched back and forth between 6.24 and 4.20 using concentrated hydrochloric acid and aqueous sodium hydroxide (10 M). The solid and dashed lines represent addition of HCl and NaOH, respectively.

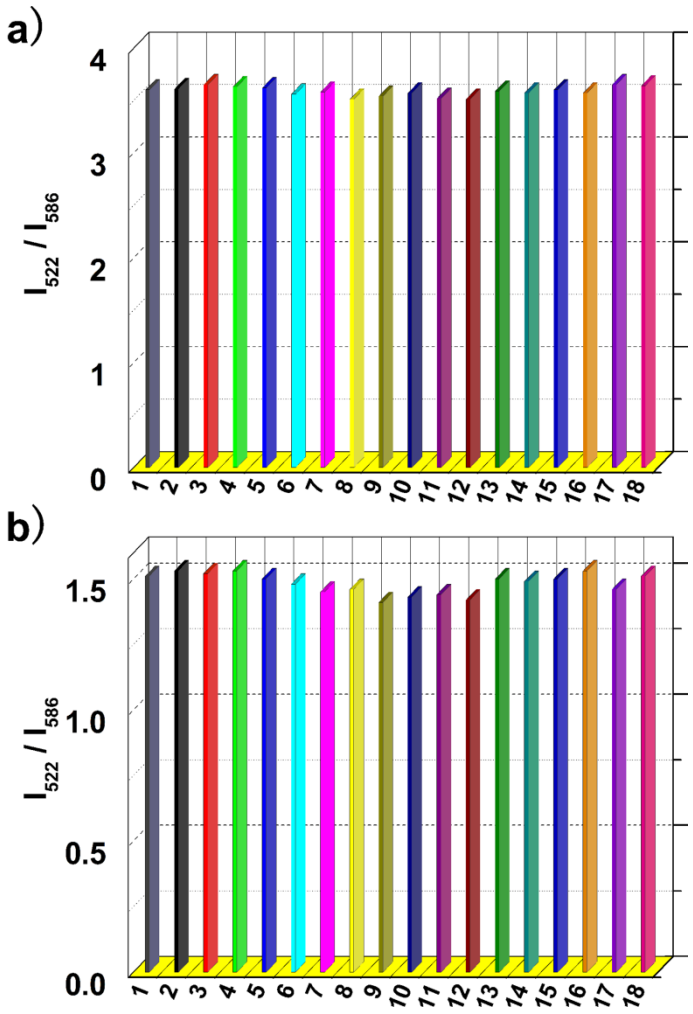

Figure S12. Fluorescence emission ratio (I_{529}/ I_{616}) of sensor **1b** (5 μM) in the presence of various species in 0.1 M potassium phosphate buffer (containing 40% ethanol as co-solvent) at pH 5.70 (a) and 4.45 (b). 1: blank, 2: Li^+ (5 mM), 3: K^+ (5 mM), 4: Ca^{2+} (5 mM), 5:Mg^{2+} (5 mM), 6: Zn^{2+} (50μM), 7: Cd^{2+} (50μM), 8: Cu^{2+} (50μM), 9: Mn^{2+} (50μM), 10: Fe^{3+} (50μM), 11: Ni^{2+} (50μM), 12: Co^{2+} (50μM), 13: Glycine (100 μM), 14: Cysteine (100 μM), 15: Tyrosine (100 μM), 16: Vitamin C (100 μM), 17: Glucose (100 μM), 18: Glutathione (100 μM). Excitation was performed at 505 nm.

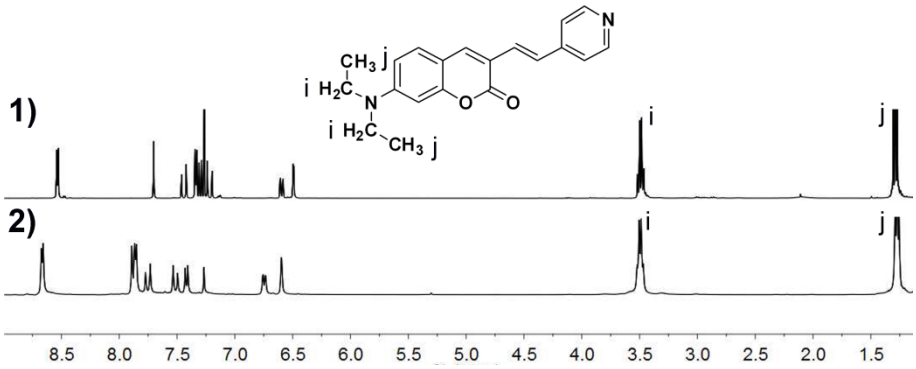

Figure S13. ^{1}H NMR (400 MHz) spectra of 1) sensor **1a**; 2) sensor **1a** + 10 equivalents of CF_3COOD.

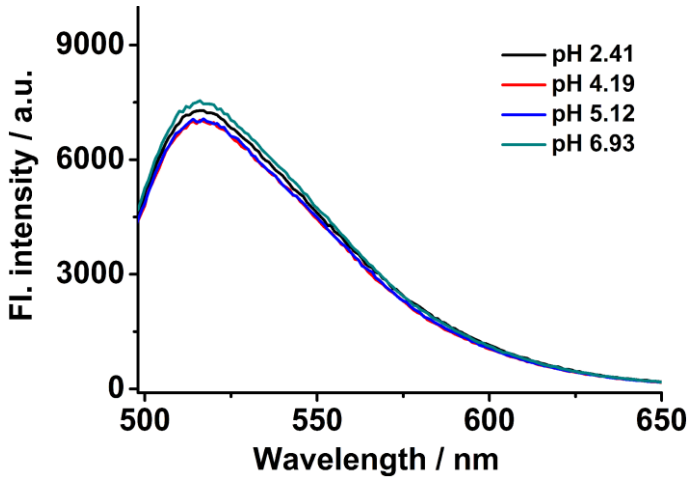

Figure S14. Fluorescence emission spectra of compound **2** (5 μM) in 0.1 M potassium phosphate buffer (containing 40% ethanol as co-solvent) with various pH values.

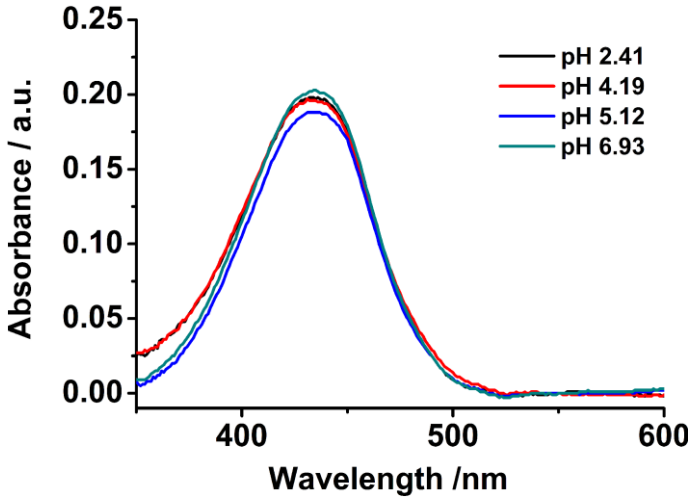

Figure S15. Absorption spectra of compound **2** (5 μM) in 0.1 M potassium phosphate buffer (containing 40% ethanol as co-solvent) with various pH values.

Determination of pH in biological fluids: The biological fluids, newborn-calf serum sample or human urine sample (the sample was taken from a healthy volunteer), were respectively adjusted to fixed pH value by addition of aliquot hydrochloric acid or aqueous sodium hydroxide. Then 3 mL of the biological fluid was added directly to 2 mL of sensor **1a** (12.5 μM) in ethanol. The resulting solution was shaken well and incubated for 20 min at room temperature. After centrifugation, the emission ratio (I_{529}/I_{616}) (λ_{ex} = 495 nm) was recorded.

Table S1. Determination of pH in biological fluids

sample	pH known [a]	pH found [b]	R.S.D.(%) [c]
serum sample 1	4.23	4.21	2.32
serum sample 2	4.91	4.89	2.01
serum sample 3	5.23	5.26	1.80
urine sample 1	4.31	4.29	1.92
urine sample 2	4.96	5.01	2.52
urine sample 3	5.36	5.38	2.74

a Measured by a pH-3c digital pH-meter.
b Obtained from the linear relationship between the ratiometric response (I_{529} /I_{616}) of **1a** and the pH value (4.0-6.5).
c Relative standard deviations of pH found when n=3.

Table S2. Crystallographic parameters for sensor **1a**

Compound	Sensor **1a**
Chemical formula	$C_{20}H_{20}N_2O_2$
formula weight	320.38
Wavelength (Å)	0.71073
Crystal system	Monoclinic
Space group	P21/c
T (K)	150(2)
a (Å)	8.2474(16)
b (Å)	17.675(3)
c (Å)	11.501(2)
α(°)	90.00
β(°)	101.32(3)
γ(°)	90.00
V(Å^3)	1643.9(5)
Z	4
D (mg/m^{-3})	1.294
F(000)	680
μ(Mo *K*a)(mm^{-1})	0.084
θ range (º)	3.41 ~ 26.02
Goodness of fit on F^2	1.105
R_1, wR_2 [$I > 2\sigma(I)$]	0.0549, 0.1128
Reflections collected / unique	7721 / 3190 [R(int) = 0.0303]
R indices (all data)	0.0741, 0.1234

$$R = \Sigma||F_0| - |F_c||/\Sigma|F_0|,\ wR_2 = \{\Sigma[w(F_0^2 - F_c^2)^2]/\Sigma[w(F_0^2)^2]\}^{1/2}$$

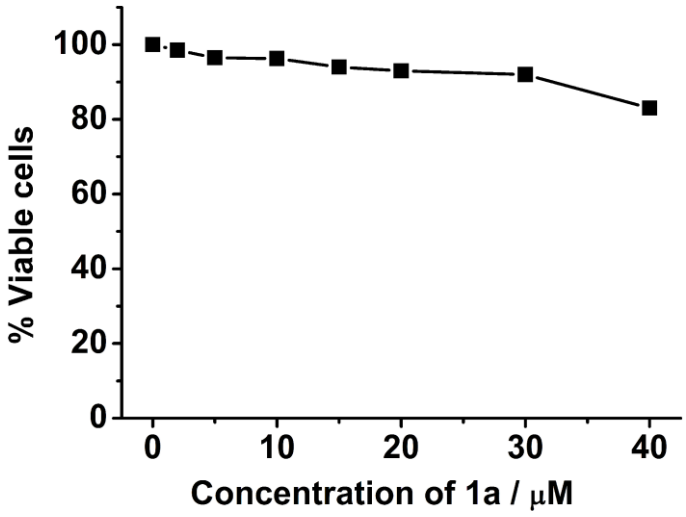

Figure S16. Cytotoxicity of sensor **1a** in cultured pancreatic cancer cells. The cells were incubated with the sensor at different concentrations for 24 h. The cell viability was measured by the MTT assay, and the data are reported as the percentage relative to the untreated cells.

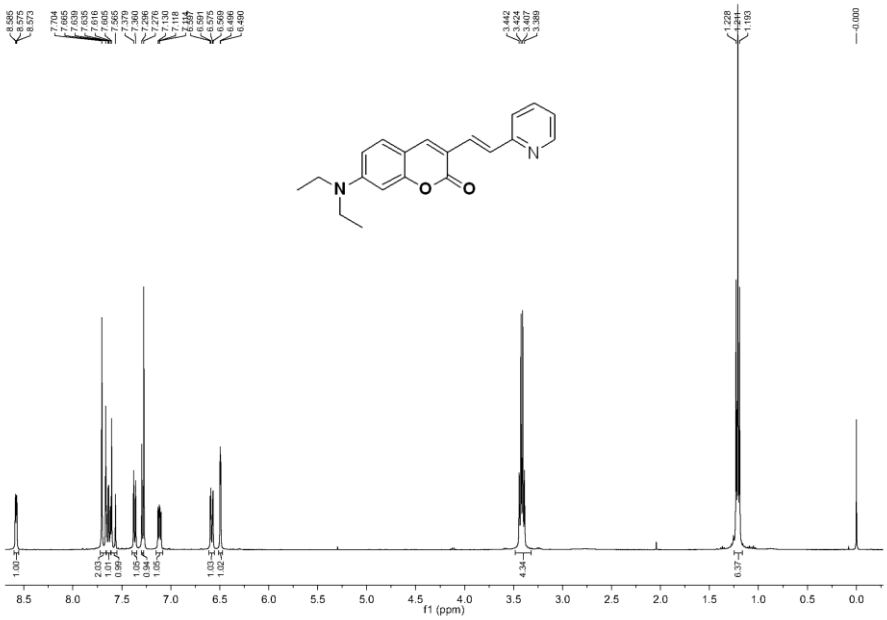

Figure S17. ^{1}H NMR spectra of **1b**.

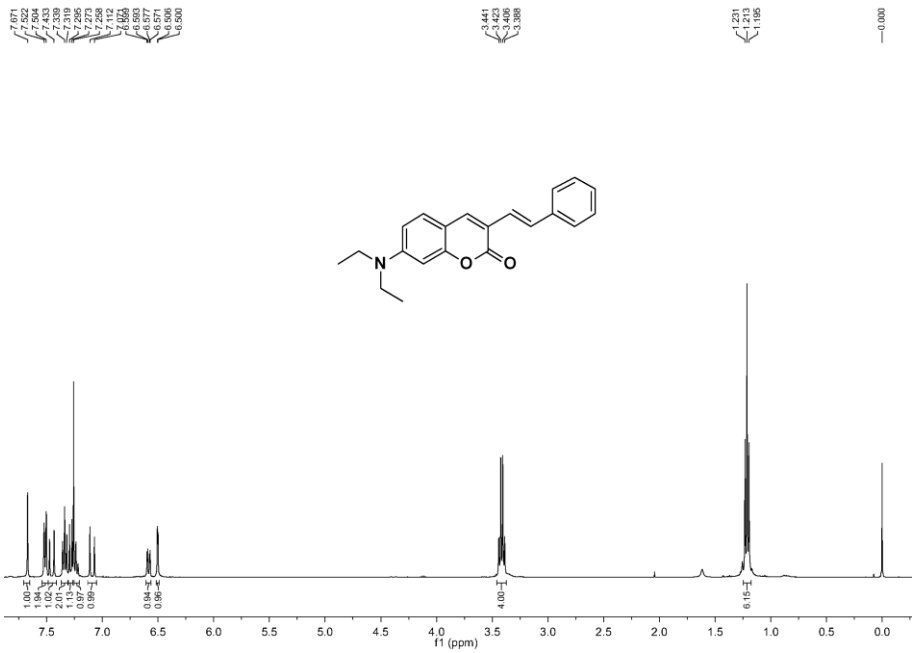

Figure S18. [1] H NMR spectra of **2**.

References

1. J. Wu, W. Liu, X. Zhuang, F. Wang, P. Wang, S. Tao, X. Zhang, S. Wu, S. Lee, *Org. lett.*, 2007, **9**, 33.
2. J.E. Whitaker, R.P. Haugland, F.G. Prendergast, *Anal. Biochem.*, 1991, **194**, 330.
3. J.N. Demas, G.A. Crosby, *J. Phys. Chem.*, 1971, **75**, 991.
4. C.A. Parker, W.T. Rees, *Analyst*, 1960, **85**, 587.
5. S.F. Forgues, D. Lavabre, *J. Chem. Educ.*, 1999, **76**, 1260.
6. A. Ajayaghosh, P. Carol, S. Sreejith, *J. Am. Chem. Soc.*, 2005, **127**, 14962.