Electronic Supplementary Information for

Novel far-visible and near-infrared pH probes based on styrylcyanine for imaging intracellular pH in live cells

Li Fan, Yue-Jun Fu, Qiao-Ling Liu, Dong-Tao Lu, Chuan Dong, Shao-Min Shuang*

College of Chemistry and Chemical Engineering, Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Shanxi University, Taiyuan 030006, PR China

E-mail: smshuang@sxu.edu.cn

Table of Contents

Experimental section

Materials

All chemicals and solvents were of analytical grade and were used without further purifications. 1,1,2-trimethyl-1H-benzo[e]indole, 2,3,3-tetramethyl-3H-indole, and 4-(diphenylamino)benzaldehyde were purchased from Sigma-Aldrich company. All other chemicals were commercially available from Beijing chemical reagent company. The stock solutions of metal ions for selectivity experiments were prepared respectively by dissolving KCl, NaCl, $CaCl_2$, $MgCl_2·6H_2O$, $FeCl_3·6H_2O$, $Zn(NO_3)_2·7H_2O$, $CuCl_2·2H_2O$, $PbCl_2$, $MnCl_2·4H_2O$, $HgCl_2$, $AgNO_3$, $CoCl_2·6H_2O$, $NiCl_2·6H_2O$, $CdCl_2·2.5H_2O$, $AlCl_3·6H_2O$, $FeCl_2·4H_2O$ in doubly distilled water. LysoTracker Green DND-26, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI-1640), fetal bovine serum (FBS), penicillin and streptomycin were ordered from Invitrogen (Carlsbad, CA). Rat glioma cells (C6) were kindly provided by Prof. Jianing Wang (Institute of Clinical Medicine, Renmin Hospital, Yunyang Medical College, Shiyan, Hubei, China). Human hepatocellular liver carcinoma cells (HepG2) were purchased from the Committee on type Culture Collection of Chinese Academy of Sciences. DMEM was used for C6 cells' culture, and RPMI 1640 was used for HepG2 cells' culture.

Instruments

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 300 and a Bruker 75 spectrometer with TMS as the internal standard in $CDCl_3$, respectively. Chemical shifts are given in parts per million downfield from tetramethylsilane (0.0 ppm). Mass spectrometric data were determined with a Bruker Autoflex MALDI-TOF mass spectrometer. Elemental analyses were obtained on a FLASH EA1112 elemental analysis recorder. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. The UV-visible spectra were recorded on a UV-2450 UV−visible spectrophotometer (SHIMADZU). Fluorescence spectra were measured on FLS-920

Edinburgh Fluorescence Spectrophotometer. All data were treated with the Origin 8.0 program. Fluorescent images were acquired on an Olympus FV1000 confocal laser-scanning microscope with a 60 objective lens.

Synthesis

Preparation of SCY-1. A mixture of 1.05 g (5.00 mmol) 1,1,2-trimethyl-1H-benzo[e] indole, 1.64 g (6.00 mmol) 4-(diphenylamino)benzaldehyde and 1.5 ml piperidine in 10 ml anhydride ethanol was refluxed for 12 h. Then reaction was cooled to room temperature. The precipitated solid was filtered and washed with cold ethanol and then purified by column chromatography using methylene chloride as the eluent to give a yellow solid of **SCY-1** (1.75 g, 3.76 mmol). Yield: 75 %. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 1.67 (s, 6H, CH_3), 6.96-7.14 (m, 9H, Ar-H), 7.24-7.25 (m, 4H, Ar-H), 7.42 -7.54 (q, 4H, Ar-H), 7.75 (s, 1H, Ar-H), 7.80-7.84 (d, 2H, Ar-H), 7.92-7.94 (d, 1H, CH), 8.02-8.05 (d, 1H, CH). ^{13}C NMR (CDCl3, 75 MHz) δ(ppm): 24.47, 118.04, 121.48, 123.89, 124.85, 125.54, 126.28, 127.46, 129.72, 130.58, 133.61, 138.37, 140.53, 148.30, 150.16, 152.62, 186.53. MS (MALDI-TOF): m/z Calcd 465.2325, found 465.2282 for $[M]^+$. Elemental Analysis: Calcd C, 87.90; H, 6.07; N, 6.03. Found C, 87.54; H, 6.05; N, 6.01. mp: 173-175℃.

Preparation of SCY-2. A mixture of 0.80 g (5.00 mmol) 2,3,3-tetramethyl-3H-indole and 1.64 g (6.00 mmol) 4-(diphenylamino)benzaldehyde were refluxed in 25 ml anhydride ethanol for 20 h. After the solvent was removed under reduced pressure, the red solid was purified by column chromatography using methylene chloride as the eluent. 1.24 g (3.00 mM) of yellow product was obtained. Yield: 60 %. 1H NMR ($CDCl_3$, 300 MHz) δ(ppm): 1.43 (s, 6H, CH3), 6.89-6.95 (d, 1H, CH), 7.01-7.13 (m, 8H, Ar-H), 7.16-7.33 (m, 8H, Ar-H), 7.43-7.46 (d, 2H, Ar-H), 7.58-7.67 (m, 1H, CH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ(ppm): 25.174, 30.846, 53.669, 118.193, 121.370, 122.240, 124.954, 162.608, 130.592, 148.194, 150.386, 154.512. MS (MALDI-TOF): m/z 413.8737 for $[M]^+$. Elemental Analysis: Calcd C, 86.92; H, 6.32; N, 6.76. Found C, 86.88; H, 6.30; N, 6.74. mp: 168-170℃.

UV-vis and fluorescence pH titrations

Stock solution of **SCY-1** and **SCY-2** (1.0 mM) were prepared in ethanol. The **SCY-1** and **SCY-2** solutions for spectroscopic determination were obtained by diluting the stock solution to 10 μM ethanol/water (2/1, v/v) medium. In the pH titrations experiments, 3 mL of **SCY-1** and **SCY-2** solutions (10 μM, ethanol/water, 2/1, v/v) were poured into a quartz optical cell of 1 cm optical path length each time and the slight pH variations of the solutions were achieved by adding the minimum volumes of NaOH (0.1 M) or HCl (0.2 M). Spectral data were recorded after each addition. Excitation and emission bandwidths were both set at 2 nm (for **SCY-1**) and 3 nm (for **SCY-2**), and the excitation wavelengths were 546 nm and 534 nm for **SCY-1** and **SCY-2**, respectively. All spectroscopic experiments were carried out at room temperature.

Calculation of quantum yield

The quantum yields of **SCY** was determined according to the literature.[R1]

$$\Phi_x = \Phi_{st}\left(D_x/D_{st}\right)\left(A_{st}/A_x\right)\left(\eta_x^{\,2}/\eta_{st}^{\,2}\right)$$

Where ***Φst*** is the reported quantum yield of the standard, ***D*** is the area under the emission spectra, ***A*** is the absorbance at the excitation wavelength and η is the refractive index of the solvent used. ***x*** subscript denotes unknown, and ***st*** means standard. We chose Rhodamine B (***Φ*** = 0.69 in MeOH) as the standard.

Culture of C6 and HepG2 cells for intracellular imaging

C6 cells were cultured in DMEM with 10% fetal bovine serum (FBS) at 37℃ in a 5% CO_2 atmosphere. HepG2 were maintained in RPMI 1640 medium supplemented with 10% FBS, $NaHCO_3$ (2 g/L), and 1% antibiotics (penicillin /streptomycin, 100 U/ml), and incubated at 37℃ in a 5% CO_2 atmosphere. Cells were seeded on 6 well chambered coverglass at a density of 1.5×10^6 and 1×10^6 cells mL^{-1} for C6 and HepG2, respectively for 24 h and 48 h. Probes dissolved in DMSO (0.5 μL, 10 mM) were added to the cells medium (500 μL) at 10 μM final concentrations. After incubating

for 30 min, excess probes were removed by gentle rinsing with cold phosphate buffered saline (PBS, pH 7.4) three times. To observe the subcellular distributions of the probes, the cells were treated with a lysosomal staining probes, LysoTracker Green DND-26 (50 nM) for additional 30 min. The media was removed and the cells were washed three times with PBS buffer (pH 7.4). Fluorescence images were collected by sequentially line scanning with an Olympus FV1000 confocal laser-scanning microscope. LysoTracker Green DND-26 was excited at 488 nm and its green emission was collected in the range of 505-550 nm; probes (SCY-1 and SCY-2) were excited at 543 nm and their red emissions were collected in the range of 620-680nm.

Cell cytotoxicity assay

According to the procedure described previously,[R1] MTT assay was used to test the cytotoxicity of new probes to HepG2 cells. HepG2 cells were plated in 96 well microplates to a total volume of 200 μL well^{-1} at a density of 1×10^{6} cells mL^{-1} at 37℃ in a 5% CO_2 atmosphere. Then different concentrations probes of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M in fresh medium were added into the HepG2 cells medium for incubating 48 h, respectively. Then 10 μL of MTT solution (10 mg/ml, PBS) was added in each well of 96 well microplates and incubated continuously for 6 h. Then the remaining MTT solution was removed from wells and 150 μL of DMSO was added into each well to dissolve the intracellular blue-violet formazan crystals. The absorbance of the solution was measured at 490 nm wavelength with a microplate reader. The cell viability was then determined by the following equation: % viability = $\left[\sum(Ai/Acontrol\times100)\right]/n$, where Ai is the absorbance of different concentrations probes of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M, respectively. $A_{control}$ is the average absorbance of the control wells in which the probes was absent, and n (= 3) is the number of the data points.

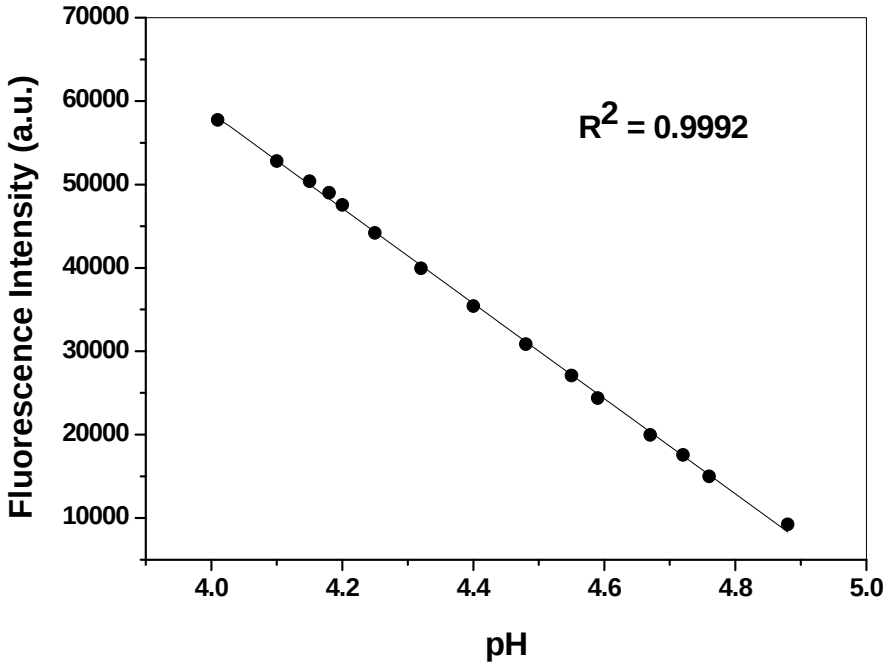
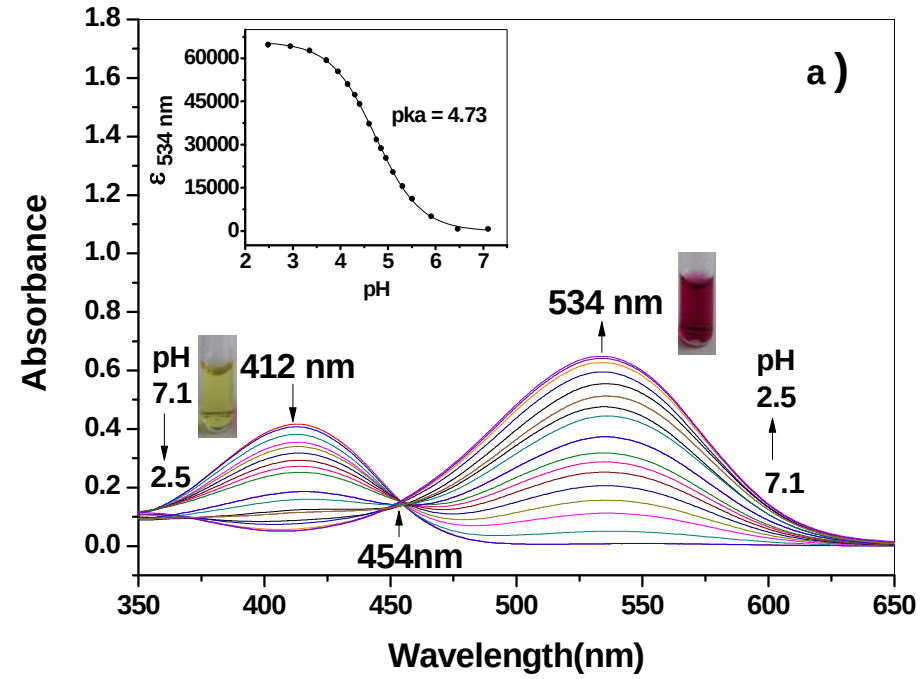


Fig. S1 The good linearity between fluorescence intensity at 655 nm for **SCY-1** and pH in the range of 4.0 - 4.9.



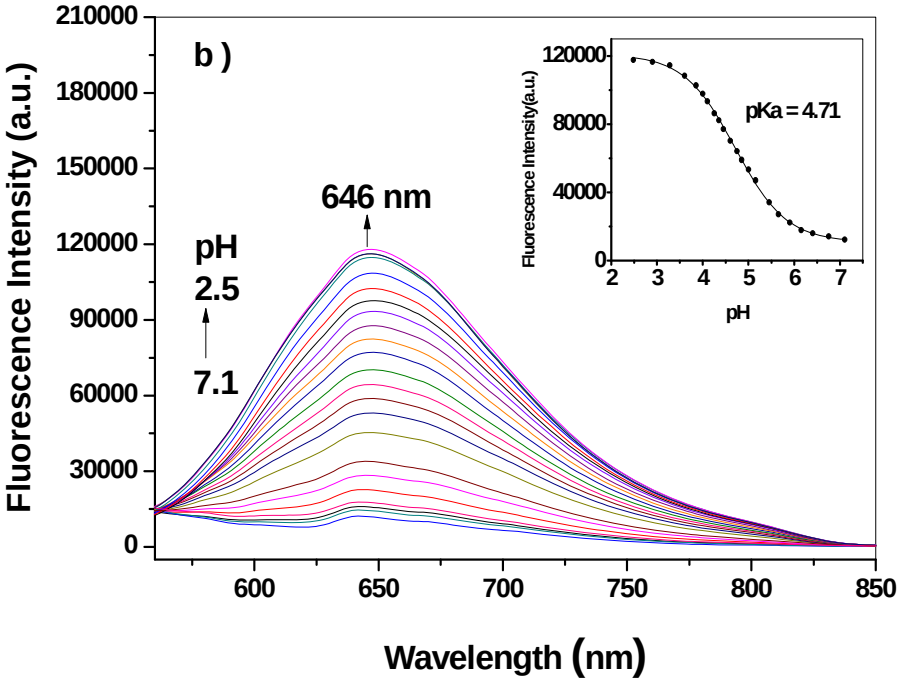


Fig. S2 (a) Change of the absorption spectra of 10 μM **SCY-2** in ethanol/ water (2/1, v/v) with pH decreased from 7.1 to 2.5. Inset: Sigmoidal fitting of the pH-dependent molar extinction coefficient ε at 534 nm. (b) Change of the fluorescence spectra of 10 μM **SCY-2** in ethanol/ water (2/1, v/v) with pH decreased from 7.1 to 2.5 (λ_{ex} = 534 nm). Inset : Sigmoidal fitting of the pH-dependent fluorescence intensity at 646 nm.

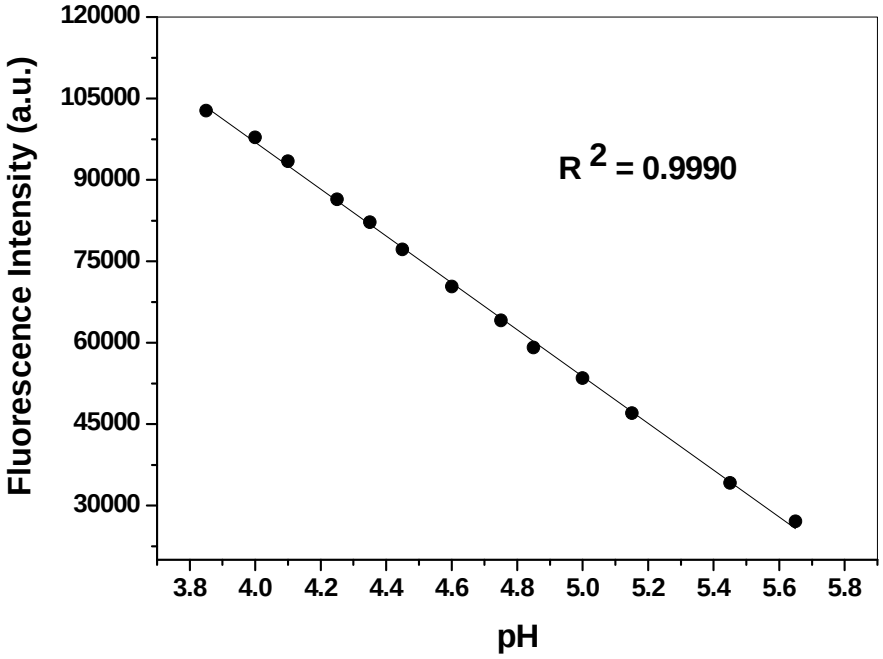


Fig. S3 The good linearity between fluorescence intensity at 646 nm for **SCY-2** and pH in the range of 3.8 - 5.7.

Table S1 Comparison of the photophysical properties of SCY-1 and SCY-2

	SCY-1	SCY-2
λmax, abs (nm)	546 (pH 2.3)	534 (pH 2.5)
	420 (pH 7.1)	412 (pH 7.1)
λmax, em (nm)	655 (pH 2.3)	646 (pH 2.5)
	655 (pH 7.1)	646 (pH 7.1)
Stokes shift (nm)[a]	109 (pH 2.3)	112 (pH 2.5)
	235 (pH 7.1)	234 (pH 7.1)
pH range	2.3 – 7.1	2.5 – 7.1
pKa	4.40	4.71
I_{max} / I_{min} [b]	～ 40	～ 10
Molar absorption coefficient ε (L mol^{-1} cm^{-1})	72800 (λ_{max} = 546 nm, pH 2.3) 41800 (λ_{max} = 420 nm, pH 7.1)	64800 (λ_{max} = 546 nm, pH 2.5) 42100 (λ_{max} = 420 nm, pH 7.1)
Quantum Yield Φ	0.22 (pH 2.3) 0.001 (pH 7.1)	0.13 (pH 2.5) 0.0005 (pH 7.1)

[a] The difference of emission maximum ($\lambda_{max, em}$) and absorption maximum ($\lambda_{max, abs}$) (e.g. $\lambda_{max, em} - \lambda_{max, abs}$) indicates the Stokes shift.

[b] The sensitivities are the ratios of the maximum fluorescence intensity (I_{max}) divided by the minimum fluorescence intensity (I_{min}).

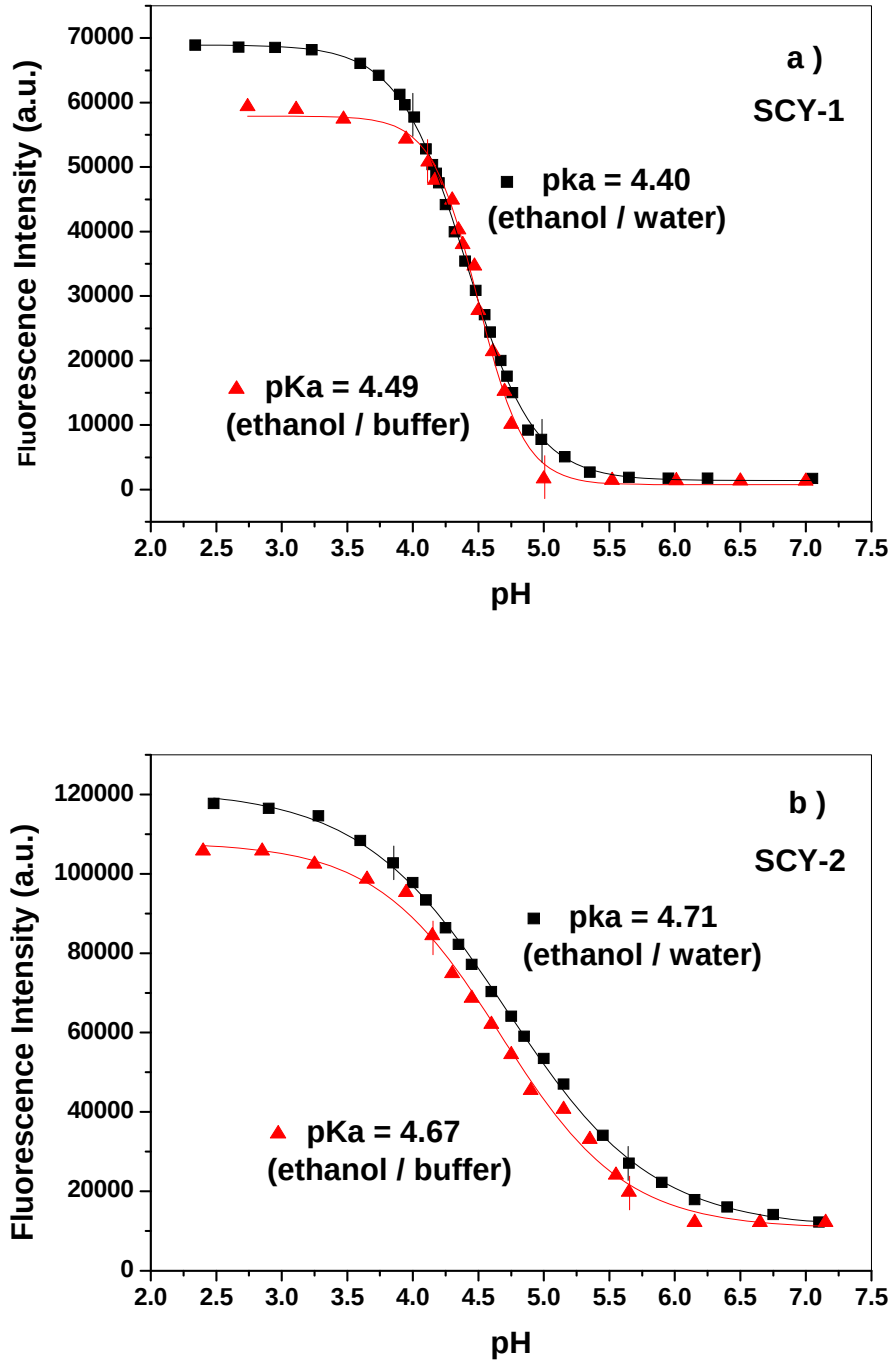


Fig. S4 (a) Sigmoidal fitting of the pH-dependent fluorescence intensity of **SCY-1** at 655 nm in ethanol/water (2/1, v/v) (squares, λ_{ex} = 546 nm) and at 652 nm in 50 mM buffer (20 mM disodium hydrogen phosphate/10 mM citric acid) with 100 mM NaCl as ionic strengths and 40% ethanol as a co-solvent (triangles, λ_{ex} = 544 nm). (b) Sigmoidal fitting of the pH-dependent fluorescence intensity of **SCY-2** at 646 nm in ethanol/water (2/1, v/v) (squares, λ_{ex} = 534 nm) and 642 nm in 50 mM buffer (20 mM disodium hydrogen phosphate/10 mM citric acid) with 100 mM NaCl as ionic strengths and 40% ethanol as a co-solvent (triangles, λ_{ex} = 530 nm).

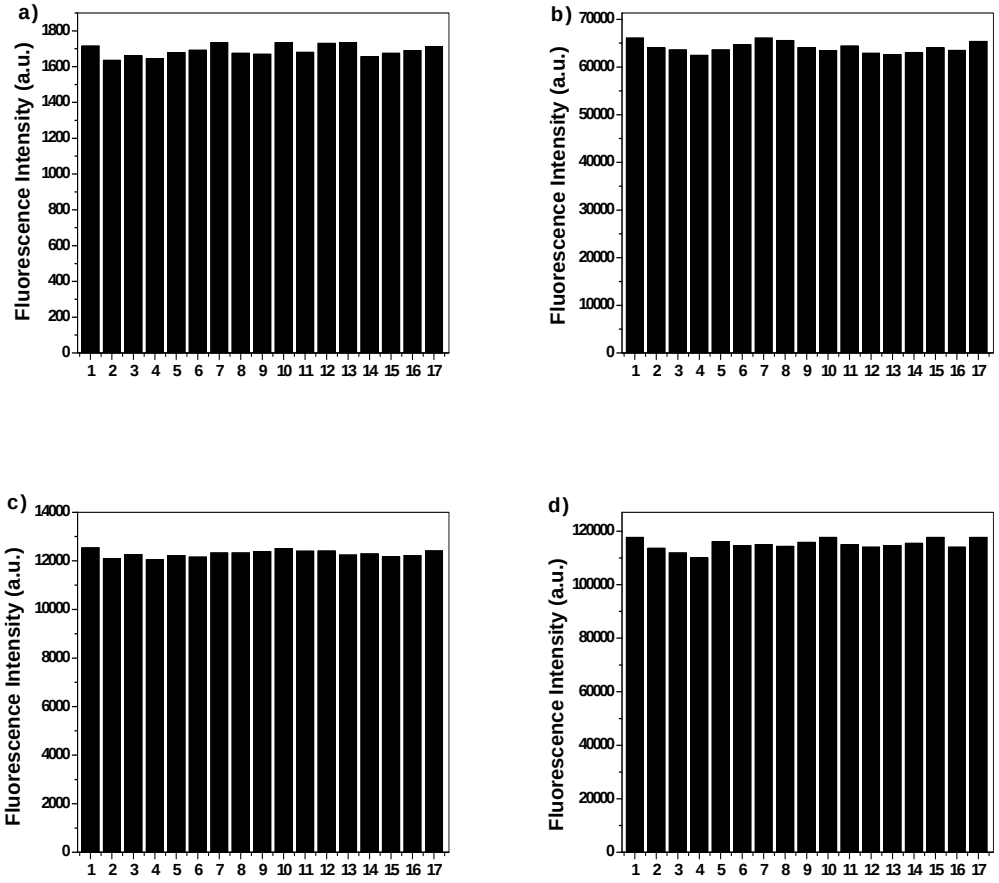


Fig. S5 The fluorescence intensity at 655 nm of 10 μM **SCY-1** (λ_{ex} = 546 nm) in ethanol/ water (2/1, v/v) at pH 7.0 (a) and pH 3.5 (b) and fluorescence intensity at 646 nm of 10 μM **SCY-2** (λ_{ex} = 534 nm) in ethanol/ water (2/1, v/v) at pH 7.0 (c) and pH 3.5 (d) to diverse metal ions, respectively. 1, blank; 2, K^+ (150 mM); 3, Na^+ (150 mM); 4, Ca^{2+} (10 mM); 5, Mg^{2+} (2 mM); 6, Fe^{3+} (2 mM); 7, Zn^{2+} (0.2 mM); 8, Cu^{2+} (0.2 mM); 9, Pb^{2+} (0.2 mM); 10, Mn^{2+} (0.2 mM); 11, Hg^{2+} (0.2 mM); 12, Ag^+ (0.2 mM); 13, Co^{2+} (0.2 mM); 14, Ni^{2+} (0.5 mM); 15, Cd^{2+} (0.5 mM); 16, Al^{3+} (0.2 mM); 17, Fe^{2+} (0.2 mM).

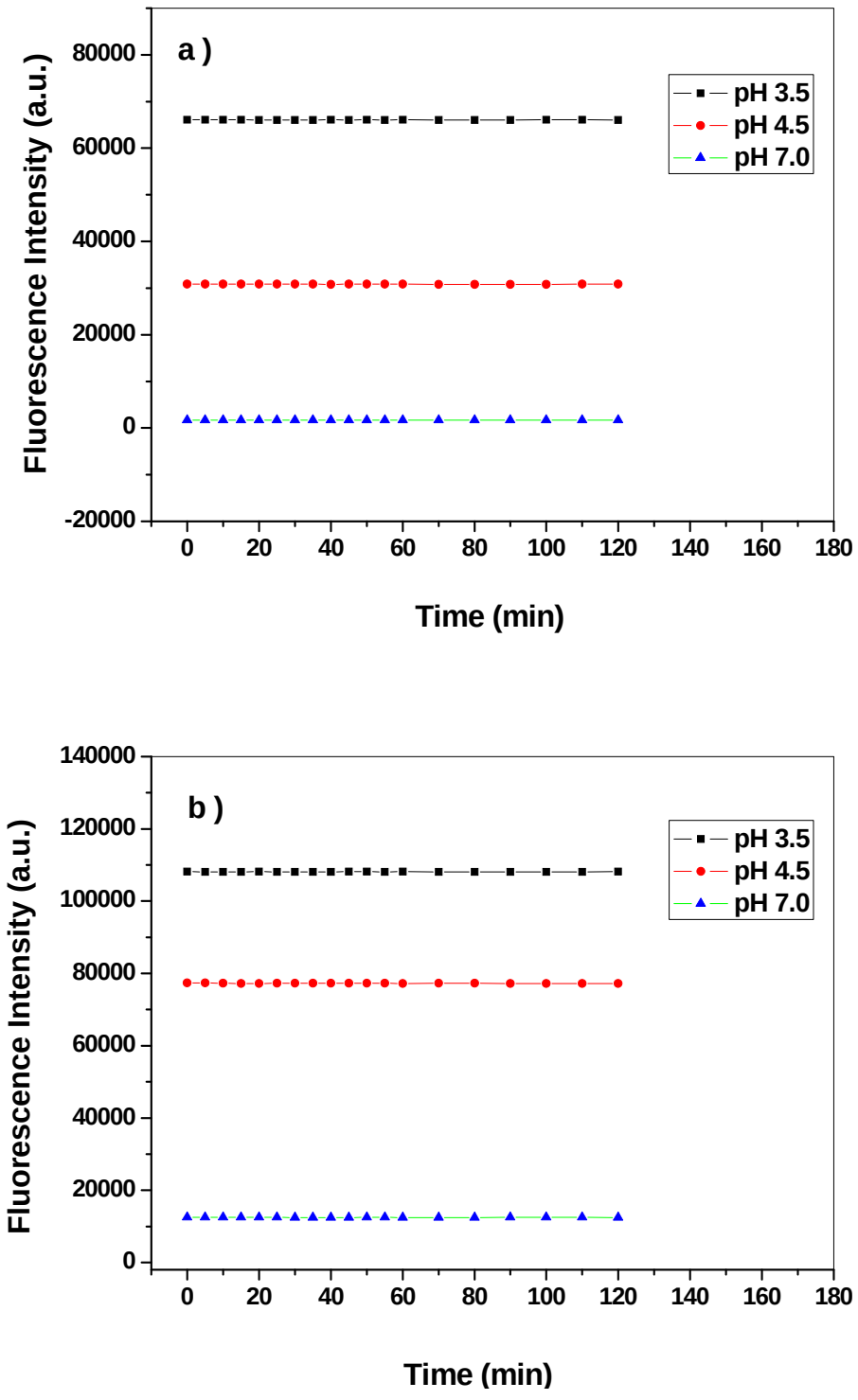


Fig. S6 Changes in the fluorescence intensity at 655 nm for **SCY-1**(a) and at 646 nm for **SCY-2** (b) with times at pH 3.5, 4.5, 7.0, respectively.

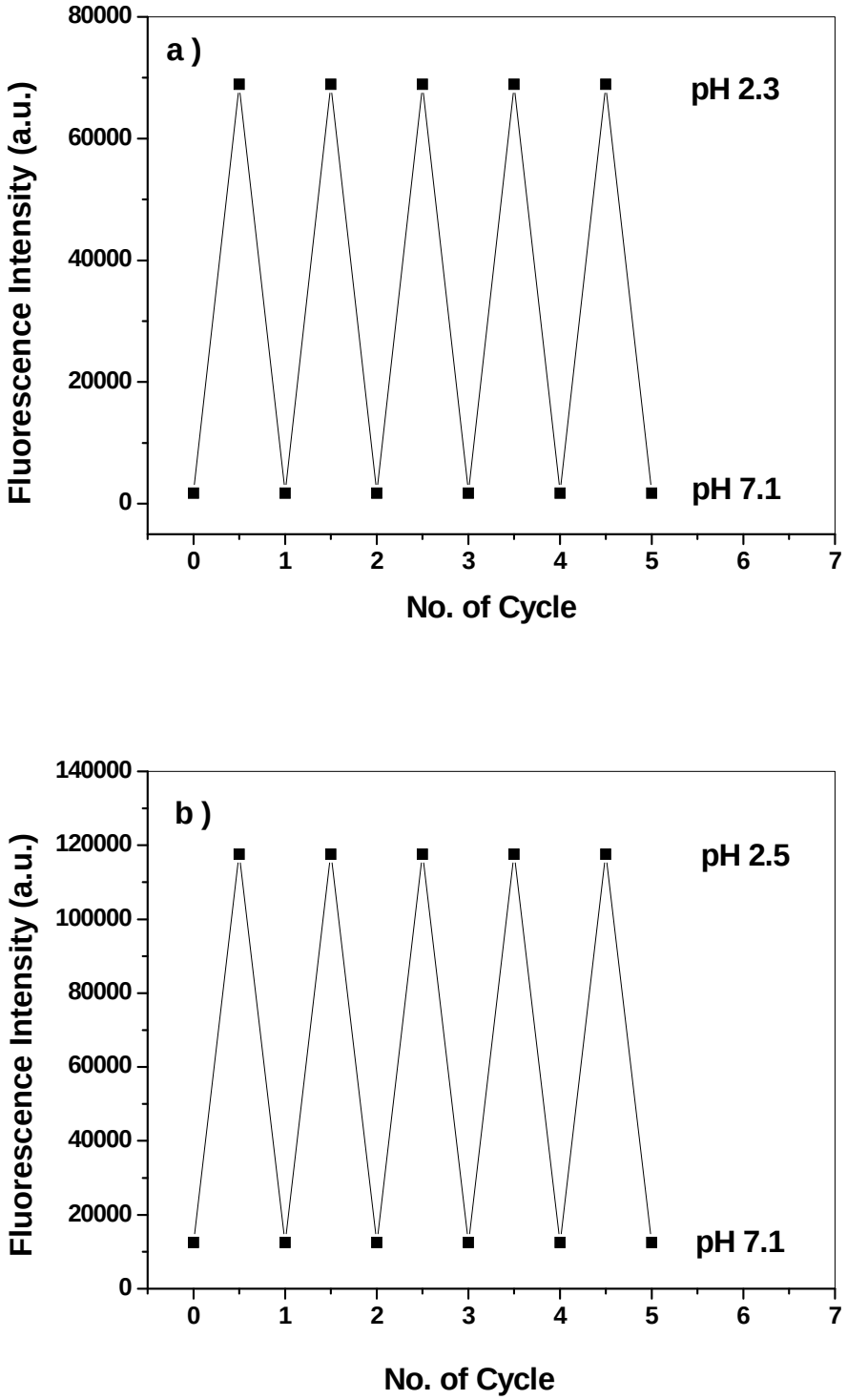


Fig. S7 Changes in the fluorescence intensity at 655 nm for **SCY-1** (a) between pH 7.1 and 2.3 and at 646 nm for **SCY-2** (b) between pH 7.1 and 2.5**.**

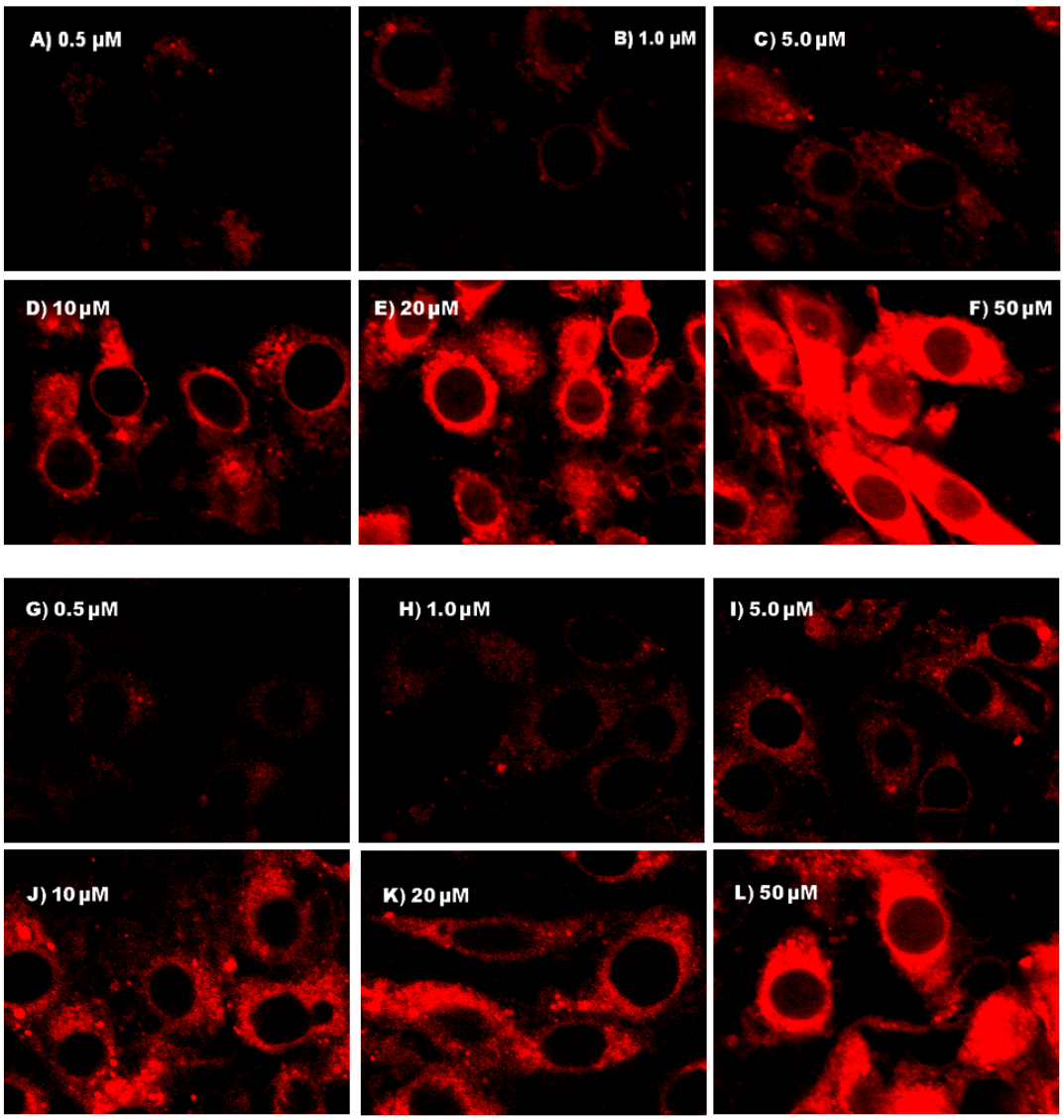


Fig. S8 Concentration dependent fluorescence bioimaging of **SCY-1** (A-F) and **SCY-2** (G-L) in C6 cells under the same optical set-up, respectively. Concentration is from 0.5 μM to 50 μM. Cell uptake time is 30 minutes.

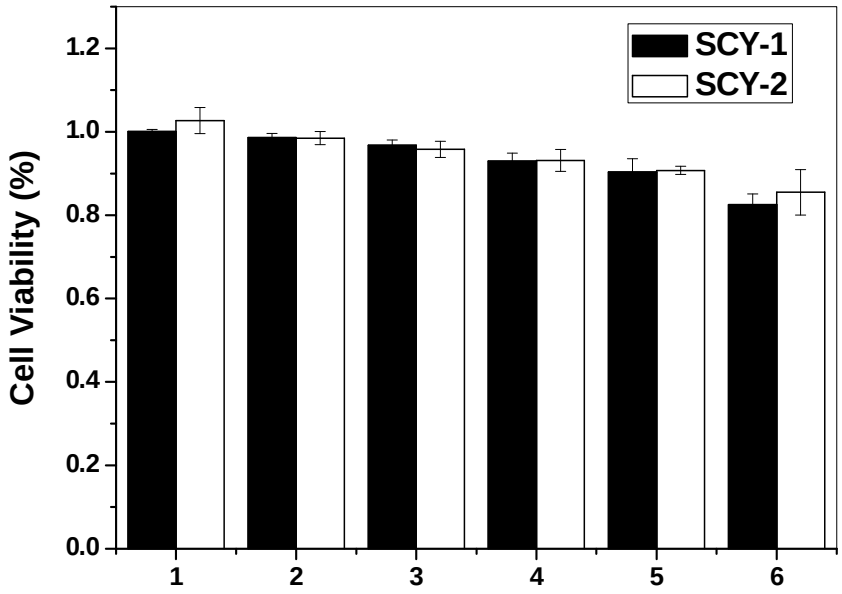


Fig. S9 Cytotoxic effects of **SCY-1** and **SCY-2** on HepG2 cells. 1, control; 2, 10^{-8} mol/L; 3, 10^{-7} mol/L; 4, 10^{-6} mol/L; 5, 10^{-5} mol/L, 6, 10^{-4} mol/L. Date are expressed as mean values±standard error of the means of three independent experiments, each performed in three triplicate.

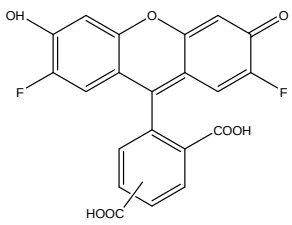
Table S2 Comparison of some commonly used probes with SCY-1 and SCY-2

Probes[a]	λ max, abs (nm)	λ max, em (nm)	Stokes shift (nm)[b]	pH range	pKa	I_{max} / I_{min}[C]	Ref.
OregonGreen 488	472 (pH 3.0)	513 (pH 3.0)	41 (pH 3.0)	3.0 – 9.0	4.7	~ 8	[R2]
Carboxylic acid	490 (pH 9.0)	515 (pH 9.0)	25 (pH 9.0)				
LysoTracker	577 (pH 4.0)	590 (pH 4.0)	13 (pH 4.0)	4.0 – 8.0	–	~ 1	[R3]
Red DND 99	577 (pH 8.0)	590 (pH 8.0)	13 (pH 8.0)				
LysoTracker	504 (pH 4.0)	511 (pH 4.0)	7 (pH 4.0)	4.0 – 8.0	–	~ 1	[R4]
Green DND 26	504 (pH 8.0)	511 (pH 8.0)	7 (pH 8.0)				
LysoSensor	373 (pH 3.1)	425 (pH 3.1)	52 (pH 3.1)	3.1 – 6.1	5.1	~ 6	[R5]
Blue DND 167	373 (pH 6.1)	425 (pH 6.1)	52 (pH 6.1)				
LysoSensor	452 (pH 2.9)	505 (pH 2.9)	53 (pH 2.9)	2.9 – 7.1	5.2	~ 7	[R3]
Green DND 189	442 (pH 7.1)	505 (pH 7.1)	63 (pH 7.1)				
SCY-1	546 (pH 2.3)	655 (pH 2.3)	109 (pH 2.3)	2.3 – 7.1	4.40	~ 40	This work
	420 (pH 7.1)	655 (pH 7.1)	235 (pH 7.1)				
SCY-2	534 (pH 2.5)	646 (pH 2.5)	112 (pH 2.5)	2.5 – 7.1	4.71	~ 10	This work
	412 (pH 7.1)	646 (pH 7.1)	234 (pH 7.1)				

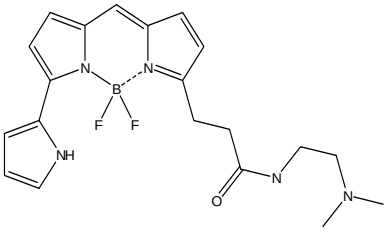
[a] Chemical structures of some commonly used probes reported by other scientists were given in Fig. S10

[b] The difference of emission maximum ($\lambda_{max, em}$) and absorption maximum ($\lambda_{max, abs}$) (e.g. $\lambda_{max, em} - \lambda_{max, abs}$) indicates the Stokes shift.

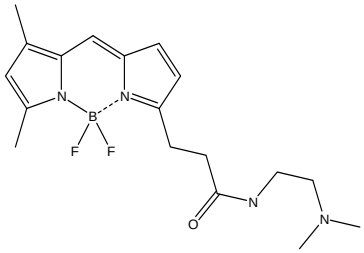
[c] The probes' sensitivities are the ratios of the maximum fluorescence intensity (I_{max}) divided by the minimum fluorescence intensity (I_{min}).



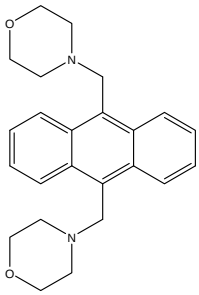
OregonGreen 488 Carboxylic acid



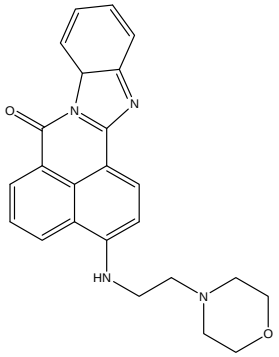
LysoTrack Red DND 99



LysoTracker Green DND 26



LysoSensor Blue DND 167



LysoSensor Green DND 189

Fig. S10 Chemical structures of the probes reported by other scientists listed in Table S1.

References

R1 (a) B. Tang, F. B. Yu, P. Li, L. Tong, X. Duan, T. Xie and X. Wang, *J. Am. Chem. Soc*., 2009, **131**, 3016; (b) X. J. Peng, J. J. Du, J. L. Fan, J. Y. Wang, Y. K. Wu, J. Z. Zhao, S. G. Sun and T. Xu, *J. Am. Chem. Soc*., 2007, **129**, 1500.

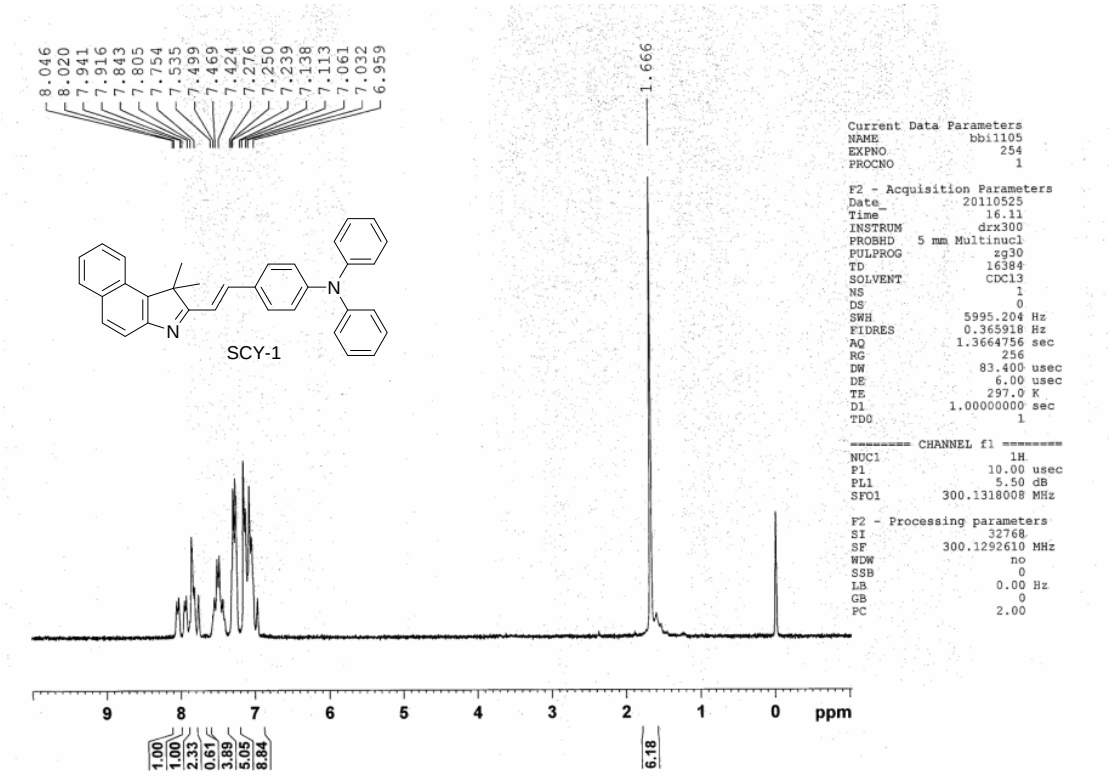
R2 W. C. Sun, K. R. Gee, D. H. Klaubert and R. P. Haugland, *J. Org. Chem.* 1997, **62**, 6469.

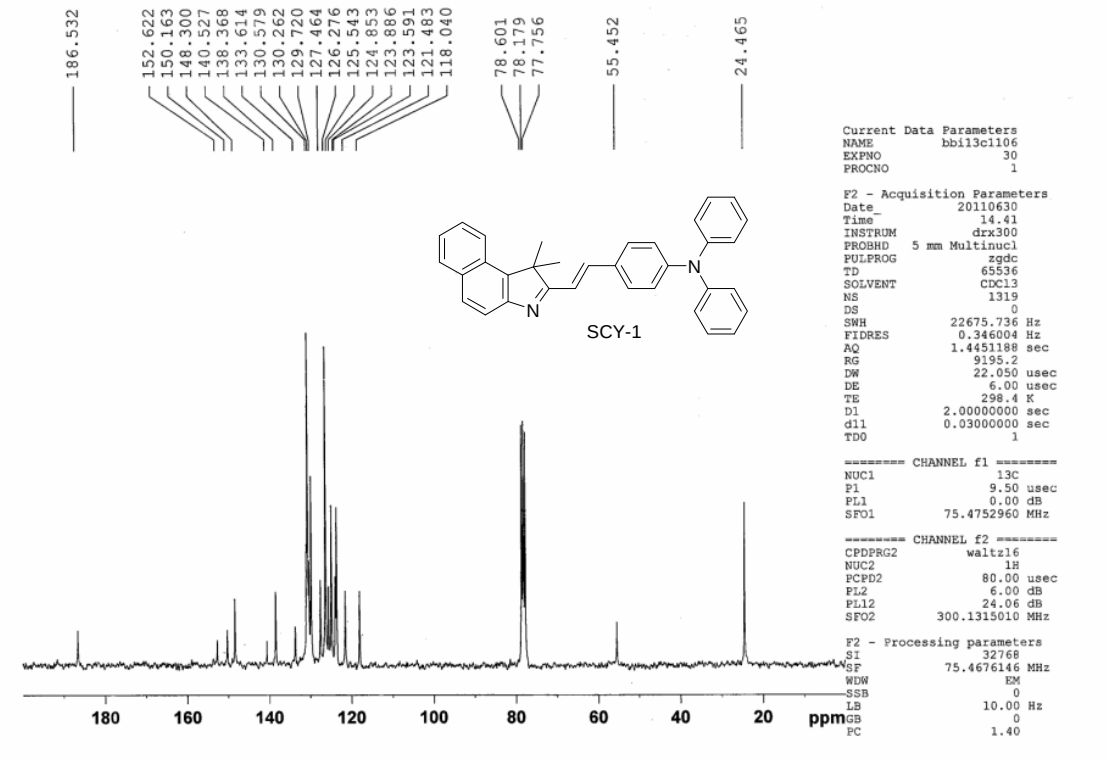
R3 J. H. Lin, P. Herman, J. S. Kang and J. R. Lakowicz, *Anal. Biochem.,* 2001, **294**, 118.

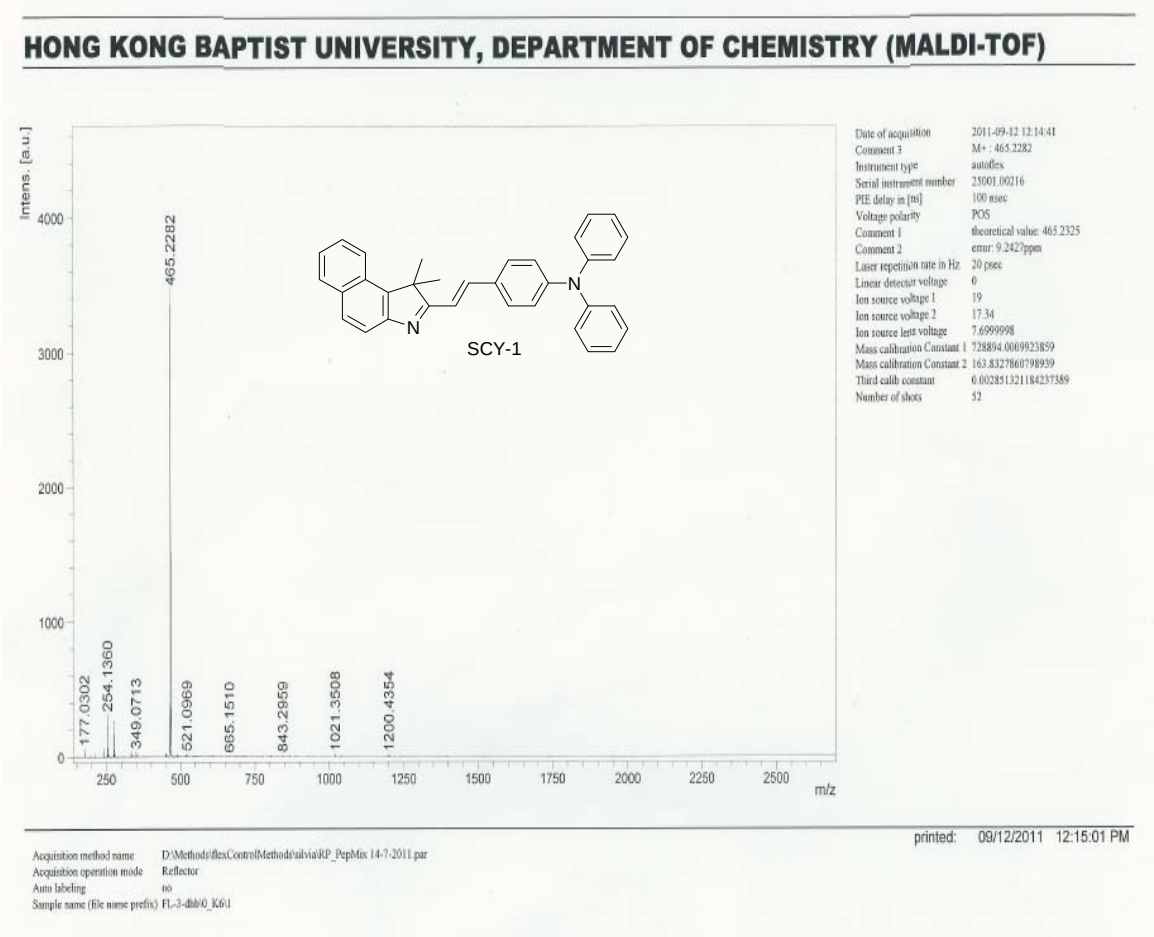
R4 F. Galindo, M. I. Burguete, L. Vigara, S. V. Luis, N. Kabir, J. Gavrilovic and D. A. Russell, *Angew. Chem., Int. Ed.,* 2005, **44**, 6504

R5 R. A. Bissell, A. J. Bryan, A. Prasannade Silva and C. P. McCoy, *J. Chem. Soc., Chem. Commun.,* 1994, **4**, 405.

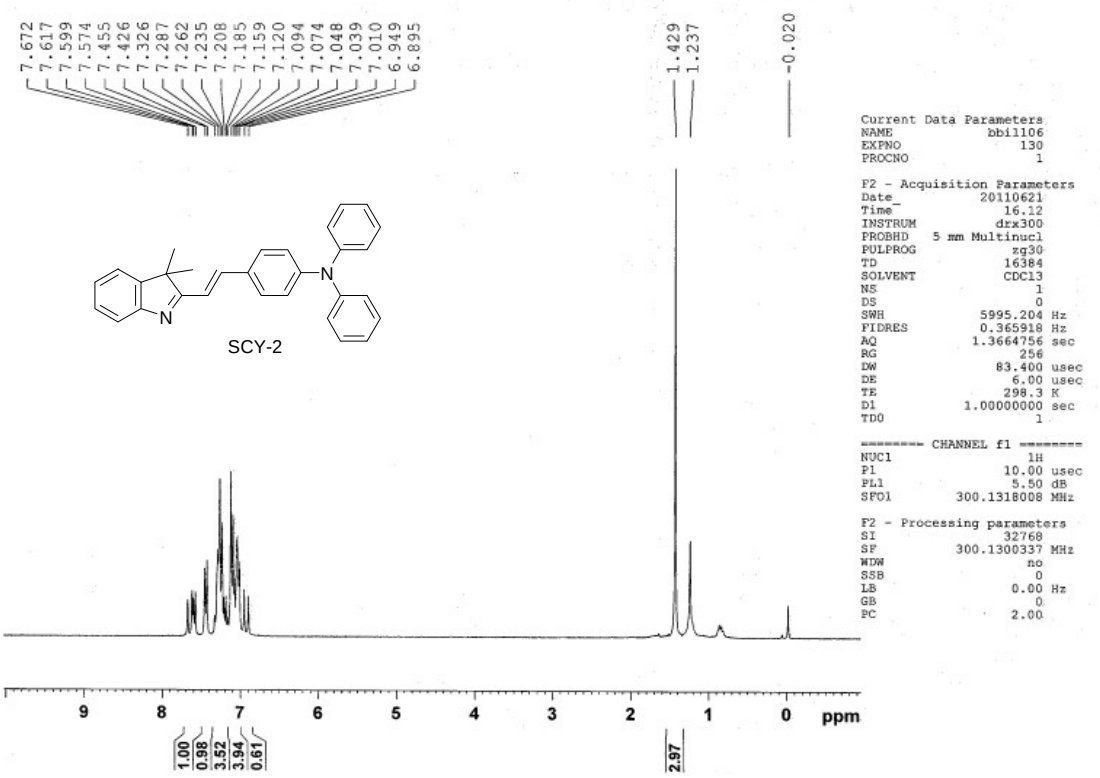
^{1}H NMR, ^{13}C NMR spectra and MS (MALDI-TOF) analysis report of SCY-1

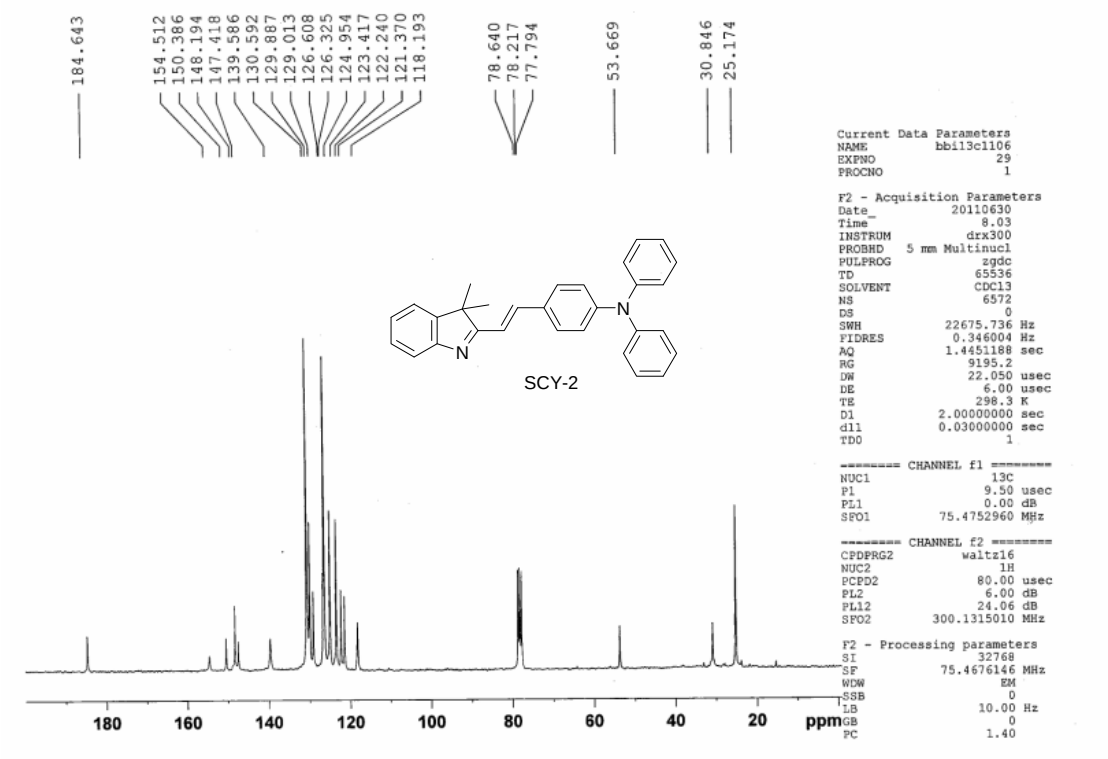


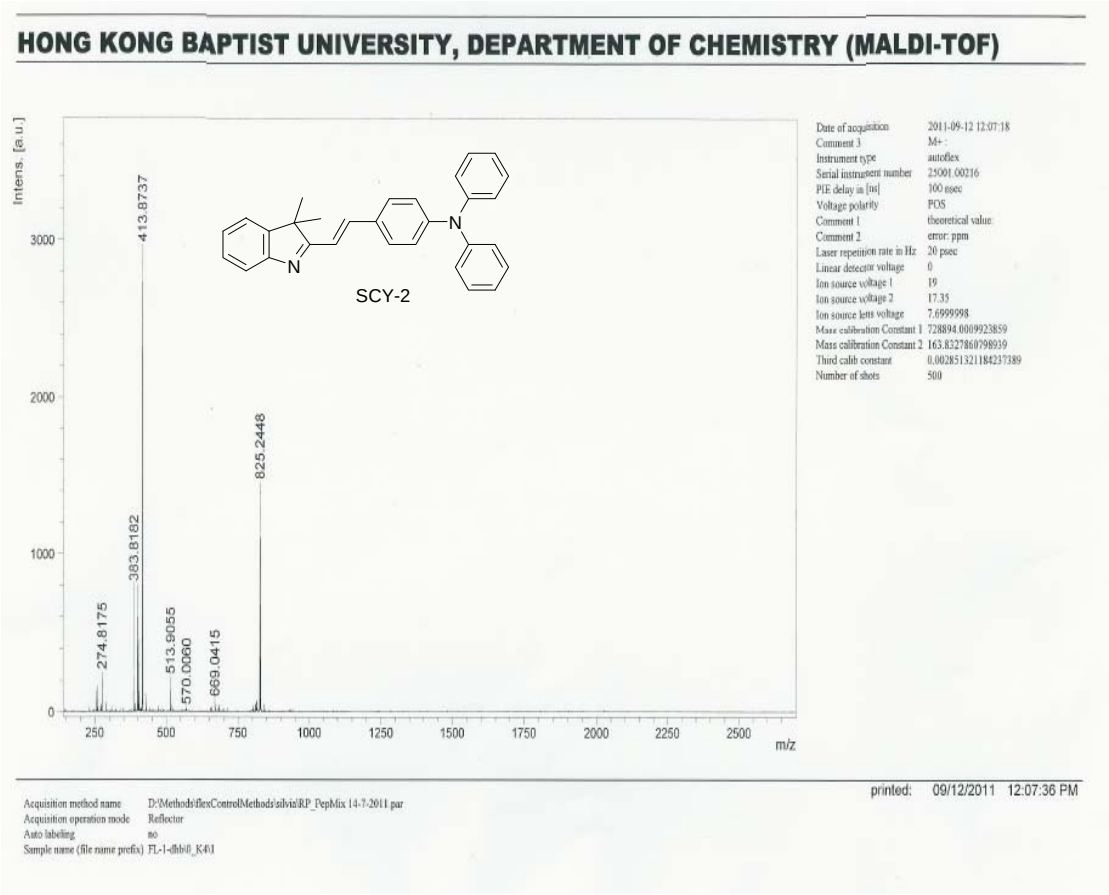



HONG KONG BAPTIST UNIVERSITY, DEPARTMENT OF CHEMISTRY (MALDI-TOF)
Intens. [a.u.]
4000
3000
2000
1000
0
465.2282
177.0302
254.1360
349.0713
521.0969
665.1510
843.2959
1021.3508
1200.4354
250
500
750
1000
1250
1500
1750
2000
2250
2500
m/z
N
N
SCY-1
Date of acquisition 2011-09-12 12:14:41
Comment 3 M+ : 465.2282
Instrument type autoflex
Serial instrument number 25001.00216
PIE delay in [ns] 100 nsec
Voltage polarity POS
Comment 1 theoretical value: 465.2325
Comment 2 error: 9.2427ppm
Laser repetition rate in Hz 20 psec
Linear detector voltage 0
Ion source voltage 1 19
Ion source voltage 2 17.34
Ion source lens voltage 7.6999998
Mass calibration Constant 1 728894.0009923859
Mass calibration Constant 2 163.8327860798939
Third calib constant 0.0028513211842373B9
Number of shots 52
printed: 09/12/2011 12:15:01 PM
Acquisition method name D:\Methods\flexControl\Methods\silvia\RP_PepMix 14-7-2011.par
Acquisition operation mode Reflector
Auto labeling no
Sample name (file name prefix) FL-3-dbb\0_K6\1

^{1}H NMR, ^{13}C NMR spectra and MS (MALDI-TOF) analysis report of SCY-2






HONG KONG BAPTIST UNIVERSITY, DEPARTMENT OF CHEMISTRY (MALDI-TOF)
Intens. [a.u.]
3000
2000
1000
0
413.8737
383.8182
825.2448
274.8175
513.9055
570.0060
669.0415
250
500
750
1000
1250
1500
1750
2000
2250
2500
m/z
N
N
SCY-2
Date of acquisition 2011-09-12 12:07:18
Comment 3 M+ :
Instrument type autoflex
Serial instrument number 25001.00216
PIE delay in [ns] 100 nsec
Voltage polarity POS
Comment 1 theoretical value:
Comment 2 error: ppm
Laser repetition rate in Hz 20 psec
Linear detector voltage 0
Ion source voltage 1 19
Ion source voltage 2 17.35
Ion source lens voltage 7.6999998
Mass calibration Constant 1 728894.0009923859
Mass calibration Constant 2 163.8327860798939
Third calib constant 0.0028513211842373 89
Number of shots 500
printed: 09/12/2011 12:07:36 PM
Acquisition method name D:\Methods\flexControlMethods\silvia\RP_PepMix 14-7-2011.par
Acquisition operation mode Reflector
Auto labeling no
Sample name (file name prefix) FL-1-dhbl0_K4\1